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MANUAL ON HARMFUL MARINE MICROALGAE

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Preface

The IOC has over the past eight years given increasing attention to activities aimed at developing capacity in research and management of harmful marine microalgae. Due to the interdisciplinary nature of the topic; taxonomy, toxin chemistry, monitoring, human health, etc, there has so far been no comprehensive source of guidance with respect to both research and management methodologies. This Manual, together with complementary IOC publications, is intended to help fill this gap, and to provide information and guidance in an easy accessible and low cost format.

One important task of the IOC and UNESCO is to synthesize the available field and laboratory research techniques for applications to help solve problems of society as well as facilitate further research and especially systematic observations and data gathering. The results include the publications in the 'IOC Manuals and Guides' series, and the UNESCO series 'Monographs on Oceanographic Methodology'. The easy access to manuals and guides of this type is essential to facilitate knowledge exchange and transfer, the related capacity building, and for the establishment of ocean and coastal area observations as envisaged in the Global Ocean Observing System.

The term 'harmful algae' is not a scientific but a societal one, determined by the increasing detrimental effects caused by these organisms to national economies. One aim of supporting research and capacity building on harmful algae is to help mitigate their negative effects on fisheries, aquaculture, human health, recreation areas, ecosystems, etc. Understanding the causes of harmful algal events and developing contingency plans directly links to other major areas of scientific and societal concern, including eutrophication, influence of climate change on marine ecosystems, integrated coastal zone management, fisheries management, and transfer of non-indigenous marine species. These linkages are important in addressing the problem of harmful algae adequately, and in a way where society will understand and appreciate the results of scientific advances within the field.

The United Nations Conference on Environment and Sustainable Development (UNCED, 1992), generated Agenda 21 and the two Conventions on Climate Change and Biological Diversity, and fully recognized the need for scientifically based information and methods for management, and specifically addressed the major areas of concern mentioned above. This Manual, together with other IOC activities, is also to be seen as a direct follow-up to UNCED, and implementation of Agenda 21.

The IOC is highly appreciative of the efforts of the 40 scientists who prepared the present volume and wishes to express its particular thanks to Dr. Gustaaf M. Hallegraeff, Editor-in-Chief, and Drs. Donald M. Anderson and Alan. D. Cembella, Co-Editors, for their devoted involvement in this project.

The scientific opinions expressed in this work are those of the authors and are not necessarily those of UNESCO and its IOC. Equipment and materials have been cited as examples of those most currently used by the authors, and their inclusion does not imply that they should be considered as preferable to others available at that time or developed since.

Gunnar Kullenberg
Executive Secretary IOC

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Introduction

G.M. Hallegraeff

Research on Harmful Algal Blooms (HAB) first emerged as a discipline in its own right at the First International Conference on Toxic Dinoflagellate Blooms which was held in Boston, Massachusetts, from 4 to 6 November 1974 (LoCicero, 1974). Of major concern at that time was the massive 1972 New England red tide caused by the toxic dinoflagellate *Alexandrium (Gonyaulax) tamarense*. This successful meeting was followed by two further International Conferences on Toxic Dinoflagellate Blooms held in Miami, Florida, in 1978 (Taylor and Seliger, 1979) and in St Andrews, Canada in 1985 (Anderson *et al.*, 1985), respectively. The First International Symposium on Red Tides in 1987 at Takamatsu, Japan (Okaichi *et al.*, 1989) broadened its scope to include bloom events caused by other algal groups (notably raphidophytes), and this tradition was followed with the Fourth, Fifth, Sixth and Seventh International Conferences on Toxic Marine Phytoplankton held in Lund, Sweden, in 1989 (Graneli *et al.*, 1990), Newport, USA, in 1991 (Smayda and Shimizu, 1993), Nantes, France, in 1993 (Lassus *et al.*, 1995) and Sendai, Japan in 1995 [future conferences in this series have been scheduled for Vigo, Spain, in 1997 and Hobart, Australia, in 2000]. At the same time a number of smaller regional meetings were convened to deal with exceptional plankton blooms in European coastal waters (Parker and Tett, 1987), the impact of algal blooms on aquaculture (Dale *et al.*, 1987), unusual "brown tides" in Long Island, USA (Cosper *et al.*, 1989), tropical red tides of *Pyrodinium bahamense* in the Indo- West Pacific (White *et al.*, 1984; Hallegraeff and MacLean, 1989), newly recognised toxic diatom blooms in Canadian waters (Bates and Worms, 1989; Gordon 1990) and an outbreak of neurotoxic shellfish poisoning in New Zealand (Jasperse, 1993).

In 1989 the Fourth International Conference on Harmful Marine Phytoplankton reached a consensus "that some human activities may be involved in increasing the intensity and global distribution of blooms and recommended that international research efforts be undertaken to evaluate the possibility of global expansion of algal blooms and man's involvement in this phenomenon". Subsequently, a number of new international programmes were created to study and manage harmful algal blooms and their linkages to environmental changes in a manner consistent with the global nature of the phenomena involved. The creation of a Harmful Algal Bloom programme by the Intergovernmental Oceanographic Commission (IOC) of UNESCO has been one such initiative. With increasing international collaboration came the unavoidable need for a standardisation of methods. More importantly, it became clear that many developing countries had very limited access to outside literature and limited finances to buy books or to travel to overseas conferences. The First Session of the IOC-FAO Intergovernmental Panel on Harmful Algal Blooms (Paris, 23 - 25 June 1992) therefore agreed to support the creation and free distribution to developing countries of a Manual on Harmful Marine Microalgae, which compiles widely different information on the taxonomy, toxicology, and epidemiology of harmful algal blooms. As one of the early proponents of such a text, I accepted the responsibility as Chief Editor [both an honour and a punishment!], capably assisted by Dr Don Anderson and Dr Allan Cembella. Most of 1993 was spent in approaching potential authors and in seeking views on the precise contents of this Manual. Authors were specifically instructed to target an audience in developing countries, and therefore avoid unnecessarily sophisticated technologies.

In the choice of the title of this Manual on "Harmful Marine Microalgae", we decided to abandon the term "bloom" since some species such as *Chrysochromulina* and *Dinophysis* can cause serious problems even at moderate biomass levels. The term microalgae is used instead of "phytoplankton" in order to include problems caused by benthic species such as *Prorocentrum* and *Gambierdiscus*. A deliberate decision was made to omit problem growths of macroalgae.

While some mention is made of toxic freshwater cyanobacteria (blue-green algae), the focus of this manual is on marine microalgae. Following a general introduction on harmful algal blooms, the Manual proper is composed of four parts: Methods, Taxonomy, Monitoring and Management, and Resources. The Methods section covers oceanographic field sampling techniques, algal culture methods, cell counting, instrumental toxin analysis techniques for PSP, DSP, ASP, ciguatoxins, cyanobacterial toxins, as well as biochemical methods and mammalian bioassays for selected algal toxins. Cyst methodologies and methods of nutrient analysis, both macronutrients and trace metal micronutrients, are also included. The Taxonomy section starts with a general introduction on "what is a species", followed by detailed accounts on the taxonomy of dinoflagellates, haptophytes, diatoms, raphidophytes, cyanobacteria and cysts. The Monitoring and Management section covers environmental monitoring, management of shellfish resources, finfish aquaculture as well as epidemiology and public health. Finally, in the Resources section the reader will find a listing of algal culture collections, and addresses of international and regional agencies involved with harmful algal bloom studies. For some related topics the reader is also referred to the handbooks by Sournia (ed.) 1978. *Phytoplankton Manual.* UNESCO monograph no. 6 (available stock nearly exhausted); Parsons *et al.*, 1984, *A Manual of Chemical and Biological Methods for Seawater Analysis,* and the conference proceedings mentioned above. In addition to the 40 authors of the various Manual chapters, numerous other scientists contributed by reviewing chapters. These include Dr Don Anderson, Mr Peter Baker, Mr Chris Bolch, Mr Ray Brown, Dr Ed Butler, Dr Allan Cembella, Dr Wenche Eikrem, Dr Edna Graneli, Dr Gustaaf M. Hallegraeff, Dr Patrick Holligan, Dr Shirley Jeffrey, Dr Pheroze Jungalwalla, Dr Serge Maestrini, Dr Harvey Marchant, Mr Jay Maclean, Dr Andrew McMinn, Dr Øjvind Moestrup, Prof. T. Okaichi, Prof. Ted Smayda, Mr Colin E. Sumner, Dr Hideaki Takano, Dr Ewen C.D. Todd and Dr Tony Watson. Henrik Enevoldsen and Lene Düwel of the IOC Science and Communication Centre on Harmful Algae at the University of Copenhagen provided invaluable assistance in the preparation of the camera-ready manuscript. I hope that this Manual will help to alleviate economic hardships caused by harmful algal blooms, especially in developing countries, but also that it will foster international research collaboration on a fascinating biodiversity of microorganisms which already produced toxins hundreds of millions of years before humans turned to the oceans for aquaculture food production.

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1. Harmful algal blooms: A global overview

G.M. Hallegraeff

The microscopic planktonic algae of the world's oceans are critical food for filter-feeding bivalve shellfish (oysters, mussels, scallops, clams) as well as the larvae of commercially important crustaceans and finfish. In most cases, the proliferation of plankton algae (so-called "algal blooms"; up to millions of cells per litre) therefore is beneficial for aquaculture and wild fisheries operations. However, in some situations algal blooms can have a negative effect, causing severe economic losses to aquaculture, fisheries and tourism operations and having major environmental and human health impacts. Among the 5 000 species of extant marine phytoplankton (Sournia *et al.*, 1991), some 300 species can at times occur in such high numbers that they obviously discolour the surface of the sea (so-called "red tides"), while only 40 or so species have the capacity to produce potent toxins that can find their way through fish and shellfish to humans (Table 1.1).

It is believed that the first written reference (1 000 years B.C.) to a harmful algal bloom appears in the Bible: "... all the waters that were in the river were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river" (Exodus 7: 20–21). In this case, a non-toxic bloom-forming alga became so densely concentrated that it generated anoxic conditions resulting in indiscriminate kills of both fish and invertebrates. Oxygen depletion can be due to high respiration by the algae (at night or in dim light during the day) but more commonly is caused by bacterial respiration during decay of the bloom.

One of the first recorded fatal cases of human poisoning after eating shellfish contaminated with dinoflagellate toxins happened in 1793, when Captain George Vancouver and his crew landed in British Columbia in an area now known as Poison Cove. He noted that for local Indian tribes it was taboo to eat shellfish when the seawater became phosphorescent due to dinoflagellate blooms (Dale and Yentsch, 1978). The causative alkaloid toxins, now called paralytic shellfish poisons (PSP) (see Chapter 5), are so potent that a pinhead-size quantity (about 500 micrograms), which can easily accumulate in just one 100 gram serving of shellfish, could be fatal to humans. On a global scale, close to 2 000 cases of human poisoning (15% mortality) through fish or shellfish consumption are reported each year and, if not controlled, the economic damage through reduced local consumption and reduced export of seafood products can be considerable. Whales and porpoises can also become victims when they receive toxins through the food chain via contaminated zooplankton or fish (Geraci *et al.*, 1989). Poisoning of manatees by dinoflagellate brevetoxins contained in salps attached to seagrass (in Florida; Anderson and White, 1989), and of pelicans by diatom domoic acid contained in anchovies have also been reported (in California; Work *et al.*, 1993).

The third type of harmful algal bloom has become apparent only as a result of our increased interest in intensive aquaculture systems for finfish. Some algal species can seriously damage fish gills, either mechanically or through production of hemolytic substances. While wild fish stocks have the freedom to swim away from problem areas, caged fish appear to be extremely vulnerable to such noxious algal blooms. In 1972 in Japan, a bloom of the raphidophyte flagellate *Chattonella antiqua* thus killed 500 million US dollars worth of caged yellowtail fish in the Seto Island Sea (Okaichi, 1989).

Table 1.1 summarises the above three different types of harmful algal bloom problems, together with representative examples of causative algal species, ranging from dinoflagellates, diatoms, prymnesiophytes and raphidophytes to cyanobacteria. Clinical symptoms of various types of fish and shellfish poisoning are listed in Table 1.2 and the diversity of chemical structures of algal toxins is discussed in Chapters 5 to 9. Unfortunately, there is no clear cut correlation between algal concentrations and their potential harmful effects. Dinoflagellate

Table 1.1. Different types of harmful algal blooms

1. Species which produce basically harmless water discolourations; however, under exceptional conditions in sheltered bays, blooms can grow so dense that they cause indiscriminate kills of fish and invertebrates due to oxygen depletion. Examples: dinoflagellates *Gonyaulax polygramma, Noctiluca scintillans, Scrippsiella trochoidea,* cyanobacterium *Trichodesmium erythraeum.*

2. Species which produce potent toxins that can find their way through the food chain to humans, causing a variety of gastrointestinal and neurological illnesses, such as:
   - Paralytic Shellfish Poisoning (PSP)
     (Examples: dinoflagellates *Alexandrium acatenella, A. catenella, A. cohorticula, A. fundyense, A. fraterculus, A. minutum, A. tamarense, Gymnodinium catenatum, Pyrodinium bahamense* var. *compressum*)
   - Diarrhetic Shellfish Poisoning (DSP)
     (Examples:dinoflagellates *Dinophysis acuta, D. acuminata, D. fortii, D. norvegica, D. mitra, D. rotundata, Prorocentrum lima*)
   - Amnesic Shellfish Poisoning (ASP)
     (Examples: diatoms *Pseudo-nitzschia multiseries , P. pseudodelicatissima, P. australis*)
   - Ciguatera Fish Poisoning
     (Examples:dinoflagellate *Gambierdiscus toxicus, ? Ostreopsis* spp., *? Prorocentrum* spp.)
   - Neurotoxic Shellfish Poisoning (NSP)
     (Example:dinoflagellate *Gymnodinium breve* , *G.* cf. *breve* (New Zealand))
   - Cyanobacterial Toxin Poisoning
     (Examples: cyanobacteria *Anabaena circinalis , Microcystis aeruginosa, Nodularia spumigena*)

3. Species, which are non-toxic to humans, but harmful to fish and invertebrates (especially in intensive aquaculture systems) by damaging or clogging their gills. Examples: diatom *Chaetoceros convolutus,* dinoflagellate *Gymnodinium mikimotoi,* prymnesiophytes *Chrysochromulina polylepis , Prymnesium parvum, P. patelliferum ,* raphidophytes *Heterosigma carterae , Chattonella antiqua.*

Table 1.2. Clinical symptoms of various types of fish and shellfish poisoning

Paralytic Shellfish Poisoning (PSP)	Diarrhetic Shellfish Poisoning (DSP)	Amnesic Shellfish Poisoning (ASP)	Neurotoxic Shellfish Poisoning (NSP)	Ciguatera
Causative organism				
Alexandrium catenella;	*Dinophysis acuminata;*	*Pseudo-nitzschia multiseries;*	*Gymnodinium breve; G.* cf *breve (New Zealand)*	*Gambierdiscus toxicus;*
Alexandrium minutum;	*Dinophysis acuta;*	*Pseudo-nitzschia pseudodelicatissima;*		*? Ostreopsis siamensis;*
Alexandrium tamarense; Gymnodinium catenatum; Pyrodinium bahamense	*Dinophysis fortii; Dinophysis norvegica Prorocentrum lima*	*Pseudo-nitzschia australis*		*? Prorocentrum lima*
Symptoms				
Mild Case Within 30 min: tingling sensation or numbness around lips, gradually spreading to face and neck; prickly sensation in fingertips and toes; headache, dizziness, nausea, vomiting, diarrhoea.	After 30 min to a few hrs (seldom more than 12 hrs): diarrhoea, nausea, vomiting, abdominal pain.	After 3–5 hrs: nausea, vomiting, diarrhoea, abdominal cramps.	After 3-6 hrs: chills,headache, diarrhoea; muscle weakness, muscle and joint pain; nausea and vomiting	Symptoms develop within 12–24 hrs of eating fish. Gastro-intestinal symptoms: diarrhoea, abdominal pain, nausea, vomiting.
Extreme Case Muscular paralysis; pronounced respiratory difficulty; choking sensation; death through respiratory paralysis may occur within 2–24 hrs after ingestion.	Chronic exposure may promote tumor formation in the digestive system.	Decreased reaction to deep pain; dizziness, hallucinations, confusion; short-term memory loss; seizures.	Paraesthesia; altered perception of hot and cold; difficulty in breathing, double vision,trouble in talking and swallowing	Neurological symptoms: numbness and tingling of hands and feet; cold objects feel hot to touch; difficulty in balance; low heart rate and blood pressure; rashes. In extreme cases, death through respiratory failure.
Treatment				
Patient has stomach pumped and is given artificial respiration. No lasting effects.	Recovery after 3 days, irrespective of medical treatment.			No antitoxin or specific treatment is available. Neurological symptoms may last for months and years. Calcium and mannitol may help relieve symptoms.

species such as *Dinophysis* and *Alexandrium* can contaminate shellfish with toxins, even at very low cell concentrations. The prymnesiophyte *Chrysochromulina* produces only moderate biomass levels but has a very high toxic potency. Finally, the prymnesiophyte *Phaeocystis* is basically non-toxic but its nuisance value is caused by very high biomass levels.

GLOBAL INCREASE OF ALGAL BLOOMS

While harmful algal blooms, in a strict sense, are completely natural phenomena which have occurred throughout recorded history, in the past two decades the public health and economic impacts of such events appear to have increased in frequency, intensity and geographic distribution. One example, the increased global distribution of paralytic shellfish poisoning, is illustrated in Fig. 1.1. Until 1970, toxic dinoflagellate blooms of *Alexandrium (Gonyaulax) tamarense* and *Alexandrium (Gonyaulax) catenella* were only known from temperate waters of Europe, North America and Japan (Dale and Yentsch, 1978). By 1990, this phenomenon was well documented from throughout the Southern Hemisphere, in South Africa, Australia, New Zealand, India, Thailand, Brunei, Sabah, the Philippines and Papua New Guinea. Other species of the dinoflagellate genus *Alexandrium,* such as *A. cohorticula* and *A. minutum,* as well as the unrelated dinoflagellates *Gymnodinium catenatum* and *Pyrodinium bahamense* var. *compressum* have now also been implicated. Unfortunately, there are very few long term records of algal blooms at any single locality. Probably the best data set refers to the concentration of PSP toxins (μg saxitoxin equivalent / 100 g shellfish meat) in Bay of Fundy clams, which has been monitored by mouse bioassay since 1944 (White, 1987). Shellfish containing more than 80 μg PSP / 100 g shellfish meat are considered unfit for human consumption. Fig. 1.2 shows evidence for a cyclic pattern of toxicity at this site with increased frequency of toxic blooms in the late 1940s, early 1960s, in the late 1970s and early 1980s, and possibly beginning again in the mid 1990s (not shown). The importance of such long-term data sets is discussed in Chapter 21.

The issue of a global increase in harmful algal blooms has been a recurrent topic of discussion at all major conferences dealing with harmful algal blooms (Anderson, 1989; Hallegraeff, 1993; Smayda, 1990). Four explanations for this apparent increase of algal blooms have been proposed: increased scientific awareness of toxic species; increased utilisation of coastal waters for aquaculture; stimulation of plankton blooms by cultural eutrophication and / or unusual climatological conditions; and transport of dinoflagellate resting cysts either in ships' ballast water or associated with translocation of shellfish stocks from one area to another.

INCREASED SCIENTIFIC AWARENESS OF TOXIC SPECIES

Reports of harmful algal blooms, associated human illnesses or damage to aquaculture operations are receiving increased attention in newspapers, the electronic media and the scientific literature. Fig. 1.3 illustrates the doubling of annual literature on algal blooms every 2 to 2.5 years (Maclean, 1993). As a result, more and more researchers are now surveying their local waters for the causative organisms. Increased reports on the occurrence of dinoflagellates of the genus *Alexandrium* are a good example. Until 1988, the type species *A. minutum* was only known from Egypt (Halim, 1960); it has now been reported from Australia, Ireland, France, Spain, Portugal, Italy, Turkey, the east coast of North America, Thailand, New Zealand, Taiwan and Japan (Hallegraeff *et al.*, 1991; Yuki, 1994). Other examples are the recent description of the newly recognised phenomena of diarrhetic shellfish poisoning (since 1976) and amnesic shellfish poisoning (since 1987).

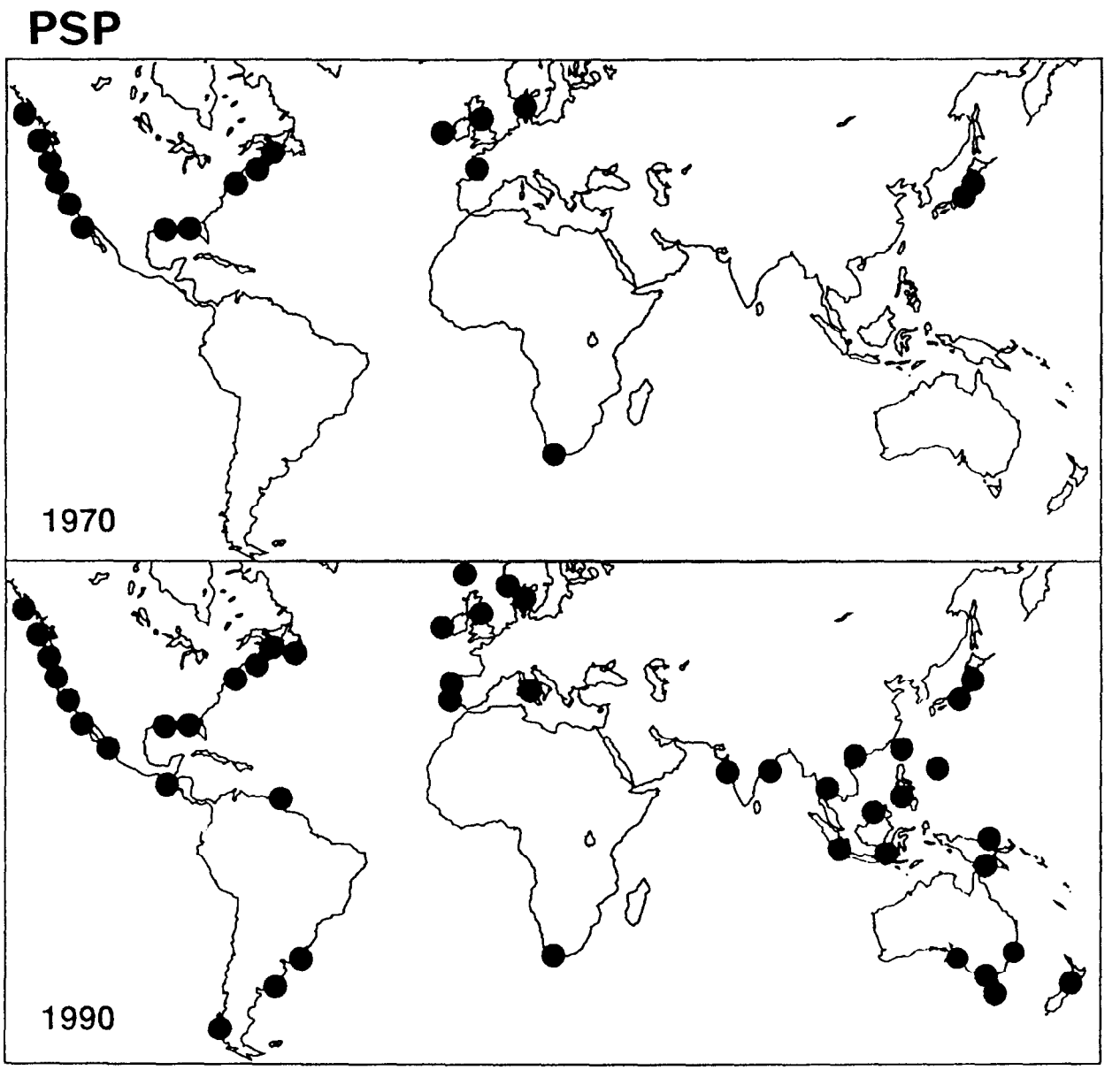

Figure 1.1. Known global distribution of paralytic shellfish poisoning (PSP) in 1970 and 1990 (From Hallegraeff, 1993).

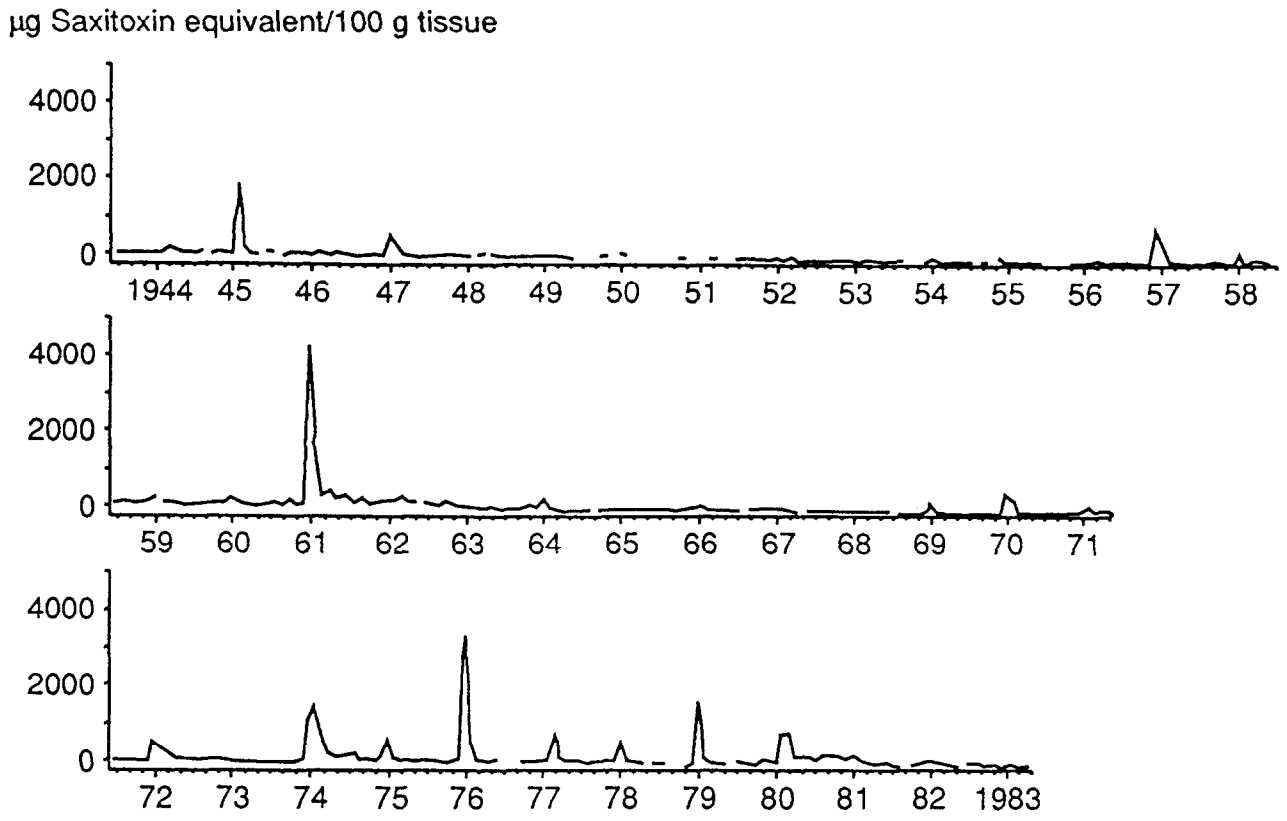

Figure 1.2. Concentration of PSP toxins in Bay of Fundy clams (µg saxitoxin equivalent/100 g tissue) in the period 1944–1983 (From White, 1987).

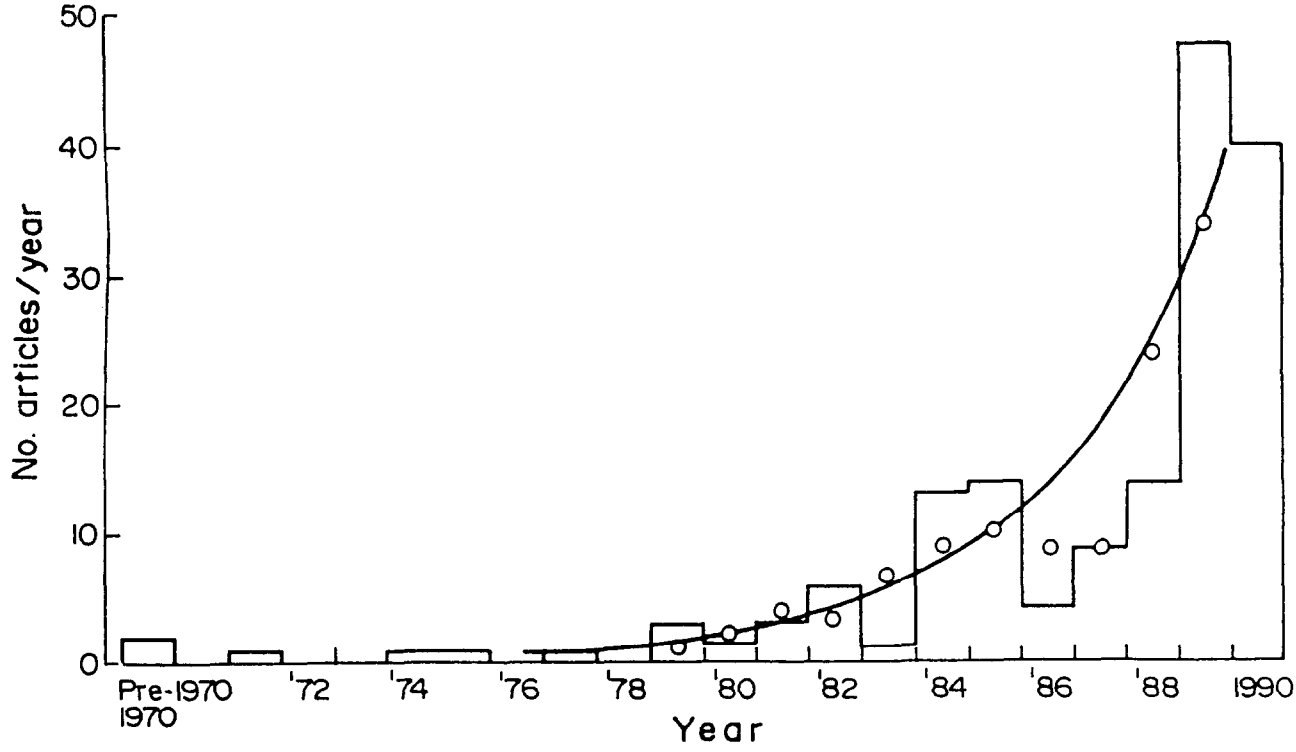

Figure 1.3. Growth of literature on harmful algal blooms, illustrated by analysing publication dates of the references used in the review by Maclean (1993).

Diarrhetic shellfish poisoning (DSP)

This phenomenon was first documented in 1976 from Japan where it caused major problems for the scallop fishery (Yasumoto *et al.*, 1978). The first dinoflagellate to be implicated was *Dinophysis fortii* (in Japan), soon followed by *D. acuminata* (in Europe), *D. acuta, D. norvegica* (in Scandinavia), *D. mitra, D. rotundata* and the benthic dinoflagellate *Prorocentrum lima*. Between 1976 and 1982, some 1 300 DSP cases were reported in Japan, in 1981 more than 5 000 cases were reported in Spain, and in 1983 some 3 300 cases were reported in France. In 1984 in Sweden, DSP problems caused a shutdown of the mussel industry for almost a year. The clinical symptoms of DSP (Table 1.2) may often have been mistaken for those of bacterial gastric infections and the problem may be much more widespread and serious than previously thought. Unlike PSP, no human fatalities have ever been reported and patients usually recover within three days. However, some of the polyether toxins involved (okadaic acid, dinophysis toxin-1; see Chapter 6) may promote stomach tumours (Suganuma *et al.*, 1988) and thus produce chronic problems in shellfish consumers. Shellfish containing more than 2 µg okadaic acid and/or 1.8 µg dinophysis toxin-1 per gram of hepatopancreas are considered unfit for human consumption (Lee *et al.*, 1987). The known global distribution of DSP (Fig. 1.4) includes Japan, Europe, Chile, Thailand, Canada (Nova Scotia) and possibly Tasmania (Australia) and New Zealand.

Amnesic shellfish poisoning (ASP)

This phenomenon was first recognised in 1987 in Prince Edward Island, Canada, where it caused 3 deaths and 105 cases of acute human poisoning following the consumption of blue mussels. The symptoms (Table 1.2) include abdominal cramps, vomiting, disorientation and memory loss (amnesia). Most unexpectedly, the causative toxin (the excitatory amino acid domoic acid; see Chapter 7) is produced by a diatom and not by a dinoflagellate. Shellfish containing more than 20 µg domoic acid per gram of shellfish meat are considered unfit for human consumption. The diatom species *Pseudo-nitzschia multiseries, P. pseudodelicatissima, P. australis (=N. pseudoseriata), P.seriata* and *P. delicatissima* have been implicated (Bates *et al.*, 1989; Garrison *et al.*, 1993; Martin *et al.*, 1990). To date, reports of domoic acid in seafood products have been mainly confined to North America (Bay of Fundy, California, Oregon, Washington, Alaska) and Canada (Prince Edward Island, British Columbia), while only insignificant concentrations have been detected in other parts of the world such as Europe, Australia, Japan and New Zealand (Fig. 1.5).

INCREASED UTILISATION OF COASTAL WATERS FOR AQUACULTURE

With increased problems of overfishing of coastal waters, more and more countries are looking towards aquaculture as an alternative. Indeed, some fisheries scientists predict that within the next 10–20 years, the increasing value of world aquaculture production may well approach the decreasing value of the total catch of wild fish and shellfish. Aquaculture operations act as sensitive "bioassay systems" for harmful algal species and can bring to light the presence in water bodies of problem organisms not known to exist there before. The increase in shellfish farming worldwide is leading to more reports of paralytic, diarrhetic, neurotoxic or amnesic shellfish poisoning. On the other hand, increased finfish culture is drawing attention to algal species which can cause damage to the fishes' delicate gill tissues.

In fish pens in British Columbia, deaths of lingcod, sockeye, coho, chinook and pink salmon have been caused by dense concentrations (5 000 cells per litre) of the diatoms *Chaetoceros convolutus* and *C. concavicorne*. The diatom's long hollow spines (setae) are studded with smaller barbs along their length. The setae can break off and penetrate the gill

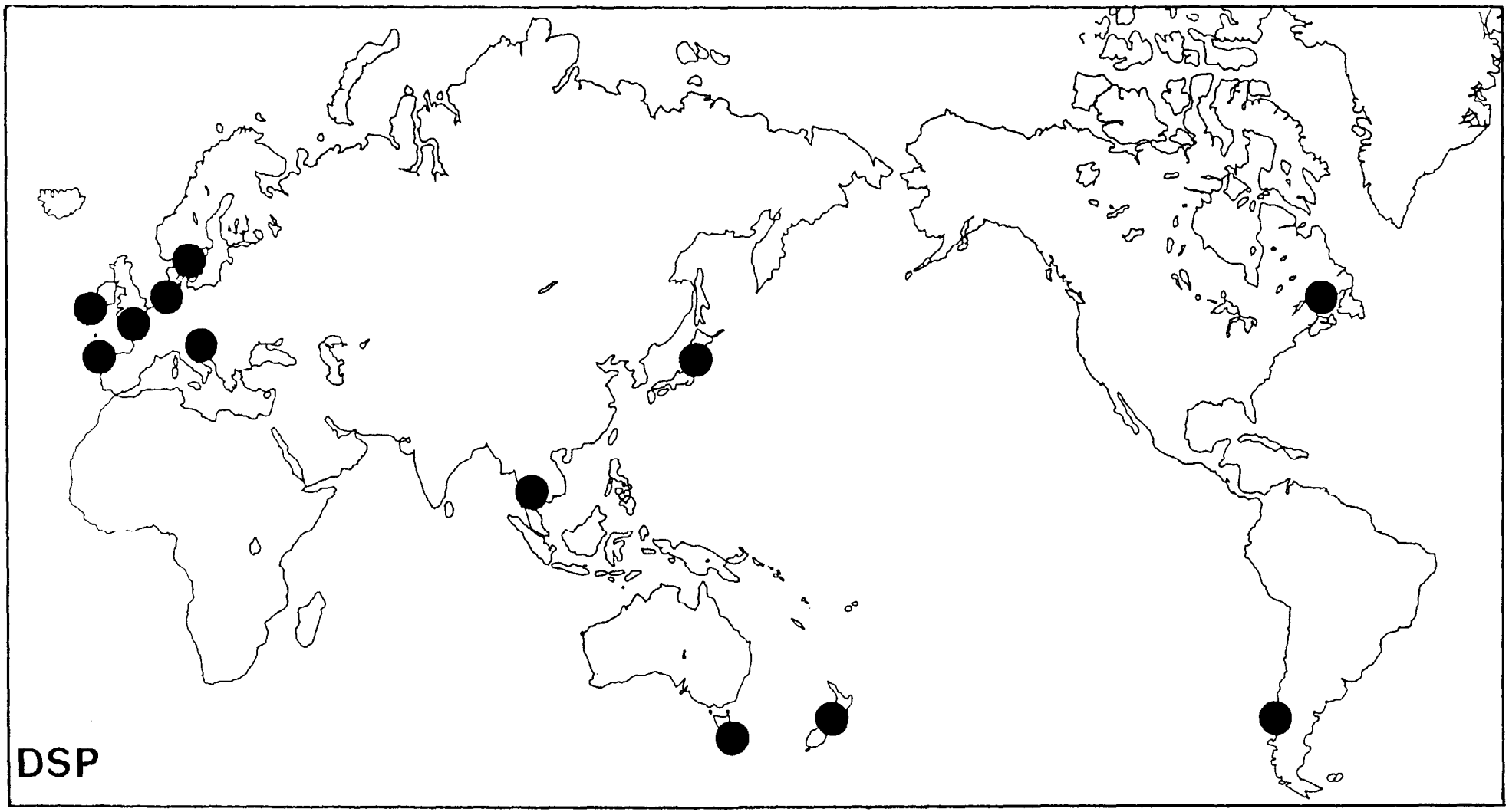

Figure 1.4. Global distribution of diarrhetic shellfish poisoning (DSP).

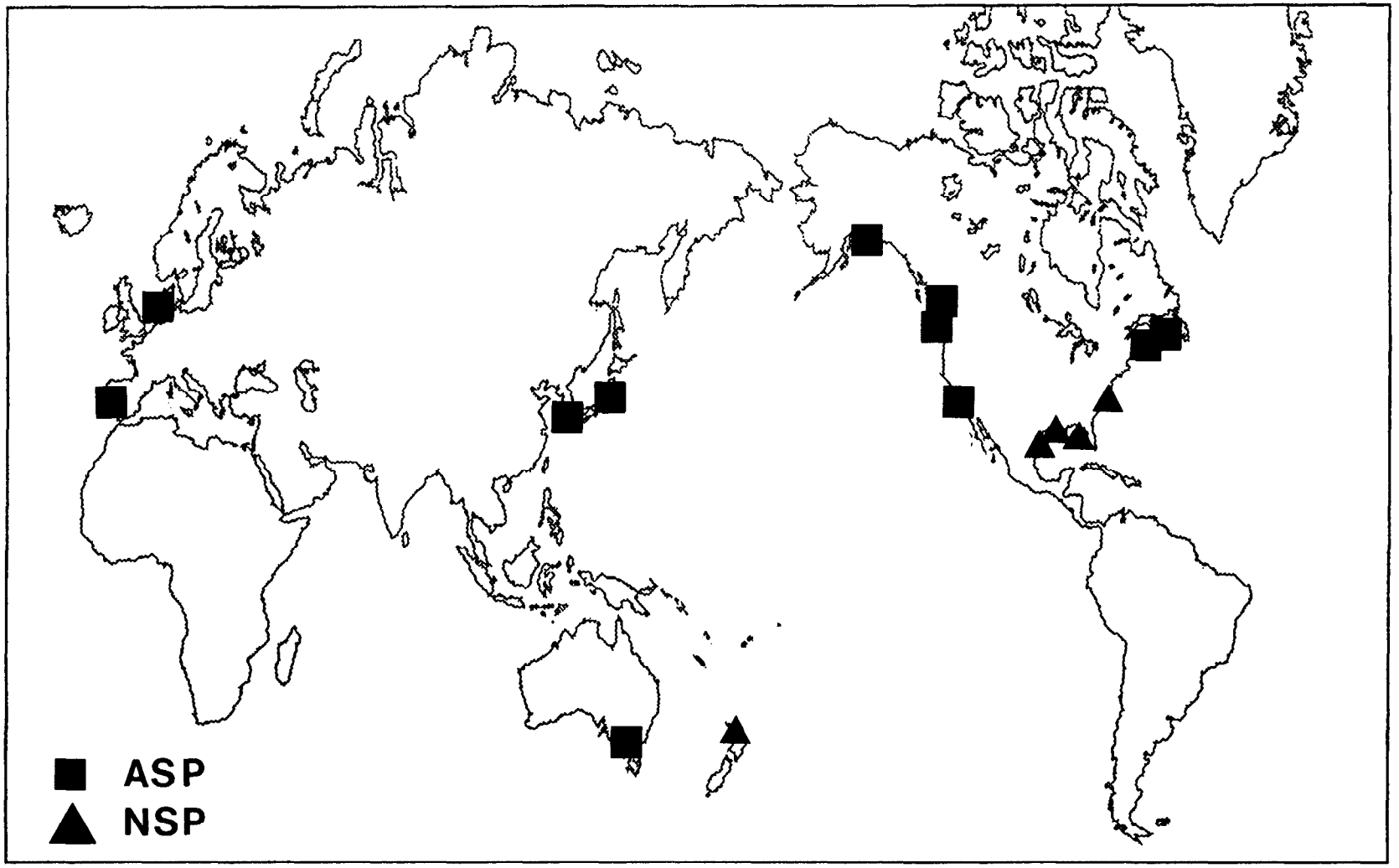

Figure 1.5 Global distrubution of amnesic shellfish poisoning (ASP) and neurotoxic shellfish poisoning (NSP).

membranes of fish, with the smaller barbs preventing them from coming out. Fish death may be caused by capillary haemorrhage, dysfunction of gas exchange at the gills, suffocation from an overproduction of mucus, or even from secondary infection of the damaged tissue (Bell, 1961; Rensel, 1993; Yang and Albright, 1992).

A more widespread problem for fish farmers is the production by various algal groups of fatty acids or galactolipids which damage the epithelial tissues of the gills. In experimental assay systems these substances destroy red blood cells and therefore have been provisionally termed "hemolysins" (Yasumoto *et al.*, 1990). Algal species as diverse as the raphidophytes *Heterosigma carterae (=H. akashiwo)* and *Chattonella antiqua* (see Chapter 18), the prymnesiophytes *Chrysochromulina polylepis* and *Prymnesium parvum* (see Chapter 16), and the dinoflagellate *Gymnodinium mikimotoi* (= *G. nagasakiense* = *?Gyrodinium aureolum;* see Chapter 15) have been implicated. *Heterosigma* has killed caged fish in Japan, Canada, Chile and New Zealand, while *Chattonella* is a fish killer confined mainly to Japan (Seto Inland Sea). With these two raphidophyte flagellates, physical clogging of gills by mucus excretion or gill damage by hemolytic substances or the production of oxygen radicals may be involved. In January 1989, a *Heterosigma* bloom in Big Glory Bay, Stuart Island (New Zealand) killed cage-reared chinook salmon worth 12 million NZ dollars (Chang *et al.*, 1990). The two prymnesiophyte flagellates *Chrysochromulina* and *Prymnesium* produce substances that affect gill permeability, which leads to a disturbed ion balance. Toxicity by these species is promoted by phosphorus deficiency. A massive bloom (60 000 km^2; 10^7 cells l^{-1}) of *Chrysochromulina polylepis* occurred in May–June 1988 in the Skagerrak, the Kattegat, the Belt and the Sound between Denmark, Norway and Sweden (Rosenberg *et al.*, 1988). Fish deaths occurred due to damage of gill membranes which produced a lethal increase in the chloride concentration in the blood, and fish cages moved into less saline fjords were therefore less affected. *Prymnesium parvum* has caused mortality of *Tilapia* fish in brackish water culture ponds in Israel (Shilo, 1981), as well as mortality of salmon and rainbow trout in net-pens in Norway. However, probably the greatest problem for Norwegian fish farms are blooms of the unarmoured dinoflagellate *Gyrodinium aureolum* (first reported in 1966) (Tangen, 1977). Similar dinoflagellates are common in Ireland and Scotland, as well as Japan and Korea (under the name *Gymnodinium nagasakiense* but now more appropriately called *G. mikimotoi).* Characteristic histopathological symptoms to fish are a severe necrosis and sloughing of epithelial tissues of the gills and digestive system (Roberts *et al.*, 1983).

Sophisticated monitoring systems using buoys with fibre optical sensors and data transfer by satellites (the MARINET system) are in place on the Norwegian coast to allow cages being towed away from bloom-affected areas. During the 1988 *Chrysochromulina* bloom, more than 26 000 tons of fish in 1 800 cages thus were moved from their permanent site into inland fjords. Fish losses in cages can also be reduced by stopping to feed fish since feeding attracts the fish to the surface and increases oxygen demand. In some cases, pumping of water to dilute the algal concentration, the administration to fish of mucolytic agents or immediate harvesting of marketable fish before they can be killed by algal blooms may also be an option. The hemolytic toxins do not accumulate in fish flesh. Virtually all algal blooms, even of non-toxic species, reduce the fishes' appetite and reduced oxygen concentrations stress the fish and make them more vulnerable to diseases (see Chapter 23).

Finally, an ichthyotoxic "phantom" dinoflagellate *Pfiesteria piscicida* was recognised in North Carolina in 1991 (Burkholder *et al.*, 1992). Its ephemeral presence (cysts germinate in the presence of life fish, and encyst again after fish death) may explain many mysterious fish kills along the southeast coast of the United States.

INCREASE OF ALGAL BLOOMS BY CULTURAL EUTROPHICATION

While some organisms such as the dinoflagellates *Gymnodinium breve, Alexandrium* and *Pyrodinium* appear to be unaffected by coastal nutrient enrichments, many other algal bloom

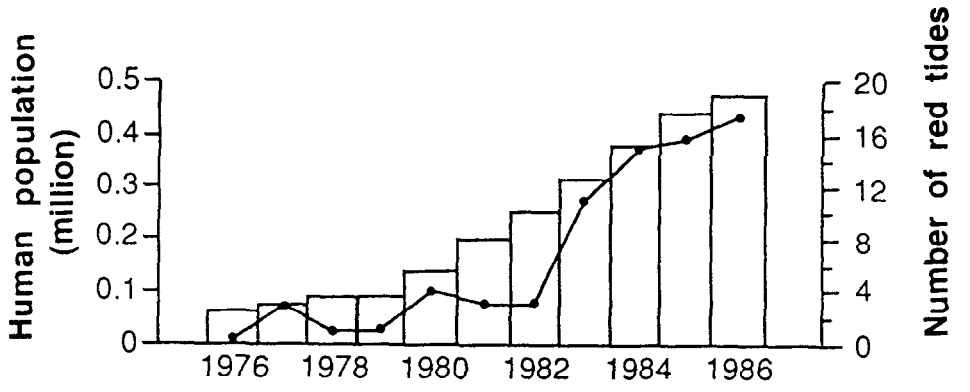

Figure 1.6. Correlation between the number of red-tide outbreaks per year in Tolo Harbour (continuous line) and the increase of human population in Hong Kong (bar diagram), in the period 1976 to 1986 (From Lam and Ho, 1989).

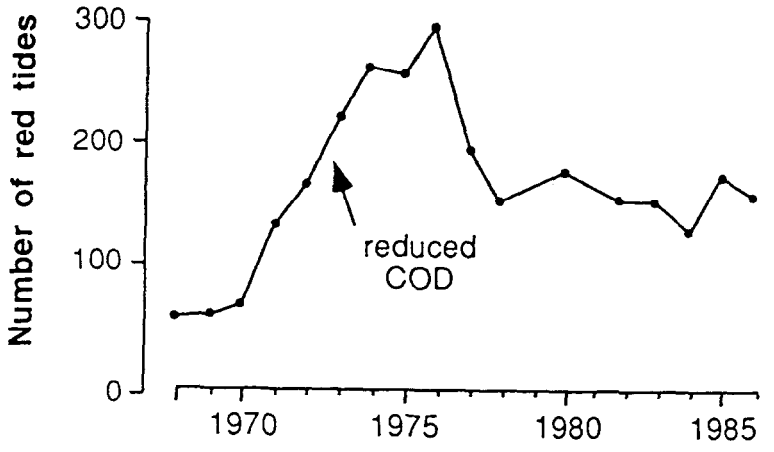

Figure 1.7. Long-term trend in the frequency of red-tide outbreaks in the Seto Inland Sea, Japan, in the period 1965–1986 (From Okaichi, 1990).

species appear to be stimulated by "cultural eutrophication" from domestic, industrial and agricultural wastes. Fig. 1.6 illustrates an 8-fold increase in the number of red tides per year in Hong Kong Harbour in the period 1976 to 1986 (Lam and Ho, 1989). This increase (mainly *Gymnodinium nagasakiense, Gonyaulax polygramma, Noctiluca scintillans* and *Prorocentrum minimum)* shows a striking relationship with the 6-fold increase in human population in Hong Kong and the concurrent 2.5–fold increase in nutrient loading, mainly contributed by untreated domestic and industrial waste. A similar experience was noted in the Seto Inland Sea, one of the major fish farm areas in Japan (Okaichi, 1989) (Fig. 1.7). Between 1965 and 1976 the number of confirmed red tide outbreaks (mainly *Chattonella antiqua*, since 1964; and *Gymnodinium nagasakiense*, since 1965) progressively increased 7-fold, concurrent with a 2-fold increase in the COD (chemical oxygen demand) loading, mainly from untreated sewage and industrial waste from pulp and paper factories. During the most severe outbreak in 1972, a *Chattonella* red tide killed 14 million cultured yellow-tail fish. Effluent controls were then initiated to reduce the chemical oxygen demand loading by about half, to introduce secondary sewage treatment, and to remove phosphate from house-hold detergents. Following a time-lag of 4 years, the frequency of red tide events in the Seto Inland Sea then decreased by about 2-fold to a more stationary level.

A similar pattern of a long-term increase in nutrient loading of coastal waters is evident for the North Sea in Europe (Smayda, 1990) (Fig. 1.8). Since 1955 the phosphate loading of the River Rhine has increased 7.5-fold, while nitrate levels have increased 3-fold. This has resulted in a significant 6-fold decline in the Si:P ratio, because long-term reactive silicate concentrations (a nutrient derived from natural land weathering) have remained constant. More recently, improved wastewater treatment has been causing decreases in the ammonia:nitrate ratio of River Rhine discharge (Riegman *et al.*, 1992). The nutrient composition of treated wastewater is never the same as that of the coastal waters in which it is being discharged. There is considerable concern (Officer and Ryther, 1980; Ryther and Dunstan, 1971; Smayda, 1990) that such altered nutrient ratios in coastal waters may favour blooms of nuisance flagellate species which replace the normal spring and autumn blooms of siliceous diatoms.

The remarkable increase of foam-producing blooms of the prymnesiophyte *Phaeocystis pouchetii,* which first appeared in Dutch coastal waters in 1978, is probably the best-studied example of this phenomenon (Lancelot *et al.*, 1987). The 1988 bloom in the Kattegat of the prymnesiophyte *Chrysochromulina polylepis*, not unusual in terms of biomass but unusual in terms of its species composition and toxicity, has been related to a change in the nutrient-status from nitrogen- to phosphorus-limitation (Maestrini and Graneli, 1991). As in Hong Kong and Japan, several North European countries have now agreed to reduce phosphate and nitrate discharges by 50% in the next several years, but their efforts will almost certainly be in vain if neighbours continue polluting. Furthermore, such indiscriminate reductions in nutrient discharges are not addressing the problem of changing nutrient ratios of coastal waters. Changed patterns of land use, such as deforestation, can also cause shifts in phytoplankton species composition by increasing the concentrations of humic substances in land run-off. Acid precipitation can further increase the mobility of humic substances and trace metals in soils. Experimental evidence from Sweden indicates that river water draining from agricultural soils (rich in N and P) stimulates diatom blooms but that river water draining from forest areas (rich in humic and fulvic acids) can stimulate dinoflagellate blooms of species such as *Prorocentrum minimum* (Graneli and Moreira, 1990). Agricultural run-off of phosphorus can also stimulate cyanobacterial blooms, for example of *Nodularia spumigena* in the Baltic Sea and in the Peel-Harvey Estuary, Australia (Fig. 1.9). These species produce hepatotoxic peptides (*Nodularia, Microcystis*) and neurotoxic alkaloids (*Anabaena, Aphanizomenon)*, which can kill domestic and wild animals that drink from the shores of eutrophic ponds, lakes and reservoirs (for example, during a 1 000 km long *Anabaena circinalis* bloom in the Darling River, Australia, in 1991). Toxicity problems from freshwater cyanobacteria have been documented from Australia, Bangladesh, China, Europe (12 countries), India, Israel, Japan, Latin America, North America, South Africa, Thailand and the USSR (Carmichael, 1989). The toxins can accumulate in the digestive system of shellfish (Falconer *et al.*, 1992) but contamination of drinking water with teratogens and tumour promoters is a more common public health risk. A neurotoxic factor has

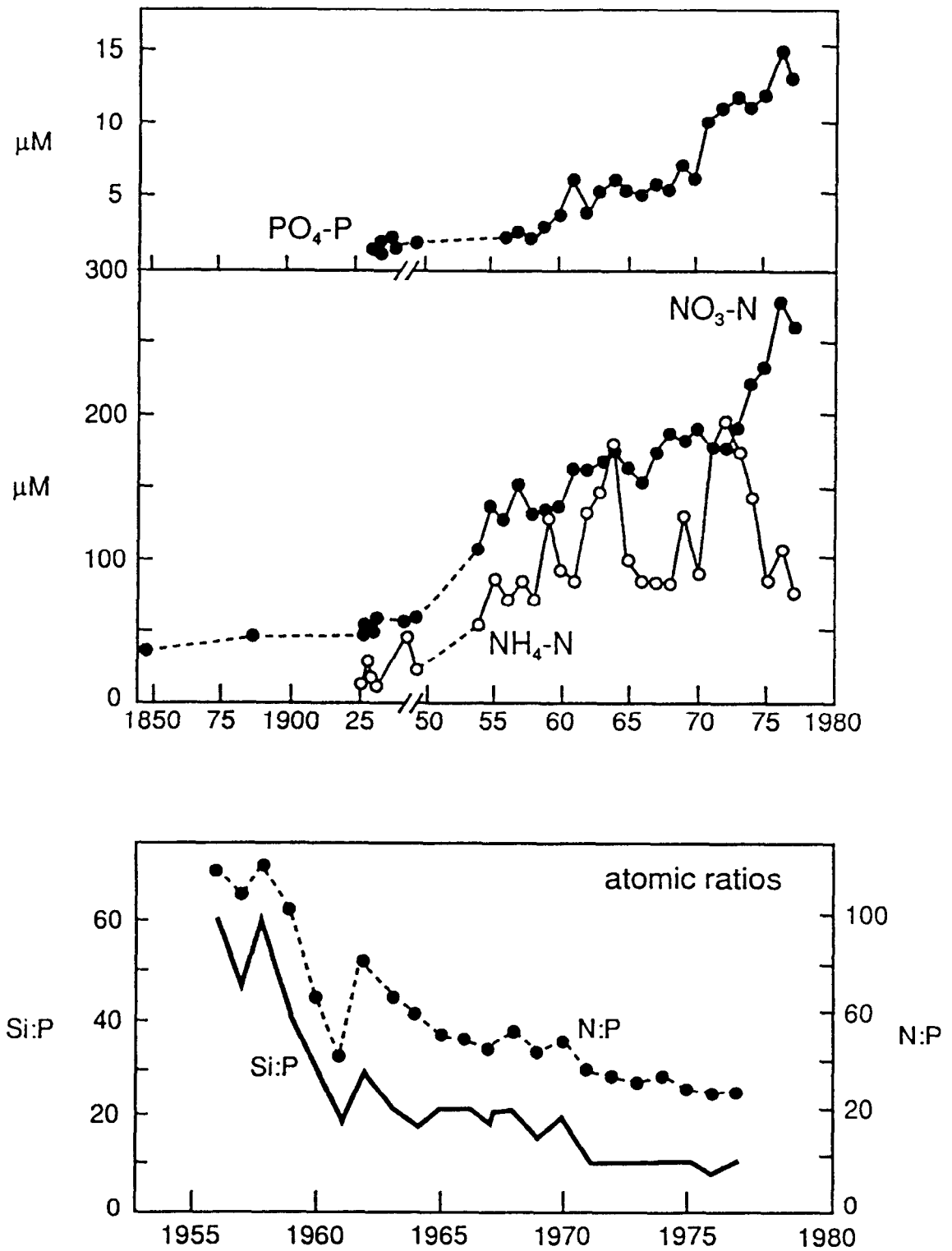

Figure 1.8. Long-term trend in the phosphate, nitrate and ammonia loading of the River Rhine (top) and concurrent changes in the N:P and Si:P nutrient ratios (bottom) (From Smayda 1990, using data by Van Bennekom & Salomons, 1981).

also been associated with some strains of the common marine, bloom-forming tropical cyanobacterium *Trichodesmium thiebautii* (Hawser *et al.*, 1991; see Chapter 9).

A much more complex "cultural eutrophication" scenario has emerged in coastal waters of New Jersey, New York and Rhode Island, USA, where an unusual "brown tide" (caused by the chrysophyte picoplankton *Aureococcus anophagefferens*) has been related to the discharge of chelators (such as citric acid) in detergents and lawn treatments, together with a suppression of zooplankton grazing by pesticides (Cosper *et al.*, 1989, 1991). This bloom was responsible for a reduction in the extent and biomass of eelgrass beds and caused starvation and recruitment failure in commercial scallop populations. A eutrophication problem like this cannot be diagnosed by routine monitoring programmes that focus on macronutrients or algal chlorophyll biomass alone (see Chapter 21).

Ciguatera fishfood poisoning and coral reef disturbance

Ciguatera is a tropical fishfood poisoning syndrome well-known from coral reef areas in the Caribbean, Australia, and especially French Polynesia. Humans consuming contaminated fish such as red bass, chinaman fish, moray eel, and paddle tail can suffer from gastro-intestinal and neurological illnesses and in extreme cases can die from respiratory failure (Table 1.2; Gillespie *et al.*, 1986). The causative organisms are benthic dinoflagellates such as *Gambierdiscus toxicus*, and possibly *Ostreopsis siamensis, Coolia monotis, Prorocentrum lima* and related species, that live in epiphytic association with bushy red, brown and green seaweeds (up to 200 000 cells / 100 g of algae) and also occur free in sediments and coral rubble. These dinoflagellates produce the potent neurotoxins gambiertoxin and maitotoxin (see Chapter 8), which accumulate through the food chain, from small fish grazing on the coral reefs into the organs of bigger fish that feed on them (the principal toxin fraction in fish is ciguatoxin). While in a strict sense this is a completely natural phenomenon (Captain Cook suffered from this illness when visiting New Caledonia in 1774), from being a rare disease two centuries ago ciguatera now has reached epidemic proportions in French Polynesia. In the period 1960 to 1984 more than 24 000 patients were reported from this area, which is more than six times the average for the Pacific as a whole. Evidence is accumulating that reef disturbance by hurricanes, military and tourist developments is increasing the risk of ciguatera by increasing benthic substrate for dinoflagellate growth (Bagnis *et al.*, 1985).

STIMULATION OF ALGAL BLOOMS BY UNUSUAL CLIMATOLOGICAL CONDITIONS

Toxic Pyrodinium bahamense blooms in the tropical Indo-West Pacific

At present the dinoflagellate *Pyrodinium bahamense* is confined to tropical, mangrove-fringed coastal waters of the Atlantic and Indo-West Pacific. A survey of fossil occurrences of its resting cyst *Polysphaeridinium zoharyi* (Fig. 1.10) (records go back to the Eocene, 50 million yrs ago) indicates a much wider range of distribution in the past. For example, in the Australasian region at present the dinoflagellate does not extend further south than Papua New Guinea, but in the Pleistocene it ranged as far south as Sydney Harbour (McMinn, 1989). There is genuine concern that with increased greenhouse effect and warming of the oceans, this species may return to Australian waters. In the tropical Atlantic, in areas such as Bahia Fosforescente in Puerto Rico and Oyster Bay in Jamaica, this species forms persistent luminescent blooms which are a major tourist attraction. Both plankton bloom material and oysters and mussels attached to mangrove roots in Bahia Fosforescente appeared to be non-toxic (Hallegraeff; Oshima, unpublished data). The first harmful implications of *Pyrodinium* blooms became evident in 1972 in Papua New Guinea. Red-brown water discolourations

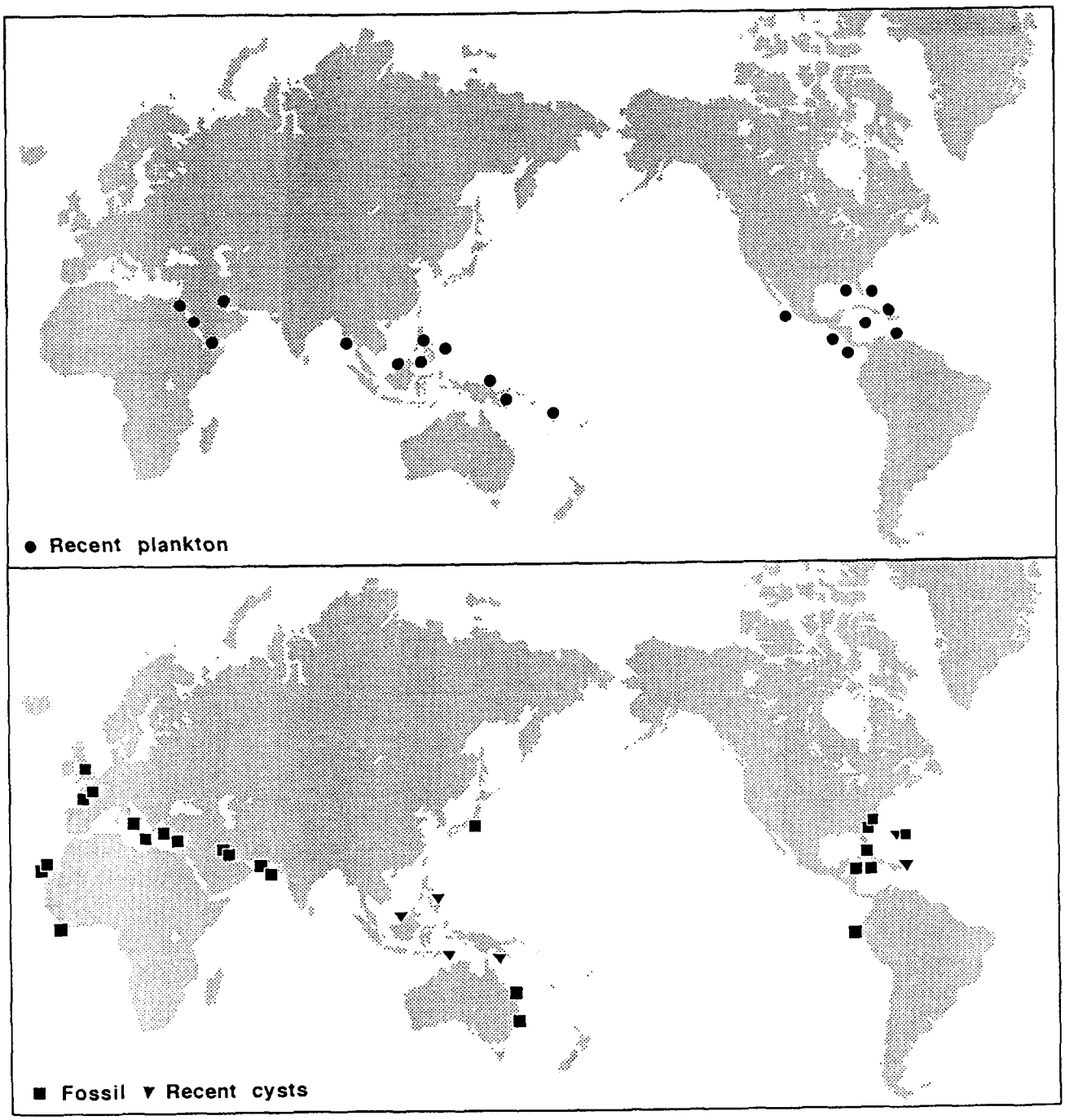

Figure 1.10. Global distribution of the tropical dinoflagellate *Pyrodinium bahamense* in Recent plankton (top) and of the fossil cyst *Polysphaeridium zoharyi* (bottom) (From Hallegraeff & Maclean, 1989).

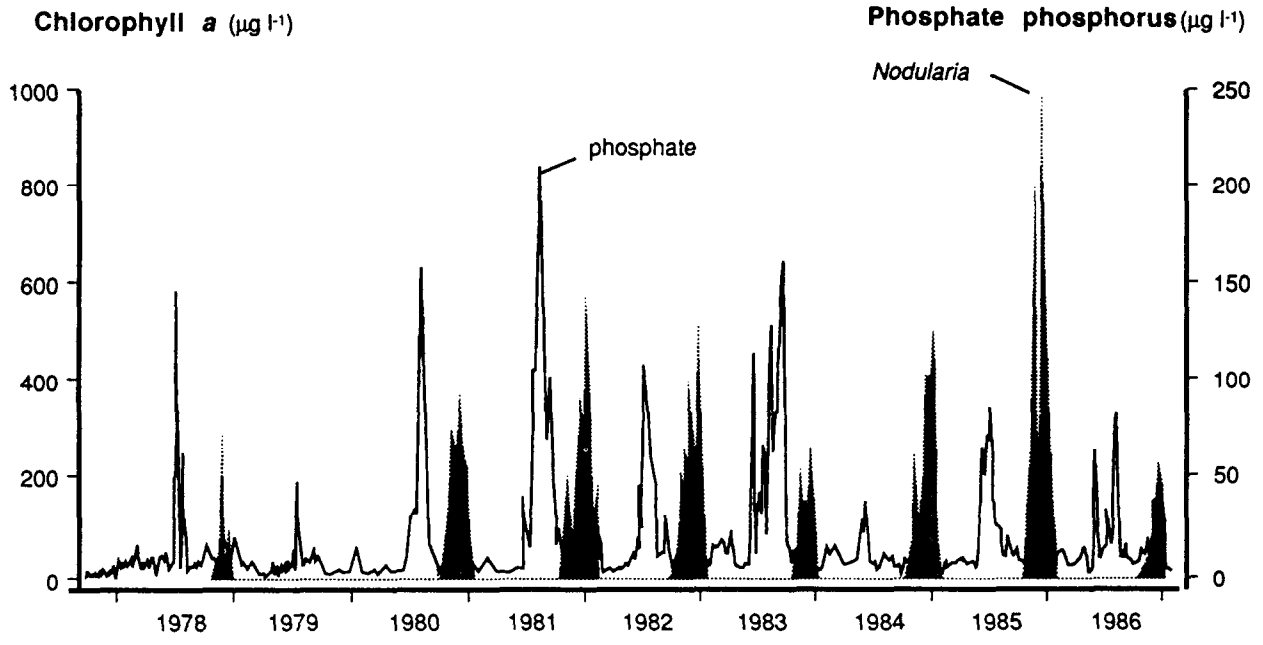

Figure 1.9. Relationship between *Nodularia spumigena* cyanobacterial blooms (as chlorophyll concentration) in the Peel-Harvey Estuary, Australia, and its relationship to riverine phosphate loading from agricultural run-off (From Hillman *et al.* , 1990).

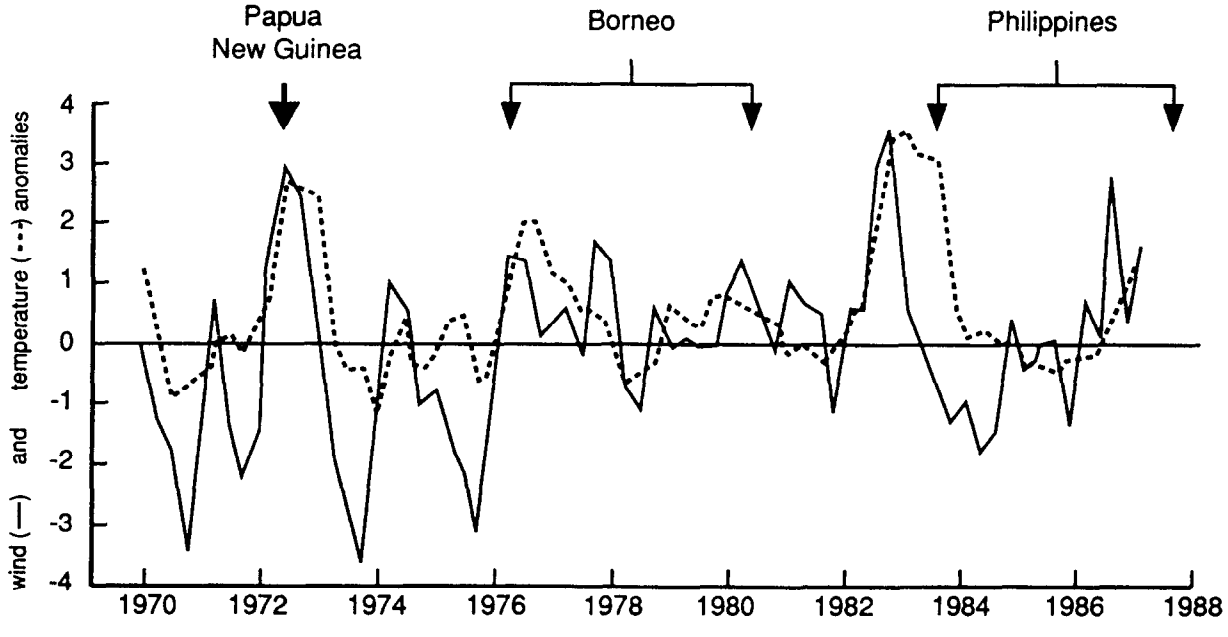

Figure 1.11. Relationship between *Pyrodinium bahamense* blooms (arrows) in the tropical Indo-West Pacific and El Nino–Southern Oscillation (ENSO) climatological events in the period 1970–1988. The graph shows empirical orthogonal functions of zonal wind anomalies and zonal mean surface temperature anomalies over the near-equatorial eastern Indian and western Pacific Oceans. Strong positive anomalies are indicative of ENSO events (From Maclean, 1989).

coincided with the fatal food poisoning of three children, and mouse bioassays on shellfish from a house in the affected village subsequently established *Pyrodinium bahamense* as a source of paralytic shellfish poisons (Maclean, 1977). Since then, toxic *Pyrodinium* blooms have apparently spread to Brunei and Sabah (1976), the central Philippines (1983), the northern Philippines (1987) and Indonesia (North Mollucas). Maclean (1989) presented strong circumstantial evidence for a coincidence between *Pyrodinium* blooms and El Nino-Southern Oscillation (ENSO) climatological events. El Nino is caused by an imbalance in atmospheric pressure and sea temperature between the eastern and western parts of the Pacific Ocean and results in a shoaling of the thermocline. The 1991-1994 ENSO event and recurrence of dinoflagellate blooms in the Philippines tend to substantiate these claims (Fig. 1.11).

Pyrodinium is a serious public health and economic problem for the tropical countries that are affected, since they depend heavily on seafoods for protein and have little prior experience in toxic dinoflagellate research. In the Philippines alone, this organism has now been responsible for more than 1 500 human illnesses and 100 fatalities resulting from the consumption of contaminated shellfish as well as planktivorous fish such as sardines and anchovies. Most unexpectedly, during a *Pyrodinium* bloom in 1987 on the Pacific coast of Guatemala 187 people had to be hospitalised and 26 persons died. In 1989 another bloom swept northward along the Pacific coast of Central America, again causing illness and death. The Guatemala populations are morphologically more similar to the Indo-West Pacific populations (sometimes distinguished as a separate variety *compressum)* than to the Caribbean morphospecies (var. *bahamense*) (Rosales-Loessener *et al.*, 1989).

Neurotoxic shellfish poisoning in Florida and New Zealand

Until recently, neurotoxic shellfish poisoning (NSP; Fig. 1.4, Table 1.2), caused by polyether brevetoxins produced by the unarmoured dinoflagellate *Gymnodinium breve*, was considered to be endemic to the Gulf of Mexico and the east coast of Florida, where "red tides" had been reported as early as 1844. An unusual feature of this organism is the formation by wave action of toxic aerosols which can lead to respiratory asthma-like symptoms in humans. In 1987 a major Florida bloom event was dispersed by the Gulf Stream northward into North Carolina waters, where it has since continued to be present (Tester *et al.*, 1991). Unexpectedly, in early 1993 more than 180 human shellfish poisonings were reported from New Zealand, caused by an organism similar (but not identical) to *Gymnodinium breve* (Jasperse *et al.*, 1993). Most likely, this was a member of the hidden plankton flora (previously present in low concentrations), which developed into bloom proportions triggered by unusual climatological conditions (higher than usual rainfall, lower than usual temperature) coinciding with an El Nino event.

Fossil blooms of Gymnodinium catenatum in the Kattegat-Skagerrak

The present-day distribution of the paralytic shellfish poison-producing dinoflagellate *Gymnodinium catenatum* includes the Gulf of California, Gulf of Mexico, Venezuela, Argentina, Japan, Korea, China, the Philippines, Palau, Tasmania (Australia), the Mediterranean and the Atlantic coast of Spain, Portugal and Morocco (Blackburn *et al.*, 1989). This species has never been recorded in the living phytoplankton of the Kattegat-Skagerrak region of Scandinavia, even though a few living cysts have recently been detected in bottom sediments from the Danish coast and German Bight (Ellegaard *et al.*, 1993; Nehring, 1994). Fossil cysts of this species were present in unusually large amounts in pollen records from Kattegat sediments (Nordberg and Bergsten, 1988). A multi-disciplinary study (Dale and Nordberg, 1993) to reconstruct the prevailing paleoenvironment has suggested the following scenario: the migration of *G. catenatum* into the area about 5 000 yrs B.P.; its establishment as part of the local plankton; a major blooming phase about 2 000 to 500 yrs B.P. of a magnitude that has not been seen since; and its disappearance during the "Little Ice Age".

TRANSPORT OF DINOFLAGELLATE CYSTS IN SHIPS' BALLAST WATER OR ASSOCIATED WITH THE TRANSLOCATION OF SHELLFISH STOCKS.

Cargo vessel ballast water was first suggested as a vector in the dispersal of non-indigenous marine plankton some 90 years ago. However, in the 1980s the problem of ballast water transport of plankton species gained considerable interest when evidence was brought forward that non-indigenous toxic dinoflagellate species had been introduced into Australian waters into sensitive aquaculture areas, with disastrous consequences for commercial shellfish farm operations (Hallegraeff and Bolch, 1992). While the planktonic stages of diatoms and dinoflagellates show only limited survival during the voyage in dark ballast tanks (Rigby and Hallegraeff, 1994), their resistant resting spores are well suited to survive these conditions. One single ballast tank thus was estimated to contain more than 300 million toxic dinoflagellate cysts which could be germinated into confirmed toxic cultures. Paralytic shellfish poisoning was unknown from the Australian region until the 1980s when the first outbreaks appeared in the ports of Hobart (*Gymnodinium catenatum*), Melbourne (*Alexandrium catenella)* and Adelaide (*A. minutum).* In Hobart, Tasmania, an examination of historic plankton samples, cyst surveys in dated sediment depth cores (McMinn *et al.*, unpublished) provided strong circumstantial evidence that the toxic dinoflagellate *G. catenatum* was introduced after 1973. Furthermore, in Melbourne and Adelaide, genetic fingerprinting using rRNA sequencing provided circumstantial evidence for the genetic affinities between Australian and Japanese strains of *A.catenella* and Australian and European strains of *A. minutum* (Scholin *et al.*, 1993).

The evidence of ballast water transfer of marine organisms other than microscopic algae is considerable and includes species of seaweeds, fish, crustaceans, polychaete worms, starfish and molluscs (Carlton, 1985). As of 1 Nov 1991 the International Maritime Organisation (IMO) has ratified the introduction of voluntary guidelines for ballast water handling procedures by bulk cargo vessels. These measures aim to reduce the risk of harmful introductions by encouraging a range of practices such as reballasting at sea (only feasible for vessels up to 40 000 dead weight tonnage), ballasting in deep water, and disposal of ballast tank sediments away from sensitive aquaculture or marine park areas. The most effective measure to prevent the spreading of dinoflagellate cysts via ships' ballast water would be to avoid ballasting during toxic dinoflagellate blooms in ports. Other options using heat, electrical shock or chemical treatment (chlorine, hydrogen peroxide) of ballast water, either in hold or in onshore facilities, are now also being explored (Bolch and Hallegraeff, 1993).

Another vector for the dispersal of algae (especially their resting cysts) is with the translocation of shellfish stocks from one area to another. The faeces and digestive tracts of bivalves can be loaded with viable dinoflagellate cells and sometimes can also contain resistant resting cysts (Scarratt *et al.*, 1993; Schwinghamer *et al.*, 1994). The Japanese seaweeds *Sargassum muticum* (England, Netherlands, Norway), *Undaria pinnatifida* and *Laminaria japonica* (Mediterranean) thus are thought to have been introduced into European waters via sporophyte stages contained with introduced Japanese oyster spat.

CONCLUSIONS

The question of whether the apparent global increase in harmful algal blooms represents a real increase or not, we probably will not be able to conclusively answer for some time to come.There is no doubt that our increasing interest in utilising coastal waters for aquaculture is leading to an increased awareness of toxic algal species. There are strong similarities between this debate and the debate on the issue of human health risks associated with cigarette smoking. In that case increased environmental and public health awareness forced medical researchers to refine their diagnostic tools, and once unambiguous scientific evidence was produced, this led to a worldwide turn-about in social attitudes towards smoking-free zones in airplanes and

government buildings. Similarly, what we are faced with today in the field of harmful algal bloom research is that the effects on public health and economic impacts of harmful algal blooms are now showing signs of a truly global "epidemic", and we should start to respond to this problem as such. In countries which pride themselves on their disease- and pollution-free status for aquaculture, every effort should be made to quarantine sensitive aquaculture areas against the unintentional introduction of non-indigenous harmful algal species.

Most importantly, people responsible for management decisions on pollutant loadings of coastal waters (including decisions on agricultural and deforestation activities in catchment areas) should be made aware that one probable outcome of increased nutrient loading will be an increase in harmful algal blooms. Finally, global climate change studies (El Nino, greenhouse, ozone depletion) need to consider possible impacts on algal bloom events. A number of new international programmes are now being created to study and manage harmful algal blooms and their linkages to environmental changes in a manner consistent with the global nature of the phenomena involved (see Chapter 26). It is hoped that this Manual on Harmful Marine Microalgae will facilitate these international efforts.

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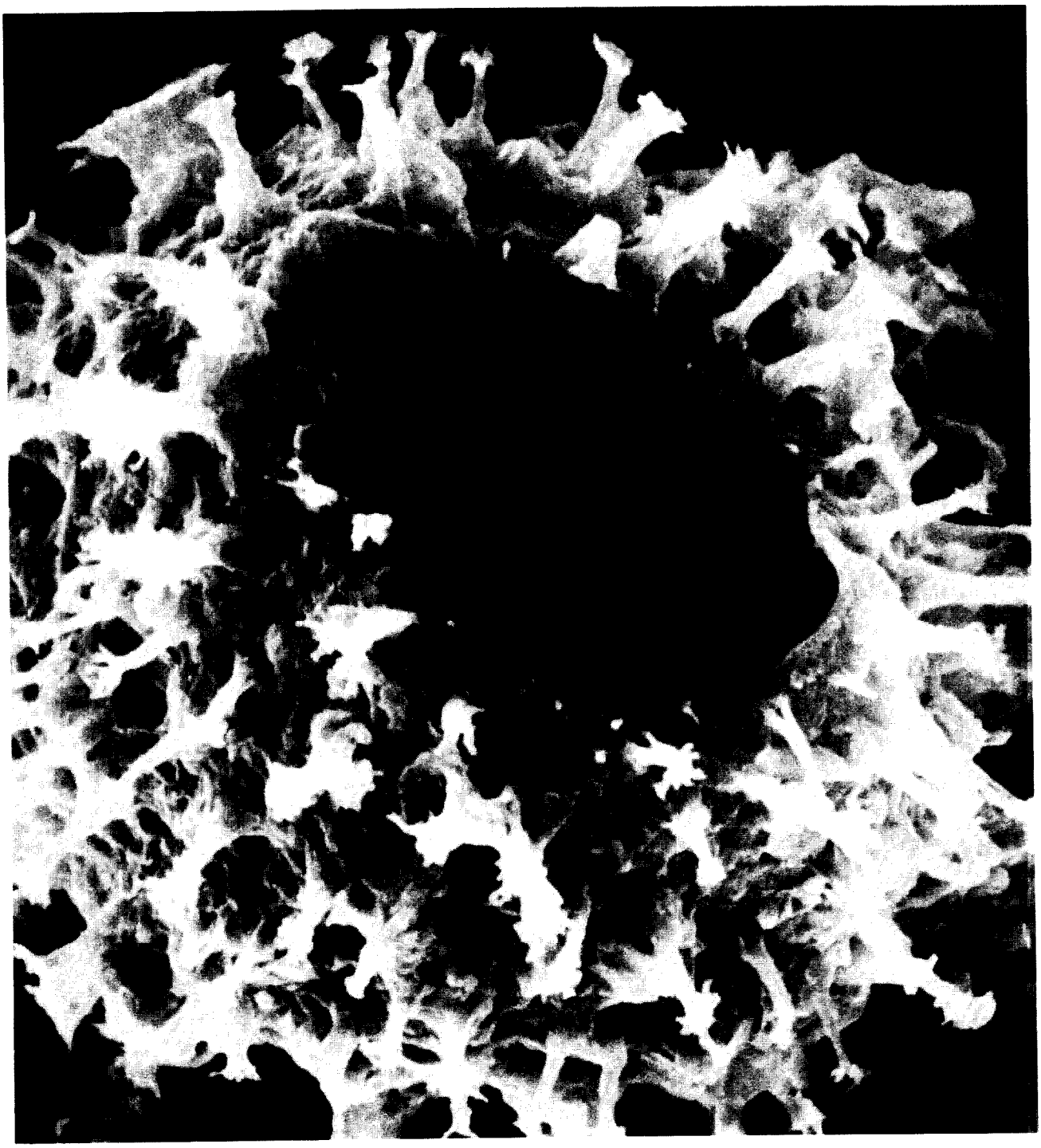
Part I

Methods



2. Sampling Techniques and Strategies for Coastal Phytoplankton Blooms

P.J.S. Franks

The primary goal of sampling for coastal toxic phytoplankton is to gain some measure of predictive capability concerning the initiation of local outbreaks. A fundamental question is whether the bloom formed *in situ,* or was advected to the region. In regions of weak physical forcing, for example embayments or lagoons with weak tidal flushing, it is likely that the bloom formed *in situ.* Prediction of toxic outbreaks in this case would depend on a thorough knowledge of the physiology, life cycle and behaviour of the phytoplankter, and the relationship of these responses to environmental forcings such as light or nutrient loading. Most coastal areas, however, are strongly influenced by physical forcings, making identification of transport mechanisms of paramount importance in predicting the timing and location of toxic outbreaks. This chapter describes sampling equipment and strategies for identifying the local causes of toxic phytoplankton blooms, and suggests techniques that may aid in gaining some predictive capability for harmful algal blooms in coastal regions.

The fundamental key to developing predictive capability for toxic outbreaks is a well-designed field sampling program. Designing a field program includes consideration of both spatial and temporal domains, with one basic point: *the sampling must begin before the bloom occurs.* There is no way to accurately identify the proximate cause of a bloom after it has begun, unless historical data are available. This point will constrain all other aspects of the sampling scheme, including spatial arrangement of sampling stations, and timing of cruises. These points will be expanded upon below, after a brief description of the equipment commonly used to sample for coastal phytoplankton. Much of the material presented here is taken from Franks and Anderson (1989). Significant typographical errors during publication of that paper rendered some of the sections unintelligible, and these have been corrected in the present contribution. Additional material has been added to expand on new techniques and new insights derived from recent studies of harmful algal blooms.

SAMPLING EQUIPMENT

Secondary in importance to the timing and spatial arrangement of stations is the equipment used to perform the sampling. This equipment can range from the very simple, e.g. nets, to relatively complex, e.g. hose-pumping systems or rosette samplers. Much of the equipment described here is more thoroughly presented in the *UNESCO Phytoplankton manual* (Sournia, 1978). In spite of its age, the information in that volume is still very pertinent today.

Nets

The most basic piece of equipment for sampling coastal phytoplankton is a net. While nets should not be used for quantitative estimation of cell numbers, and cannot be used to determine vertical distributions of cells, they are very useful for gathering qualitative presence/absence information. The main consideration when using a net is the mesh-size relative to the species of phytoplankton being sampled. Too large a mesh will eliminate small species from the samples. Other aspects of net sampling are well covered by Tangen (1978) in the above *UNESCO Phytoplankton manual.*

Tube samplers

Second to the net in simplicity are the tube and segmented tube samplers. The tube sampler (Franks and Anderson, 1989; Fig. 2.1a) is suitable for obtaining integrated samples of shallow (<5 m deep) water columns, or integrated samples from the surface waters of deeper regions. It consists of a 2 - 3 m length of PVC pipe, fitted with a cork attached to a line threaded through the pipe to a handle at the top. A short foot at the bottom keeps the sampler from being pushed into the benthos. The sampler is slid vertically into the water until the foot rests on the bottom. Once the depth to the bottom is known, the tube is raised and moved to a new location nearby, and lowered until the foot is just off the bottom. This procedure prevents the water column samples from being contaminated with resuspended material. The line is then pulled to seal the tube with the cork. The tube is emptied by pouring from the top, since pouring from the bottom causes spillage around the cork. The integrated vertical sample can be poured into a carboy or bucket for further subsampling (see below). The advantages of this type of sampler are: 1) low cost; 2) ease of construction; 3) one sample integrates any vertical heterogeneity of the organism; and 4) ease of deployment. The disadvantages include: 1) no vertical resolution; 2) relatively small volume; and 3) no real-time (i.e. instantaneous) vertical information.

In shallow areas, and relatively calm seas, the segmented tube sampler described by Lindahl (1986) and Sutherland *et al.* (1992) may be the most appropriate sampling device. This low-cost sampler consists of lengths of PVC pipe or garden hose, linked with valves and easily-separated connectors (Fig. 2.1b). The length of a section determines the vertical resolution of sampling, typically 1 - 3 m, with a total length of up to 20 m. The sampler is slowly lowered with all valves open until the hose is filled. The top valve is then closed, and the hose raised until the next valve down can be closed. The upper section can now be removed, and its water drained. This procedure can be repeated until the whole length of hose has been raised. Hydrostatic forces within the hose will hold the water within the hose while it is being raised. Thus a small-volume vertical profile of the water column is obtained. The advantages of this sampler are similar to those of the tube sampler described above, although vertical resolution is obtained. The disadvantages are: 1) small volume; 2) no real-time vertical information; and 3) smearing of vertical structure within the pipe due to its narrow diameter.

Bottles and rosettes

Approximately half of all studies published in the last three years in the main oceanographic journals, which examined the distribution of noxious phytoplankton species, relied on bottles for obtaining samples. The reason for this is that bottles are relatively inexpensive, they are very robust and can be deployed from almost any vessel, and they give an accurate quantitative representation of species densities within the depth sampled. Several bottles suspended from a line, or bottles in a single rosette formation, can give adequate vertical spatial coverage for most studies. Again, a good description of the types of bottles and their use can be found in Venrick (1978) in the *UNESCO Phytoplankton manual.* Bottles are especially recommended for use in heavy seas, as they are easy to deploy, and have no components to tangle or kink as with pumping systems. Some studies (e.g. Legovic *et al.*, 1991) have used bottles deployed by divers to obtain samples from visible strata of dinoflagellate accumulations.

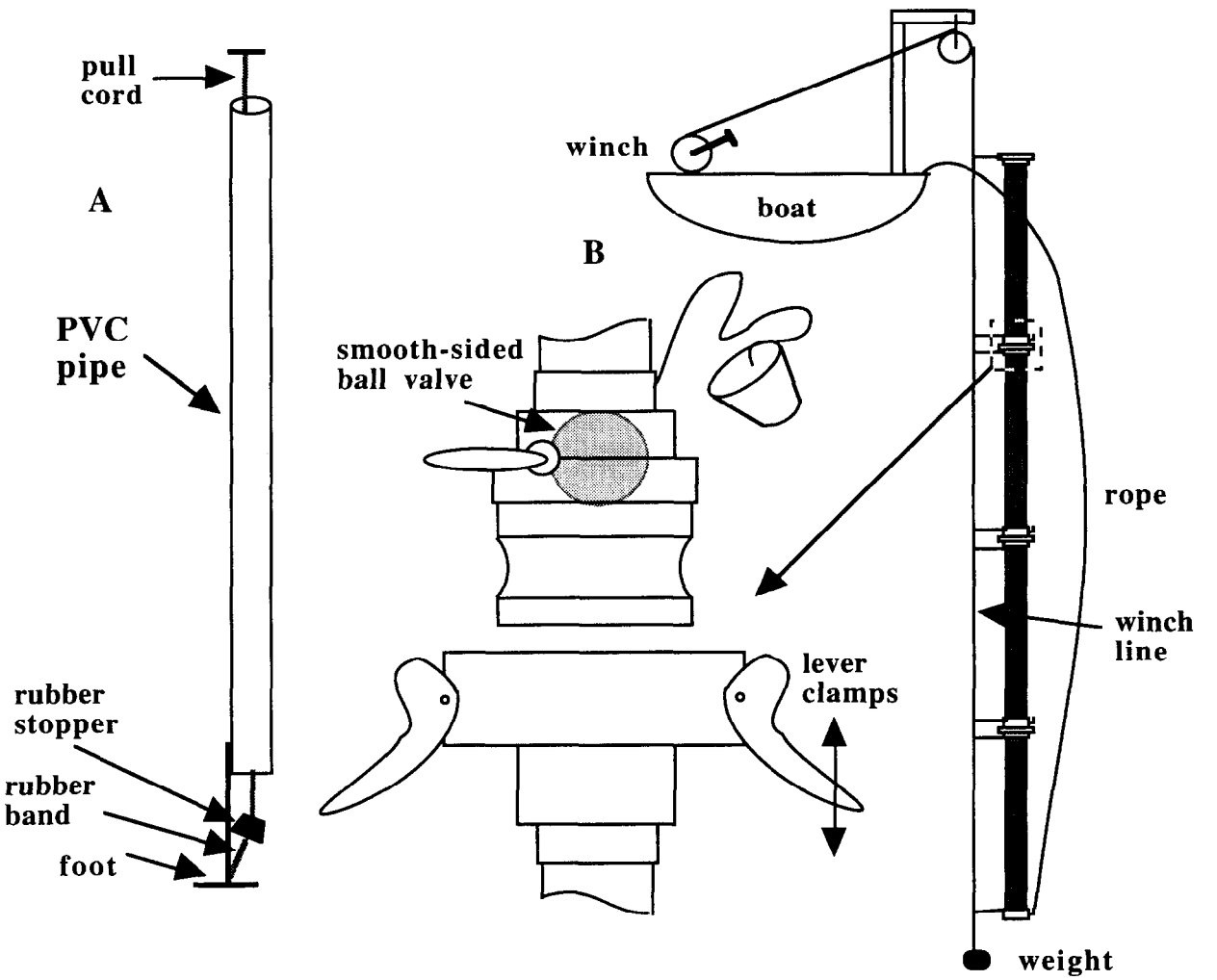


Figure 2.1. a) Tube sampler. b) Segmented tube sampler, and detail of connector (after Sutherland *et al.*, 1992).

Pumping systems

Most coastal areas are dynamically complicated, requiring detailed coverage of both the biology and the hydrography. While bottle samplers are usually adequate, they are nevertheless somewhat limiting due to their small volume and inability to provide continuous vertical profiles. If more detailed vertical profiles are required, for example if the phytoplankton are aggregated into a thin layer which is difficult to sample with a bottle, a pump profiling system may be an appropriate tool. These systems have the advantages of: 1) high volume, permitting sampling of a number of variables; 2) they give continuous vertical profiles; and 3) they provide the possibility of real-time observations of vertical features. The disadvantages are: 1) relatively high cost (generally >$200 US); 2) more difficult to deploy than the samplers described above; and 3) require AC or DC power source for the pump and associated instruments.

The pump profiling system at its simplest is a pump and one or more lengths of hose. Aspects of the system design which must be evaluated include the position of the pump (at surface or at depth), the pump head (the vertical distance the pump can push water), the insertion of various flow regulators and a bubble trap, and the inclusion of subsidiary sampling devices such as fluorometers, autoanalyzers, etc. (Fig. 2.2). These aspects are well described by Beers (1978), Franks and Anderson (1989) and Powlik *et al.* (1991). Pumps can range from a peristaltic pump (Voltolina, 1993), a centrifugal diaphragm pump (Taggart and Legget, 1984) or a water-tight submersible pump (Miller and Judkins, 1981). Swimming-pool pumps and sump pumps are relatively inexpensive, waterproof, and available through most hardware or plumbing stores.

Many of the pumping systems described in the literature have been designed for the sampling of zooplankton, and therefore pump large volumes of water through a mesh of some sort to concentrate the samples. Since phytoplankton are so abundant, this will usually not be a problem and sample volumes can be smaller and flow rates less. Systems designed for sampling phytoplankton typically have a junction beyond the pump to allow splitting of the pump discharge: the bulk will be discarded overboard, while a relatively small stream will be passed through instruments or used for integration or subsampling (Franks and Anderson, 1989).

The protocol for sampling with the pump profiling system is complicated by the transit time of the water through the hose. The water that is being pumped on deck was obtained from the depth the hose inlet occupied some time ago. To correct for this requires measurement of the transit time of water through the hose. This is typically done using coloured dye or a flow-through fluorometer to detect a spike of chlorophyll introduced at the hose inlet (e.g. Franks and Anderson, 1989). The calculated transit time should be at the first appearance of the dye at the end of the hose: smearing within the hose will cause an initial spike to be spread over a considerable distance within the hose.

Bubbles within the hose can be a serious problem with certain instruments such as fluorometers and autoanalyzers. To mitigate the problem, a bubble trap can be included before the water reaches the instruments (Fig. 2.2). One design of bubble trap consists of a 1 m length of 10 cm internal diameter acrylic pipe, fitted with stoppers and hose connectors at the top and bottom. A small chimney tube at the top allows air to escape. A length of clear tubing joined to the top and bottom with right-angled connectors allows one to see the water level within the pipe. A 20 cm length of hose extends from the top hose connector into the bubble trap. The level of water within the bubble trap should always be kept above the level of this hose so that no more bubbles are created. The bubble trap itself should be located at the highest point of the pumping system, so that all bubbles within the system may escape. The height of the bubble trap will largely be determined by the head of the pump and the geometry of the ship.

From the bubble trap, the flow feeds by gravity into the attached instruments, and back to the deck. The water may be collected in carboys to obtain samples integrated over any desired depth interval as the hose is raised or lowered. This is known as "binning". For certain

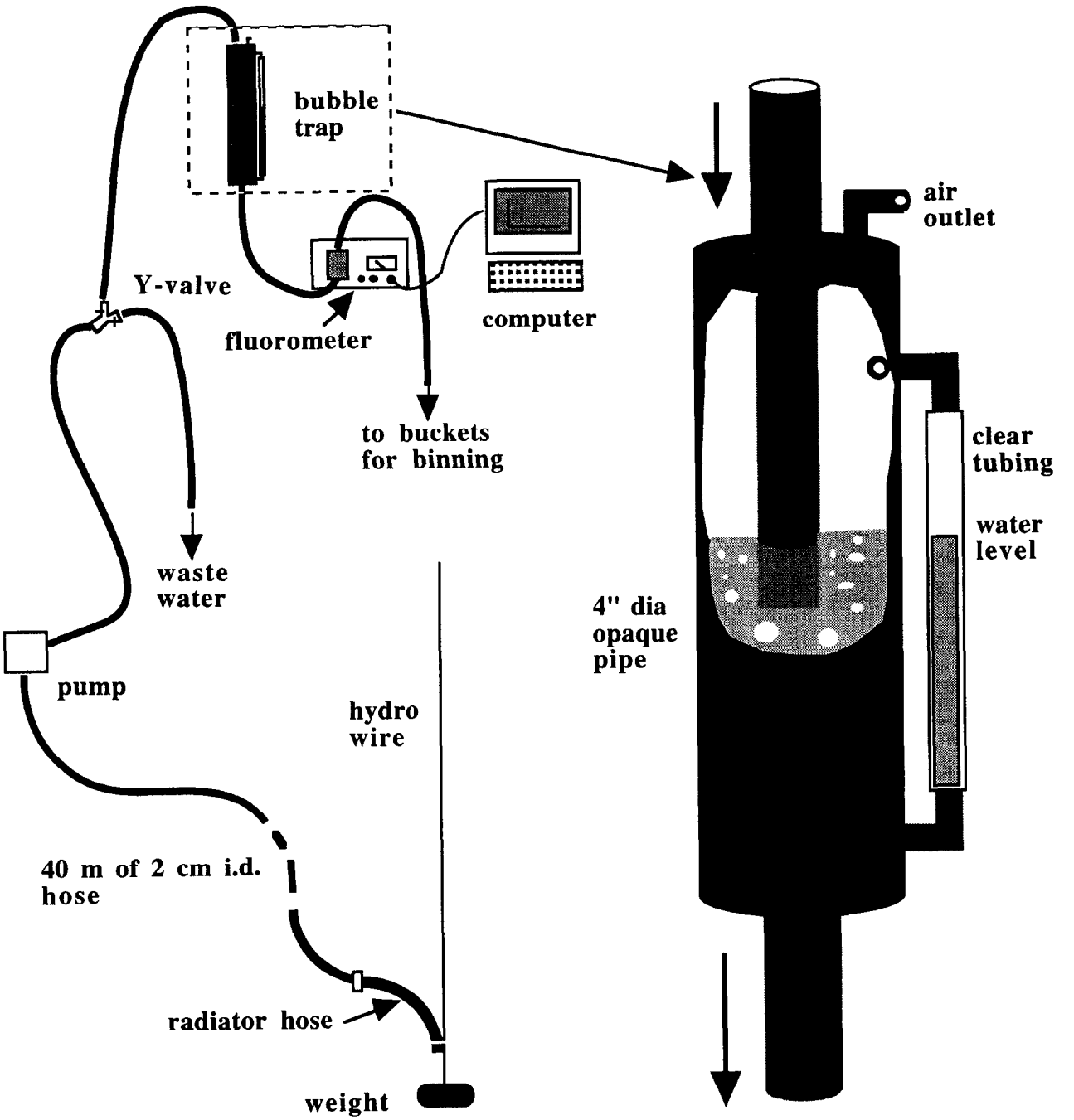


Figure 2.2. An example of a hose-pumping system, and detail of the bubble trap (after Franks and Anderson, 1989).

analyses (e.g. productivity or chlorophyll) the sample containers should be opaque or acid-washed, although a seawater rinse is sufficient for species counts.

Subsampling from the bins depends on the information needed. For example, Franks and Anderson (1989) sieved one litre from each 5 m depth bin through 20 μm Nitex mesh (epoxied onto a cylinder of 8 cm PVC pipe) and preserved it in 5% formalin for cell counts. An additional 500 ml was filtered through GF/A filters. The filters were used for chlorophyll analyses and the filtrate frozen for nutrient analyses. The flow rate should be adjusted at the post-pump junction so that sufficient water is obtained for all these analyses, as well as for the auxiliary, on-line instruments.

Hydrographic samplers

An essential part of any sampling program is the acquisition of hydrographic data, specifically temperature and salinity versus depth. This importance of this has been stressed for at least a decade (e.g. Seliger and Holligan, 1985), and will be repeated here: coincident biological and physical data are necessary for the identification of transport mechanisms for noxious phytoplankton blooms. In regions of strong freshwater input or thermal forcing, such basic instruments as reversing thermometers and a hand-held refractometer may suffice. The refractometer has an accuracy of about 0.2 psu (practical salinity units; 1 psu = 1 ppt), which is reasonable for most coastal areas with freshwater influence. If no more sophisticated instruments are available, temperature readings, measured with a thermometer, and salinity values, measured with a refractometer, should be taken at least at 0.5 m intervals. These measurements can be made on water exiting the hose if a pumping system is used.

In dynamic regions with weaker variations in properties, a small, self-contained CTD (Conductivity-Temperature-Depth) is an invaluable tool. While such instruments may be expensive for a small laboratory, the investment may be worthwhile in the excellent quality of data returned, and the ease of deployment and data reduction. Inexpensive battery-operated temperature/conductivity probes, such as those manufactured by InterOcean Systems Inc. (3540 Aero Ct., San Diego, CA USA 92123) can be lowered to learn details of water column structure, but accuracy is limited. A more sophisticated system might include a thermosalinograph (available through InterOcean Systems, Inc.) integrated into a hose-pumping system on deck. This instrument may also be interfaced to the computer, allowing rapid data acquisition and storage. The quality and durability of such instruments is of paramount importance: nothing is more frustrating than trying to collect data with unreliable instruments. Always try to test an instrument in the field before purchase, and talk to others who have used the instrument.

The instrument I recommend is a CTD profiler. Many oceanographers now use the "Sea Cat Profiler" (Sea Bird Electronics, 1808-136th Pl. NE, Bellevue WA, USA 98005). This small CTD stores data internally during a cast, thus no extra electrical wires are required over the side of the ship. The data can be subsequently transferred to a personal computer using the various programs supplied with the CTD. The instrument itself is practically indestructible and fool proof. This CTD can be hung from a hydrowire, mounted below the hose inlet of a pumping system, alongside a water bottle, or in a rosette array. An additional advantage of many CTD profilers is that they may be expanded to include *in situ* fluorometers, transmissometers, light meters, O_2 sensors, etc. These instrument packages are easily deployed even in fairly rough seas, when deployment of a pumping system may be impossible. Because deployment is relatively easy, they also allow dense sampling of a variety of fields.

One final word of advice on sampling equipment: always plan for the worst. Bring spares of all pieces of equipment: hose, pumps, valves, connectors, radiator hose, hose clamps, etc. A supply of duct tape is a necessity. Always make contingency plans if any aspect of the sampling program should fail at sea, e.g. if the pump loses its prime, the computer fails, or the fluorometer breaks. Planning ahead for such emergencies will help to make the best out of a bad situation, and may prevent the waste of a lot of time and money.

SAMPLING SCHEME

A feature of noxious blooms which has been noted for decades is that the blooms are often associated with distinct water masses (e.g. Mead, 1898; Slobodkin, 1953; Kierstead and Slobodkin, 1953; Conover, 1954; Ryther, 1955; Cullen *et al.*, 1982; Carreto *et al.*, 1986; Fraga *et al.*, 1988; Dundas *et al.*, 1989; Franks and Anderson, 1992a). This attribute is central to the identification of transport mechanisms, and the prediction of local outbreaks. Once the association between toxic phytoplankton and water mass has been identified, sampling schemes can concentrate on elucidating the processes influencing the timing, size, location and direction of travel of the water mass (e.g. Tyler and Seliger, 1978; Dundas *et al.*, 1989; Franks and Anderson, 1992a,b). In this section I will discuss physical mechanisms known to be important in the regional transport of toxic phytoplankton in coastal regions, and optimal temporal and spatial arrangement of sampling schemes to identify the important physical dynamics.

The most important transport mechanisms in coastal regions are those forced by tides, buoyancy (e.g. freshwater outflows), wind, and topography (Franks, 1992; Fig. 2.3). In any one area, it is quite likely that several of these forcings are operating at any one time. However, usually only one mechanism will be responsible for the initial formation of the water mass that supports the toxic algae. Other forcings may come into play in the transport of this water mass. It is important to distinguish between the formation and the transport of the water mass for the prediction of local outbreaks.

Buoyant plumes

Central to the understanding of coastal currents is the Coriolis effect, which causes a deflection of currents to their right in the Northern Hemisphere (to their left in the Southern Hemisphere). Many alongshore flows are created as a result of a balance between a pressure gradient (warm, fresh light water that tends to flow over cold, salty dense water) and the Coriolis effect. A buoyant plume formed by freshwater discharge from an estuary will deflect to its right in the Northern Hemisphere upon exiting the estuary, and flow along the coast (Beardsley and Hart, 1978; Woods and Beardsley, 1988; Chao, 1988a) if no other forces are acting on it. With no Coriolis effect, the fresh water would spread radially from the estuary mouth. The deflection of the current to its right creates a relatively narrow hydrographic feature which is trapped at the coast, and propagates along the coast at a speed determined by the volume of freshwater discharge, and external forcing such as remotely forced alongshore flows (Beardsley and Hart, 1978; Woods and Beardsley, 1988), wind (Chao, 1988b), tides or topography (Butman, 1976; Franks and Anderson, 1992a).

Wind-forced flows

Wind-forced flows also deflect to their right in the Northern Hemisphere (left in the Southern Hemisphere). This, combined with the constraint that the water does not flow through the coast, leads to the strong along- and across-shelf flows created by coastal winds. The phenomena of wind-driven upwelling and downwelling are well documented (e.g. Richards, 1981). While these flows are typically visualized in a cross-shelf plane, it is often forgotten that any across-shelf motion is accompanied by strong along-shore flows. A poleward wind blowing along the eastern coast of a land mass will generate an offshore flow of water (Fig. 2.4a). This offshore surface flow must be balanced by an onshore flow of deeper water, which creates the upwelling front. At the same time, however, strong alongshore flows are generated, predominantly in the direction of the wind. A buoyant plume propagating along the coast would be forced offshore by the upwelling, and backwards against its natural direction of propagation by the wind (Fig. 2.4c). On the other hand, an equatorward wind

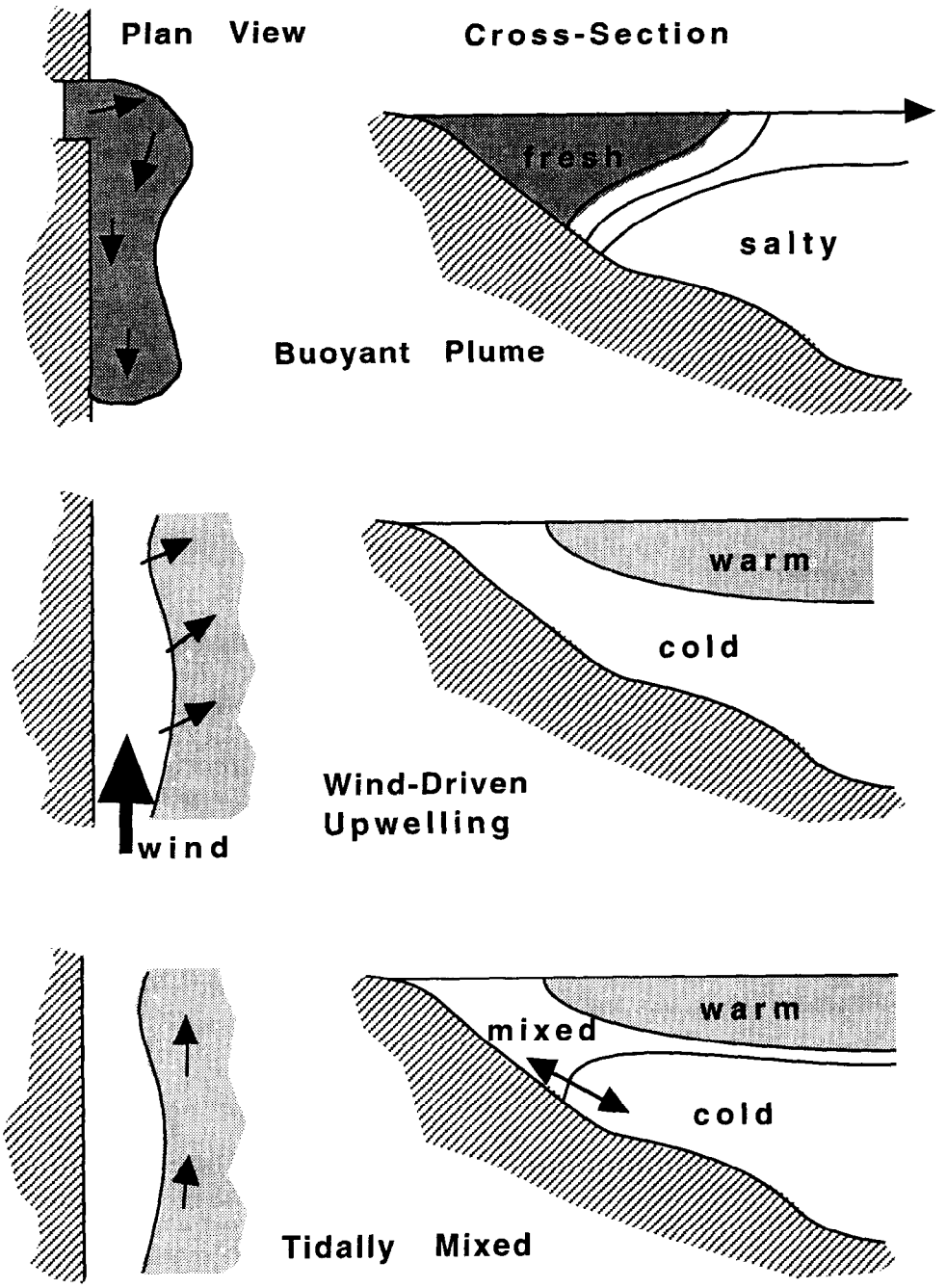


Figure 2.3. Plan and cross-sectional (cross-shelf) schematic views of a buoyant plume (top), wind-driven upwelling (middle), and a tidal front (bottom). Cross-hatched regions represent land, grey shades indicate less-dense water.

along the same coast would force surface waters into the coast (downwelling), generating a strong flow of water in the direction of the wind along the coast (Fig. 2.4b). The same buoyant plume caught in this wind-forced flow would be pushed against the coast, and accelerated in its natural direction of propagation (Fig. 2.4d). It was found that this combination of buoyant plume and wind-forced dynamics explained the timing and location of Paralytic Shellfish Poisoning toxicity along the coast of the Gulf of Maine (Franks and Anderson, 1992a, b).

Tidal currents

Coastal tidal currents may transport material both parallel and perpendicular to the shoreline. While tides give particle displacements which are approximately elliptical in the horizontal (when seen from above), the sloping bottom and irregular topography in coastal regions can lead to residual tidal currents that produce a net alongshore transport of material (Greenberg, 1979; Pingree and Maddock, 1985; Geyer, 1993). This transport will vary in strength depending on the lunar cycle. Tides also interact with buoyant plumes and wind-forced flows in that they advect hydrographic features back and forth. Because of this, a sampling station at a fixed geographic location may be in quite different water masses over the course of a tidal cycle.

Energetic tides in shallow waters may cause complete homogenization of the water column inshore, while offshore waters remain stratified. The boundary between these two water masses is often very sharp, and is termed a tidal front (e.g. Bowman *et al.*, 1986). Tidal fronts have been shown to be the site of enhanced dinoflagellate populations around the British Isles (Pingree *et al.*, 1975, 1979), the Patagonian Shelf (Carreto *et al.*, 1986), and may be the formation sites for noxious blooms in other regions of the world (Yentsch *et al.*, 1986).

Topographic features

The influence of sharp topographic features, such as capes and islands, on coastal flows is not well understood. Some studies have examined the separation of buoyant plumes from the coast at capes (Butman, 1976; Bormans and Garrett, 1989; Franks and Anderson, 1992a, b), while others have examined the influence of capes on wind-forced flows (Suginohara, 1974; Crepon *et al.*, 1984). Pingree and Maddock (1979) and Wolanski *et al.* (1984) have examined the flows generated in the lee of an island embedded in a larger scale flow. In general, the topographic feature may cause separation of the flow from the land, with the consequent formation of eddies and upwelling cells. If the toxic cells are carried in the coastal flow, this separation mechanism may shield the downstream side of the cape or island from toxicity (e.g. Franks and Anderson, 1992a,b). On the other hand, the upwelling created by the eddies may stimulate phytoplankton growth, or create accumulation zones in which noxious populations may grow and eventually be advected downstream.

Station location

Given the variety and complexity of physical forcings in coastal oceans, a critical requirement of any sampling program is that *sampling must be done at more than one location.* It is surprising how often this simple rule is broken, almost always with dire consequences. Even detailed hydrographic and biological samples taken frequently at a single point cannot tell you the direction of water motion or the scale of hydrographic variability. This is because *in situ* changes in properties cannot be distinguished from horizontal advection of gradients of properties. Any attempt to perform a mass balance in a coastal region will almost certainly conclude that the majority of changes in properties at a single station can be accounted for by horizontal advection. For this reason, any sampling program must include a minimum of two,

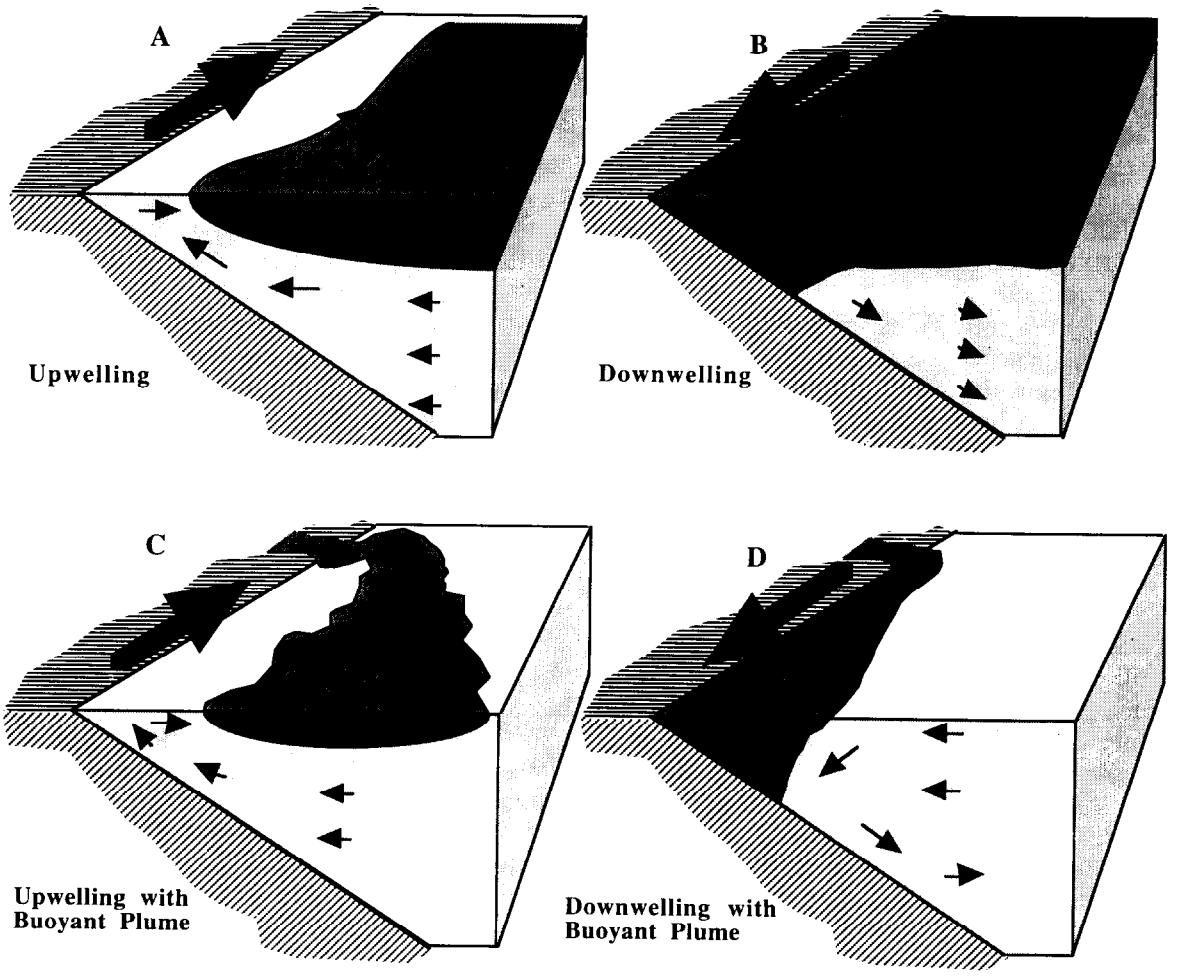


Figure 2.4. a) Three-dimensional schematic representation of currents and water masses generated by wind-driven upwelling. b) Currents and water masses generated by wind-driven downwelling. c) A buoyant plume forced offshore by wind-driven upwelling. d) A buoyant plume forced against the coast by wind-driven downwelling. Heavy arrow indicates wind direction, small arrows indicate water currents. Darker water masses are less dense (either warmer or less salty).

and preferably several more, stations in the *across-shelf* direction. This is because across-shelf gradients in hydrographic properties give a great deal of information about the along-shelf flows. As mentioned above, horizontal pressure gradients in one direction lead to currents in the perpendicular direction due to the Coriolis effect (the geostrophic balance). Thus a sampling scheme must be able to resolve sloping isopycnals in the across-shelf direction, which can give information on the direction of water motion.

The spacing between stations in the across-shelf plane is determined by the across-shelf scale of the hydrographic features which dominate the coastal region (Fig. 2.4). A good first estimate of this scale is the internal Rossby radius of deformation, R_i, often simply referred to as the Rossby radius (see Franks, 1992, for a thorough explanation of this scale, and its relation to a variety of fronts). The Rossby radius can be thought of as the radius of curvature of a current under the influence of the Coriolis effect and a pressure gradient. It is a natural scale for the curvature or width of wind-driven, topographic and buoyant plume fronts.

The Rossby radius, R_i, is calculated using the density difference between the surface and deep water masses, the thicknesses of the two water masses, the Coriolis frequency and gravity. The Coriolis frequency, f (units: s^{-1}), is calculated from the latitude, ϕ, and day length, and is given by:

$$f = \frac{4\pi}{86400} \sin \phi.$$

If the surface waters have density ρ_S (units: kg m^{-3}) and thickness h_s (units: m), while the deep waters have density ρ_d and thickness h_d, and the acceleration due to gravity is g (~10 m s^{-2}), then the Rossby radius, R_i (units: m), is:

$$R_i = \frac{\sqrt{g \frac{\rho_d - \rho_s}{\rho_d} \frac{h_s h_d}{h_s + h_d}}}{f}$$

This radius is usually between 5 and 20 km in coastal regions. Since R_i gives the natural scale of horizontal variation for many hydrographic features, sampling should always be on scales smaller than R_i in order to resolve the natural variability.

The number of stations in the across-shelf direction will usually be determined by factors other than the hydrography, for instance the shelf width, the ability of the vessel to sample far offshore, available manpower, etc. A good rule to follow, however, is that samples should be taken out to at least 4 to $6R_i$ offshore, giving a transect of at least five stations.

Because the coastline presents such a strong barrier to flow, most coastal flows are aligned along the coast. Thus stations oriented in a line perpendicular to the coast will generally sample the strongest hydrographic, and therefore biological, gradients. Where the curvature of the coast is very sharp, a coastal current may separate from the coast and move into open water. The criterion for whether or not this separation will occur (Bormans and Garrett, 1989) is given roughly by the Rossby number, R_o:

$$R_o = \frac{u}{fr_c}$$

Here u is the speed of the current, f is the Coriolis frequency for that latitude, and r_c is the radius of curvature of the coast (or the bathymetry). If this number can be calculated, and it is greater than 1, the coastal current is likely to separate from the coast and move offshore (Fig. 2.5).

If one has prior knowledge of the local toxic phytoplankton distribution, the sampling strategy may be tailored as follows. If toxic phytoplankton are known to be associated with

the coastal current, the stations should be moved offshore in the lee of the topographic feature, in order to sample the waters that separated from the coast. On the other hand, if the toxic phytoplankton grow in the shelter of the topographic feature, for example in an eddy (Wolanski and Hamner, 1988), the stations should be moved inshore to follow the coastline.

If multiple transects are to be run, it is preferable that they be oriented parallel to each other. This will give even data coverage, with no poorly sampled areas (Fig. 2.5a, c). It may seem more efficient, in terms of navigation, to orient the transects into a Z shape. However, relatively large gaps in coverage occur at the top and bottom of the Z (Fig. 2.5b). A more even coverage is obtained with an E-shaped cruise pattern (Fig. 2.5c). This will be found to be important when trying to contour the data and make surface maps of features.

It is often feasible to alternate cruise patterns to obtain dense vertical coverage along one transect during one cruise, and extensive horizontal coverage with coarse vertical resolution during another cruise. The timing and planning of these efforts should be coordinated with available data on toxicity patterns, and known associations of toxic phytoplankton with hydrographic features in the region.

In the vertical, the most dense coverage possible is always preferable. Some workers advocate binning of samples into three categories: below the thermocline, within the thermocline, and above the thermocline. I do not suggest using this procedure for several reasons: 1) the thermocline may not correlate with the dinoflagellate peak or the nutricline; 2) the sample resolution is low; 3) the variable depth of the thermocline will make data reduction and interpretation difficult; and 4) unexpected features may be missed. Rather, I recommend obtaining as many evenly-spaced samples as is feasible given sample processing time. The even spacing allows for quick and easy plotting of data, with good resolution of most vertical features. I suggest beginning with a vertical spacing no greater than 5 m. It is often possible to elucidate the relationship of toxic phytoplankton with water masses based solely on surface samples of phytoplankton. This reduced vertical sampling should not be used, however, until after a more thorough survey has allowed documentation of the subsurface patterns of phytoplankton and hydrography.

The timing of sampling cruises will depend on many factors, including the organism being studied, the manpower available, the size of the boat, the oceanographic and meteorological conditions, and the amount of money available for cruises and data reduction. Two important generalizations can be made, however: 1) always begin sampling before a toxic species becomes dominant, and 2) sample as frequently as possible. The timing of cruises is determined by several factors including the growth rate of the phytoplankter, vertical migration behaviour, and the speed of alongshore currents. Cruises should be spaced so that a cruise is not totally independent of the previous one. For example, if a coastal current, u, is, on average, 10 cm s^{-1}, and R_i is 10 km, then cruises should ideally be $R_i/u \sim 1$ d apart if a single transect is sampled. If two parallel transects spanning 10 km are used, then cruises can be less often. This is seldom practical, so cruises are often one or two weeks apart, leading to problems in data interpretation. Any data available between cruises, such as mooring data or shore-based sampling, can be very useful in interpolating between cruises. In this regard, regional shellfish monitoring programs can be invaluable in providing a consistent temporal record of coastal toxicity. Again, the more extensive the spatial coverage of cruises, the longer the time interval can be between them.

In the absence of a monitoring program, there are some environmental cues which correlate well with dinoflagellate blooms. Most dinoflagellate blooms are found in the pycnocline of a well-stratified water column. This stratification can be caused either by salinity differences, or by heating. Thus strong rains, or several sunny days in a row can be important in bloom formation. Keeping a close eye on the weather is important when sampling for dinoflagellates.

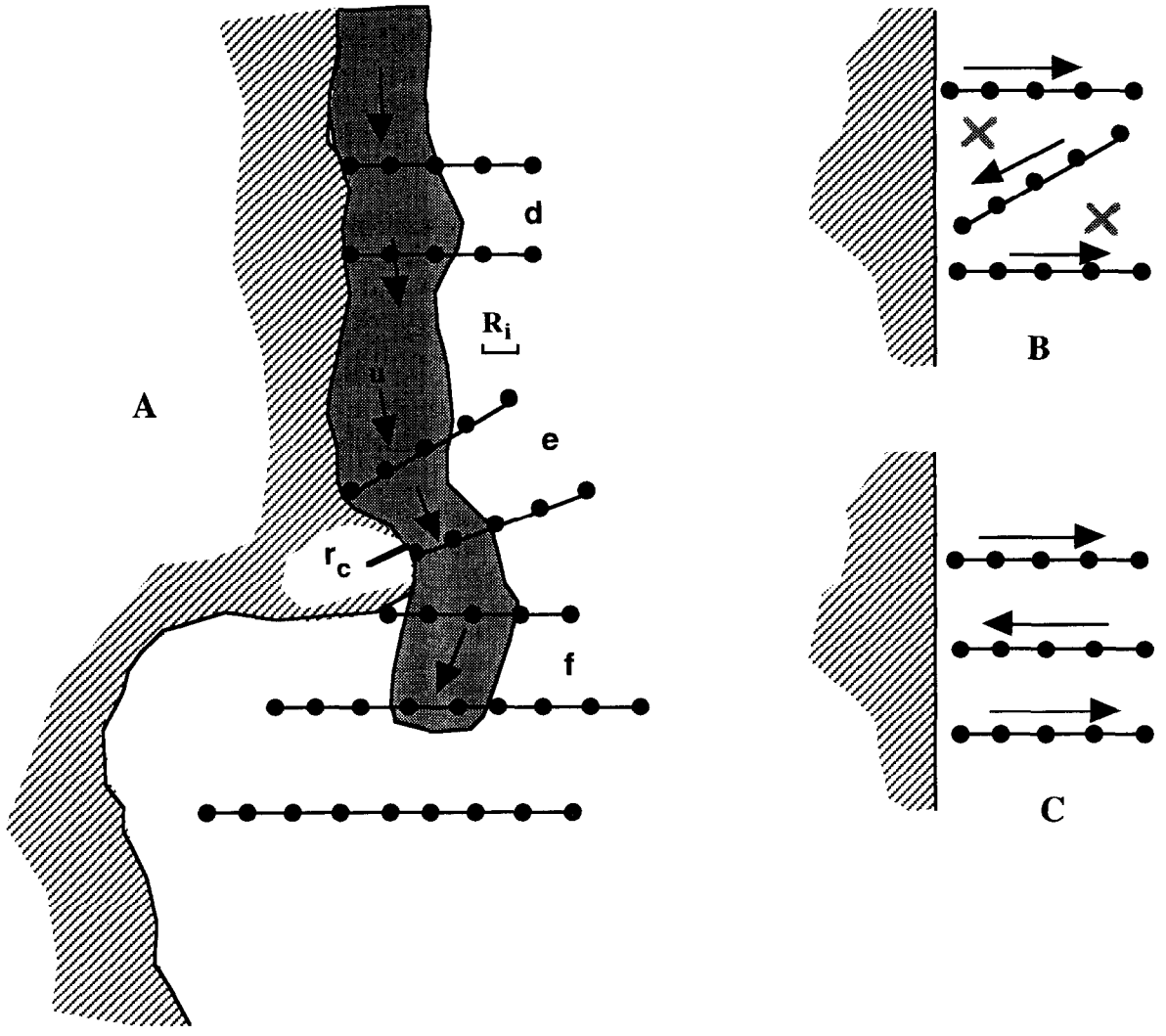


Figure 2.5. a) Optimal station arrangement for sampling of a coastal current. In region (d), the coastline is straight, and transects should be parallel. In region (e), the coastline is bending, so transects should be oriented perpendicular to the shoreline. In region (f), the coastal current has separated from the coast as it passed the cape with radius of curvature, r_c. Transect here should be moved offshore in order to sample the coastal current in open water. b) Transects arranged in a Z formation. The X's indicate regions of low sample density. c) Transects arranged in an E formation. Sample spacing is even.

Satellite data

The first study to use remote sensing to understand the distribution of toxic phytoplankton was probably that of Murphy *et al.* (1975). Several recent studies of harmful algal blooms have used remotely sensed images of sea-surface temperature to aid in their prediction of transport of toxic phytoplankton (Johannessen *et al.*, 1989; Tester *et al.*, 1991; Franks and Anderson, 1992a; Keafer and Anderson, 1993). These images were useful only because the researchers had previously identified the association of noxious phytoplankton with particular water masses. Images give the boundaries of water masses; successive images may give some indication of water mass movement, although currents along temperature gradients cannot be estimated. However, toxic phytoplankton cannot be identified remotely; indeed, toxic outbreaks may occur with extremely low concentrations of cells (<1000 cells l^{-1}), which are difficult to identify even with a microscope. In addition, satellite sensors sample a very thin layer of water for temperature, and so may not give an accurate picture of the absolute temperatures in a local area. Remote sensing, then, is only useful as a supplement to a well-designed field program.

The main utility of remote sensing is in providing a larger-scale synoptic picture of the sea-surface temperature than can be achieved through shipboard sampling. Knowledge of insolation, wind speed and direction, freshwater discharge and dynamic response to local topography are all essential elements in the interpretation of satellite images. Satellite images are extremely useful tools in gaining understanding of the regional influences of processes affecting local phytoplankton populations. In particular, they can aid in understanding the geographical extent of hydrographic features, the large-scale response of hydrography to forcings, and the upstream conditions relative to a particular location.

Supplementary data

Biological and hydrographic data gathered during a field program indicate only the instantaneous state of the waters at the time of sampling. For information on the dynamic response, more consecutive data are necessary. In this regard, time series of wind speed and direction, and freshwater discharge of local rivers may be invaluable. Wind speed and direction are often available from the local airport; occasionally offshore buoy information can be obtained from the national weather service. Comparison of records from several locations along the coast should be made, in order to assess the spatial and temporal scales of weather systems and correlation between locations. Freshwater discharge rates are important along any coastline that has a reasonably sized river. The stratification created by the low-salinity water can be an important refuge for dinoflagellates. Continuous records of tidal height and bottom pressure are often made along coastlines. This information can be used to infer geostrophic currents or propagation speeds of water masses along a coast (e.g. Brown and Irish, 1993).

DATA INTERPRETATION

Once biological and hydrographic data have been gathered, it is necessary to process and collate them in a form that will make dynamics and correlations apparent. Two plotting formats are central to this data reduction: profiles and contour plots (Fig. 2.6). Profiles are plots of properties determined by vertical sampling at a given station (Fig. 2.6a). Associations between variables at a station can be assessed by plotting several variables on the same set of vertical axes. Thus subsurface peaks in species concentrations may be seen to be associated with the shallow diurnal pycnocline, for example. Or a lack of association of toxic species with the vertical profile of chlorophyll may become apparent.

A limitation of vertical profile plots is that they give no horizontal spatial information. Contour plots address this limitation, by providing a two-dimensional view, in either a vertical or horizontal plane, of the variability of properties along two spatial axes (Fig. 2.6b). While profile plots may show an association of toxic cells with the pycnocline, contour plots in a vertical plane may show the pycnocline to have a slope, indicating a flow perpendicular to the plane. From this we could infer that the toxic cells are being transported in that direction. Contour plots can be even more useful than profile plots in showing the relationship between two variables. By plotting contours of the two variables on the same axes, and overlaying the plots, a two-dimensional picture of the patchiness and spatial correlations between properties becomes apparent. Many computer programs are available for making contour plots, however doing contours by hand at least once is a good exercise for any scientist.

DISCUSSION

Several studies of harmful algal blooms have brought together many of the sampling techniques and strategies described above to elucidate the factors leading to local outbreaks and transport mechanisms in coastal regions. Good examples are the studies by Tyler and Seliger (Tyler, 1984; Tyler and Seliger, 1978, 1981) of *Prorocentrum mariae-lebouriae* in the Chesapeake Bay estuary (USA), the field work by Franks and Anderson (Franks and Anderson, 1992a, b) examining *Alexandrium tamarense* in the Gulf of Maine (USA), the work by many scientists to determine the factors leading to the 1988 *Chrysochromulina polylepis* bloom along the Scandinavian coasts (e.g. Dundas *et al.*, 1989), and the studies of *Gymnodinium catenatum* and *Alexandrium affine* in the rias of Spain (e.g. Fraga *et al.*, 1988). While this list is not close to being exhaustive, it is representative of the types of studies which can ultimately lead to some limited predictive capability for toxic phytoplankton blooms.

A feature common to all these studies is that they incorporate a variety of data bases and types of information. While a single cruise may not yield much information, several cruises under different conditions, along with good insights into the local physical forcings, supplementary data to interpolate conditions between cruises, and a variety of biological samples allow the researchers to formulate strong, testable hypotheses from which the course of future blooms may be predicted. A finding common to most, if not all studies of toxic algal blooms is that their occurrence is strongly linked to recent and prevailing meteorological conditions. This immediately suggests that blooms are probably no more predictable than the weather, limiting their forecast to, at most, about a week. This inherent limitation in the predictability of harmful algal blooms demands that sampling be done on a short enough timescale that important events are not missed. This strong constraint will largely determine the timing of cruises, and arrangement of sampling stations.

While the sampling strategies described may aid in the determination of how a toxic bloom arrived at a given locale along a coast, they will not necessarily allow identification of the processes that began the bloom. Steidinger (1983) suggests four stages for a phytoplankton bloom: initiation, growth, concentration and dispersal. The sampling techniques described here will aid in elucidating the factors leading to growth, concentration and dispersal. The factors allowing the initiation of a harmful algal bloom fall squarely within the realm of the physiologist, who can give information concerning growth response to temperature, salinity, light, nutrients, turbulence, and life-history traits such as encystment or sexual stages. However, it is the transport of toxic blooms along coastlines that can lead to widespread ecological devastation, economic hardship and health risk. It is imperative, therefore, that we design and implement useful and efficient coastal sampling schemes, in order to relate local toxic phytoplankton blooms to the physical systems that influence their distribution.

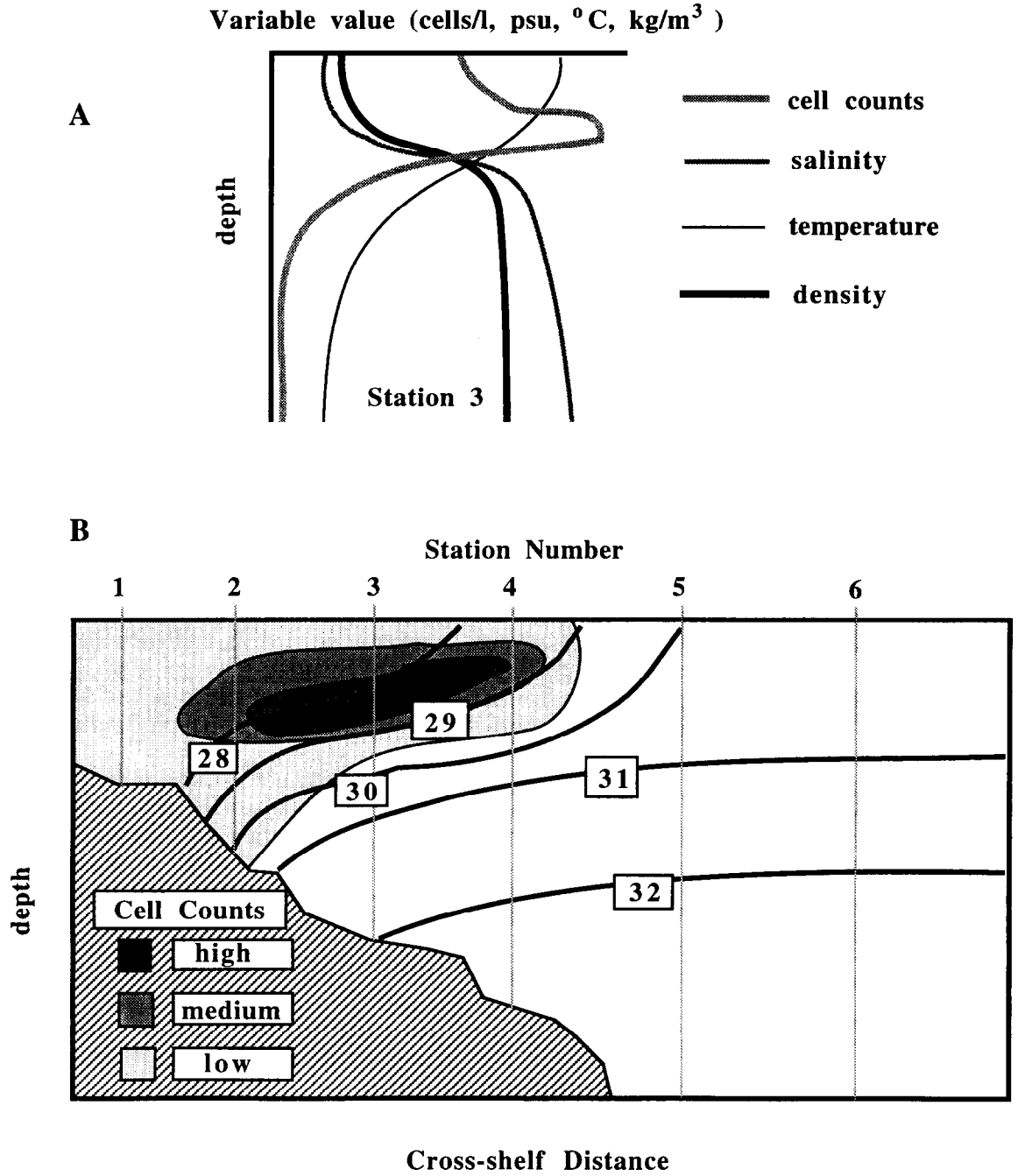


Figure 2.6. a) An example of a vertical profile plot, with four variables (cell density, salinity, temperature and density) all plotted on the same vertical axis. b) The data from six stations (1-6) contoured into a cross-sectional view. Two properties have been plotted on the same axes: salinity and cell counts. The sloping isohalines (heavy black lines) with freshwater inshore indicates that there is probably a buoyant plume present inshore. The slope of the isohalines suggests (via geostrophy in the Northern Hemisphere) that the water currents are out of the page at stations 2, 3, 4 and 5. The highest cell densities (shades of grey) are associated with the salinity front, indicating that the cells are being advected alongshore (out of the page) with the water.

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3. Culture Methods

R.R.L. Guillard

This chapter deals with the culture of marine microalgae of known or presumed toxicity to humans or animals of commercial interest. Virtually all phytoplankters suspected of producing toxic substances are photosynthetic, which is why this chapter deals exclusively with autotrophic (and auxotrophic) modes of nutrition. It should be noted that some related forms, notably of the dinoflagellates or golden-brown flagellate groups, are wholly or partially phagotrophic. For culture purposes these should be treated as small photosynthetic animals, with suitable prey made available. For some research it is sufficient to use strains of microalgae already in culture. Some of these may be obtained in unialgal or even axenic condition from various sources - university departments, research institutes, government laboratories, or formal culture collections (see Chapter 25). It is usually desirable and often essential to obtain cultures of harmful or nuisance species from the local waters they infest. Such cultures not only help in identifying organisms as species previously described, but can be critical in determining the systematic position of a previously unrecognized organism. Properties that can be studied in culture include pigment content, ultrastructure, genetics, life cycles, storage products or other organic products, and toxin production. Techniques of molecular biology also may require cultured material. Any attempts to correlate the occurrence of a species with the ecology of its habitat through culture experiments should obviously employ strains from the local population of interest. For growing crops of phytoplankton, natural seawater contains an abundance of many elements, but far too little of others. Thus various enrichments of seawater have been designed to supply the scarce materials in usable form at levels that are neither toxic nor severely growth-rate limiting. Sections that follow treat various aspects of culture techniques and cite readily available book chapters or papers that offer more specific instructions or alternate methods. The emphasis here is on methods that apply to harmful marine species of interest, or on aspects not covered in the references cited.

GENERAL LABORATORY PROCEDURES

A population started from a single specimen isolated from nature is allowed to increase in a favorable environment until some factor, usually nutrient depletion, stops cell multiplication. Well before the half-life of the culture is reached, a daughter culture is inoculated from it under aseptic conditions and the parent culture is kept as a "back-up" culture and as a source of inoculum. While some species can be stored as cysts, spores, or resting cells, or even in cryogenic storage, this is not usual practice with strains in active use. In order to avoid contaminating local waters, all cultures of known or presumed toxic species should be kept and discarded with precautions like those used with pathogens. Discarded cultures should be treated by heat (autoclaving) or chemical sterilization (*e.g.* aqueous solution of sodium hypochlorite, which can be in the form of commercial household bleach) before being released into waste lines.

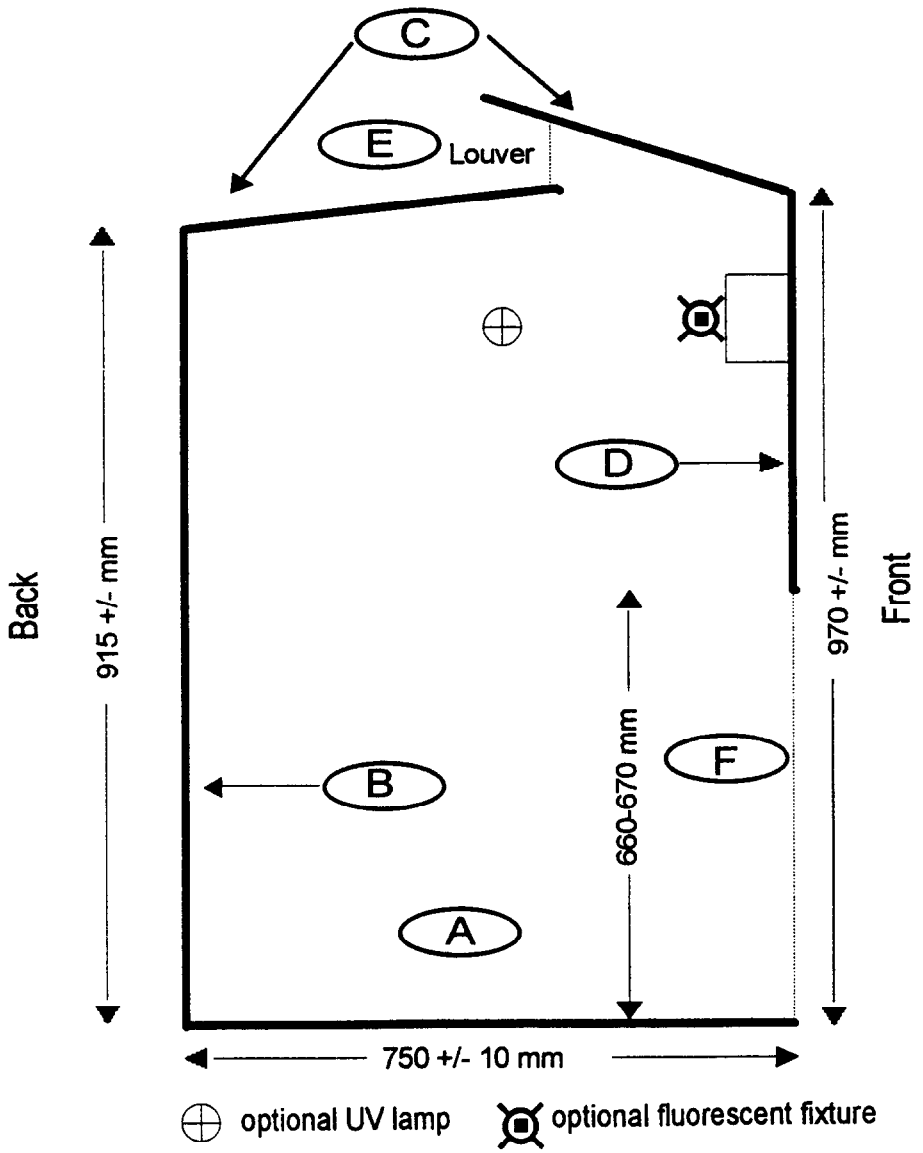

Figure 3.1. Side view of a simple hood. A) sides of 3/4" plywood; adjust depth (front to back) to fit bench. B) Back of 3/4" plywood. C) Top pieces of 3/8" plywood or 1/4" clear plastic. D) Front of 3/8" plywood or 1/4" clear plastic. E) Louver open from side to side. F) Front opening. Make width of hood (left) to right) 950-1050mm.

MATERIALS AND APPARATUS

Bench facilities

The outlines and dimensions of a suitable bench design are shown in Fig. 3.1. The bench top should be wiped with 80% ethanol or isopropanol before use to remove dust and microorganisms and the whole hood interior so wiped occasionally.

Culture vessels, enrichment

For enrichment or selective cultures made from water samples, the liquid volumes should not be less than 100 ml for small species and should be at least a liter for large species. Glass or polycarbonate Erlenmeyer flasks of 250 ml to 2 liter are suitable, covered by cotton plugs or inverted beakers of glass or polypropylene. Fernbach flasks (2800 ml) or borosilicate bottles are also satisfactory for large volumes. The liquid-air surface should be large. Cleaning culture vessels involves soaking (optional), followed by scrubbing with a brush when necessary to dislodge solid algal remains or other material, then rinsing with tap water, dilute HCl (10%, which is *ca.* 1.2 N) to remove carbonate deposits, then a final rinse with tap water and "purified water" (distilled or deionized). Final rinsing with tap water should include filling and emptying smaller vessels 4-6 times and swirling larger vessels with several changes of smaller volume.

Isolation tubes, culture tubes, culture flasks

Borosilicate tubes are usually used for both single cell isolation work and dilution cultures. Screw-capped tubes are usually 16 x 120 mm or 20 x 150 mm in size. Caps should be teflon-lined or made of polypropylene. Delicate species may do better in polycarbonate centrifuge tubes (Oak Ridge type) in 92 x 23.5 mm or 92 x 25.5 mm sizes. The 25 x 150 (or 20 x 150) mm threaded tubes are adequate for small volume maintenance cultures of certain small flagellate species. The standard maintenance vessels for years have been cotton-plugged 125 ml borosilicate flasks containing 40-50 ml of autoclaved medium. Good surface-to-volume ratios are provided by 100 ml of liquid in 250 ml flasks and 1 l in 2 l flasks. Polycarbonate Erlenmeyer flasks have excellent properties but cannot be autoclaved often with seawater in them because of the high pH reached during autoclaving.

Reagent bottles

Borosilicate bottles of 100 ml to 1 liter having ground glass stoppers or polypropylene screw caps are suitable for most primary and working stock solutions. Polycarbonate screw-capped Erlenmeyer flasks or square bottles are excellent for neutral or acidic solutions. Teflon, though expensive, is best for sodium metasilicate and sodium carbonate stock solutions, glass is next best, polypropylene is not indicated.

Transfer pipettes, isolation equipment

For transferring stock cultures or starting cultures used as inoculum, sterile cotton plugged 9 inch Pasteur pipettes are best. Isolation pipettes are made by drawing out sterilised commercial 9 inch (230 cm) Pasteur pipettes over a flame into capillaries of diameter suited to the species involved (See Fig. 3-3 in Hoshaw and Rosowski (1973) and discussions in this reference and in Guillard (1973)). Fig. 3.2 shows the general construction of an isolating pipette with mouthpiece tube. Small cells can be isolated from and washed through drops of water on 12-

Table 3.1. Sterility test medium L1pm Methylamine added according to Sieburth and Keller (1988/1989)

Algal enrichment L1[1]	1 liter
Bacto peptone[2]	1 g
Methylamine. HCl[3]	0.675g

Dissolve the peptone and dispense medium in 5-7 ml lots in tubes for testing algal cultures and in screw-capped glass or polycarbonate bottles for uses of larger volumes.

(1) Seawater algal medium L1 according to Table 3.2. Dilute (with seawater) to 1/2 or 1/4 strength if desired. Keep Fe and EDTA above 10^{-6}M in any dilution.

(2) Bacto-peptone, Neopeptone, Trypticase, and Hy-case do not precipitate in seawater; Proteose Peptone and Tryptone do. Test other commercial preparations. If it is necessary to use ones that precipitate, an additional possibility is to autoclave them separately in distilled water, add aseptically to the rest and dispense aseptically.

(3) This amount provides methylamine HCl (Formula Wt. 67.52) at 10^{-2}M; it has a negligible effect on pH of the medium. It is convenient to make a molar stock solution in distilled water (6.75 g per 100 ml), autoclave in a screw capped bottle and use 10 ml l^{-1} of L1pm test solution before final autoclaving.

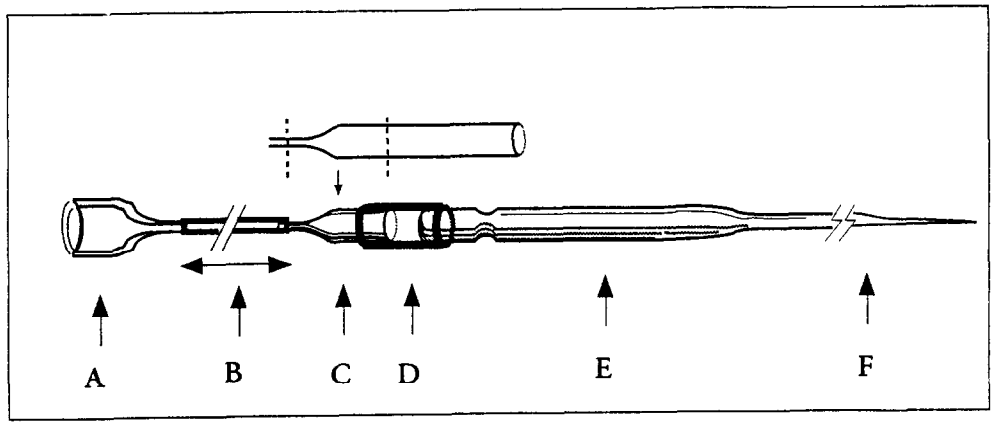

Figure 3.2. Diagram of isolating pipette mouthpiece tube.
A. Mouthpiece as used for blood diluting pipettes, or improvised; B. Latex tubing 1/8 inch (3.2 mm) bore, 1/32 inch (0.8mm) wall thickness, 30-40 cm long; C. Portion of Pasteur pipette, scored with a file and broken off; D. Latex tubing 3/16 inch (4.8 mm) inside diameter, 1/16 inch (1.6mm) wall thickness, 12-15 mm long; E. Drawn out Pasteur pipette being used for isolation; F. Capillary end of pipette.

well borosilicate glass commercial chemical spot plates, or on depression slides (25 x 75 mm, 2 or 3 depressions per slide) or even on ordinary glass slides. A bunsen burner with pilot light, fine and medium forceps, wire tube racks, and tape for labelling tubes and flasks are needed. For further description of useful materials and arrangement of facilities see Hoshaw and Rosowski (1973), Guillard (1973) and Guillard (1975 or 1983). Plankton nets and sieves made of nylon plankton netting (see Tangen, 1978 for sources) are widely used to get rid of the larger species in order to deal with the smaller ones, to wash away the smaller ones, or just to remove the larger grazers. Reverse (upward) filtration exerts very little pressure on the cells being concentrated (Dodson and Thomas, 1964). Polycarbonate filters with holes of 0.8 µm to 12 µm can serve the same purpose as nets for separating small cells. Small contaminating cells such as those of *Synechococcus* or *Nannochloropsis* can sometimes be greatly reduced in numbers relative to the species desired by washing them through the filters using repeated additions of sterile medium (the filter surface must not be allowed to dry).

Optical equipment

Any compound microscope is suitable for general observation. The most versatile stereomicroscopes have a base for transmitted light illumination and are provided with a mirror on a gimbeled fork to allow rotation in all directions. The magnification used for isolation seldom exceeds 100x, however good resolution is desirable for distinguishing cells from detritus. It is possible to isolate cells only 2-4 µm in size with a dissecting microscope using darkfield illumination from a ring light.

Apparatus for filtration

Filtration has three different purposes in algal culture. The first is to remove detritus and larger planktonic organisms from the seawater used for routine culture and experiments. This is ordinarily done in 10 - 20 l carboy lots using 47 µm glass fiber filters having porosities of one to a few micrometers (*e.g.*, Whatman GF/C, Gelman A/E) or even fine filter paper. The apparatus shown in Fig. 3.3 is convenient. The second purpose is to provide diluent either for serial dilution isolations or for dilution of seawater samples brought into the laboratory for acclimation or selective enrichment prior to isolation efforts. For this purpose filtration may be through membrane filters of 45 - 50 mm diameter and about 0.4 µm porosity, which permit only some heterotrophic bacteria and prochlorophytes to pass. Membrane filters of 0.2 µm porosity are generally considered to yield water free of bacteria, but not viruses. The third purpose for filtering is to sterilize liquids that should not be subjected to autoclaving.

Apparatus for sterilization by heat

A commercial autoclave is best, but pressure cookers of various sizes are also suitable. Sterility requires 15 minutes at 121°C in the entire volume of liquid. Routine culture safety is helped by using indicator tapes that change color upon autoclaving to identify flasks, pipette tubes etc. that have been sterilized. Heating to 90 - 95°C for at least 30 minutes, and cooling, and repeating on two successive days is an alternative process ("tyndallization"). It is assumed that vegetative cells are killed by heat and that heat resistant spores will germinate in the following cool periods and be killed by subsequent heatings. Sterilization by commercial microwave apparatus has been explored by Keller *et al.* (1988) and Price *et al.* (1988/1989). The method is best suited to treating two liter Teflon or polycarbonate bottles (up to three at one time in the microwave) holding 1.5 l of seawater or medium. Some materials cannot be microwaved without damage. Test tube racks must be plastic.

Autoclaving medium is still the ultimate guarantee of sterility; it includes the destruction of viruses. Autoclaving in Teflon would reduce losses and avoid leaching of materials (silicon

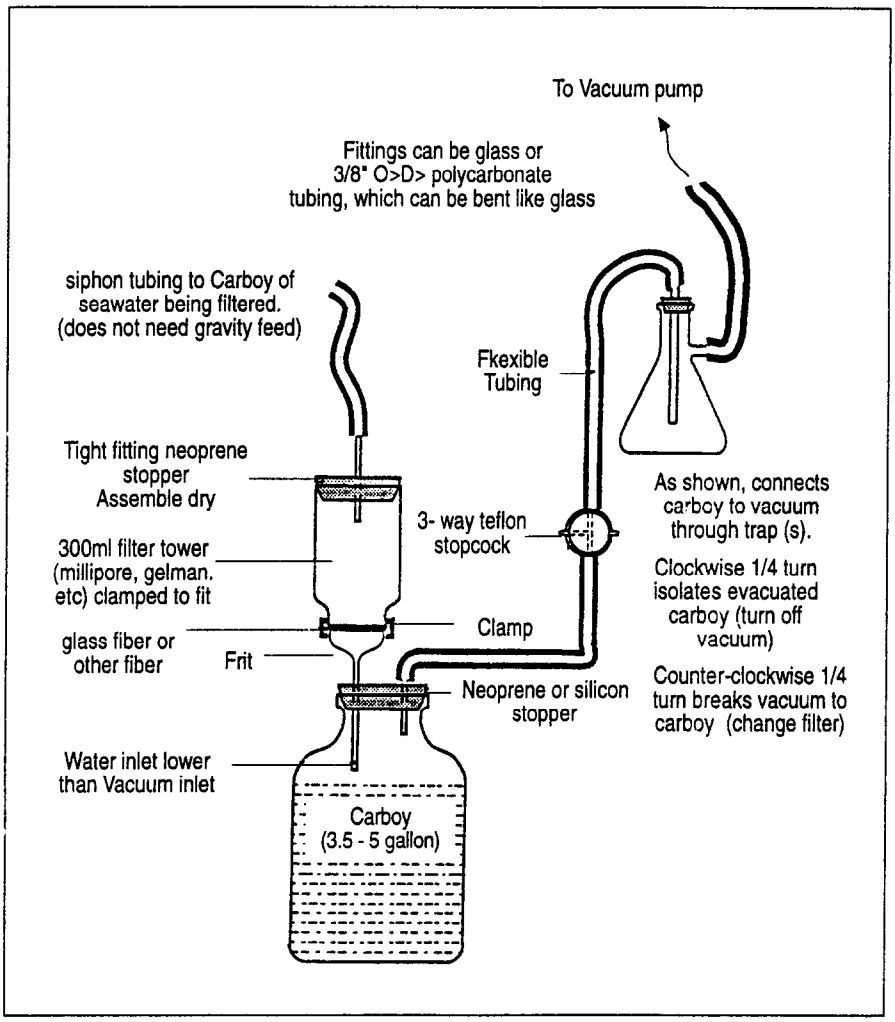

Figure 3.3. Diagram of filtration set-up to permit intermittent use of vacuum pump while maintaining vacuum.

especially) from glass. In many media, including L1 (see later), a precipitate forms upon autoclaving, some of which may disappear upon re-equilibration of the liquid with CO_2 of the air. Concerning the nature of the precipitate, that which is formed from f/2 (and doubtless L1 and other media), contains much of the iron and phosphate added in the medium. There is a body of circumstantial evidence that the level of availability of iron is crucial to algae of different habitats, with coastal and estuarine algae needing easily available iron and perhaps even having high iron cellular quotas, while many oceanic species can easily obtain iron from forms (in culture media) totally inaccessible to the coastal ones.

Illumination

The problem of temperature control in a culture facility may influence the lighting technology chosen. The important considerations regarding light are intensity (irradiance), color (spectral distribution) and duration (daylength). Daylight does very well for most cultures, but the intensity, spectrum, and daylength are dependent on geography and weather. Screens or gauze will do to attenuate daylight if needed. Maximum irradiance experienced by cultures during the day should be approximately 5-10% of full summer sunlight (170 μ Einsteins $sec^{-1}m^{-2}$). Many algae grow well at 1-2% of full sunlight, and some only then, and back-up cultures always survive better at low intensities. It is noteworthy that a strain (from the Pacific) of the pelagic cyanobacterium *Trichodesmium* only grew successfully under dim daylight (1% of full sunlight PAR) and could not be grown under fluorescent light (Ohki and Fujita, 1982; Ohki *et all.*, 1986). On the other hand, natural light may have too much red and far red light for algae that live in deep water (bad red to blue ratio; Brand, 1986). Fluorescent bulbs remain the light source of choice, employing colored filters when felt necessary. The type "cool white" is somewhat favored because of a better ratio of light output to energy consumed, and general availability. Its spectral distribution of energy is reasonably good for coastal species and perhaps others living in surface waters. "Daylight" bulbs have more of their output in the blue, but less total light. Fluorescent lights emit roughly 2% of their radiant energy in the near ultraviolet (<380 nm), and there is suggestive evidence that this can inhibit dinoflagellates (reviewed by Guillard and Keller, 1984). Use polycarbonate or lucite to absorb ultraviolet. While it is true that some algae tolerate or thrive in continuous light, many species have an absolute requirement for a dark period, even if the irradiance is low (Brand and Guillard, 1981). Light/dark cycles of 16h /8h, 14h /10h, and 12h /12h are commonly used.

Temperature control

Uncontrolled room temperature is seldom satisfactory for marine algae. Rooms and culture boxes should have safety systems to guard against overheating or chilling (recommendations in Guillard, 1975 or 1983). Excursions of temperature, sometimes too low, but usually too high, cause by far the most losses of cultures. It is most important to have alternate safe locations.

ISOLATION PROCEDURES - SAMPLING AND INCUBATION

Fig. 3.4 outlines possible subsequent treatments of the sample collected (Fig. 3.4.A). Water samples should be collected in clean vessels (ca.1 liter) and protected from bright light and changes of temperature. The well-known agar plate technique is not included because the only marine nuisance algae readily amenable to it are species of *Nannochloropsis, Nannochloris,Chlorella* and possibly *Pseudonitzschia.* Screening to remove larger predators is always desirable because populations of desired cells can be depleted in just hours. Predator removal is essential in paths 3.4.B and C. Selective screening is often helpful *via* any path to

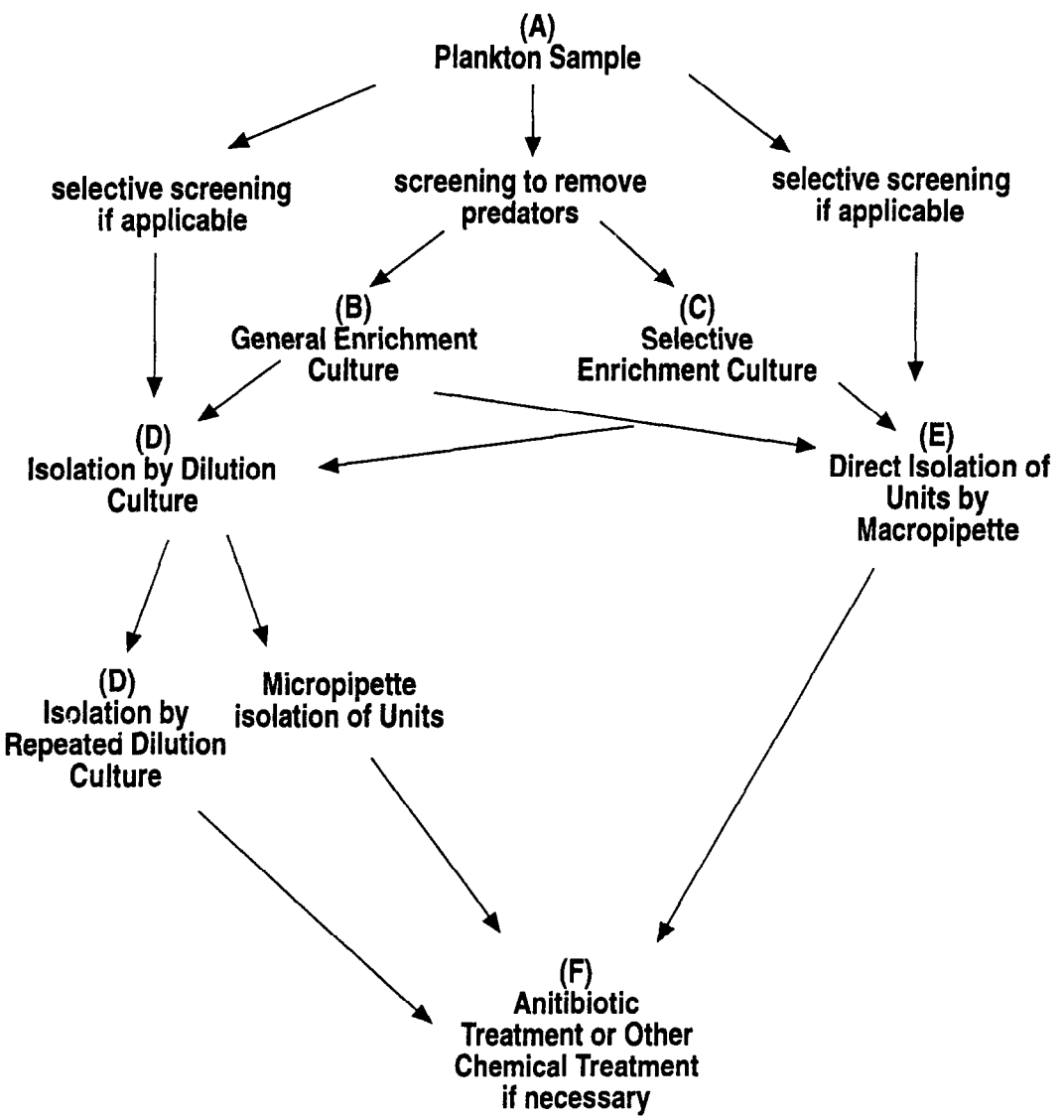

Figure 3.4. Alternate treatments of a phytoplankton sample collected for establishing cultures derived from single cells or colonies.

reduce the numbers of organisms larger or smaller than the ones desired. At any of the steps 3.4.B, C, D, or E the whole algal population, some part of it, or single cells, will have been put into a limited volume of culture and moved into a new physical environment. Light and temperature are the manipulable physical factors that can change the rates or timing of cell processes. The ideal for protocols B and especially D and E would be to duplicate natural circumstances unless there were *a priori* knowledge that something was less than favorable at the time of collection. Salinity is not usually changed from that of the sample. The basic dilution culture technique is described by Throndsen (1978).

MANIPULATIONS - ENRICHMENT CULTURES

General enrichment cultures (Fig. 3.4.B) are intended to support the entire phytoplankton population to provide adequate material for isolation by protocols D or E. The following nutrient additions are recommended, concentrations not to exceed those given: (inorganic) nitrate, 35 µm; ammonium (NH_4Cl), 10 µm; urea, 10 µm; sodium glycerophosphate, 3 µm; silicate (for diatoms or silicoflagellates only) 50 µm; trace metals, 1/10 the level in L1 (see Table 3.2); vitamins, 1/10 the level in L1 (see Tables 3.2 and 3.3). Because inorganic phosphate salts have occasionally been found inhibitory, presumably because of impurities, glycerophosphate is suggested (see Dzurica *et al.* (1989) for *Aureococcus anophagefferens* and Ohki *et al.*, (1986) for Pacific isolates of *Trichodesmium* species). Soil extracts are called for in some recipes and often found of benefit in isolation efforts. One procedure for making soil extract is essentially that of Gross (1932). For soil, use a good garden soil not recently fertilized, or a commercial potting soil, which may be easier to obtain in some locations. Autoclave (15-30 min) approximately equal weights of soil and (fresh) water and allow to stand a few days for sediment to settle to some extent. Clarify by decanting, filtering or centrifuging (or all three). The extract (supernatant) is re-autoclaved if necessary and stored sterile in the cold. Use 10-50 (usually 20) ml l^{-1} of final culture medium.

Selective enrichment cultures (Fig. 3.4.C) employ either physical or nutritional treatments to influence differentially the growth rates of various species in the sample. Hundreds of combinations are thus possible but logistically unmanageable, calling for educated guesses based on observations of the natural population. Nutritional aspects are easier to predict in some cases. Diatoms can be suppressed by omitting silicate and incubating in polycarbonate; if the sample water is very high in silicon then 1-10 mg/l (10^{-5} to 10^{-4}M) of germanium dioxide may be used to inhibit diatoms selectively (also silicoflagellates, presumably). Omitting ammonium will discriminate against species that cannot use nitrate, such as certain cryptomonads, euglenoids, and chlamydomonads. Some of these species tolerate ammonium concentrations toxic to most other algae - as high as 0.5mM. Thus ammonium (NH_4Cl) addition can be a positive selective agent at low levels (*ca.* 25mM) or a negative one, even at levels as low as 100 µM. Additions of glutamate, arginine, asparagine or other organic nitrogen compounds (10^{-6} to 10^{-8} M) are potentially useful additions (Mihnea, 1993; Dzurica *et al.*, 1989). Strains of *Synechococcus* may develop as contaminants in cultures of eukaryotic species when rigorously "clean" techniques are used; low levels of streptomycin (<25 mg l^{-1}) or penicillin eliminates them (as well as other cyanobacteria). Other selective treatments are referred to in Guillard (1973).

Special factors in enrichment. Trace elements, chelators, and vitamins call for special attention when making enrichment cultures. Both selenium and molybdenum are influenced little if at all by chelation. Selenium, as selenite (SeO_3^{2-}) is present in (undiluted) "L1" medium at 10^{-8} M, almost ten times the content of all selenium species in average seawater, estimated at 1.7×10^{-9} M (Brand, 1986). There is reason to suspect that for some species a ten-fold dilution can result in growth rate or crop limitations, or both. Species of the prymnesiophyte genus *Chrysochromulina* have long been known to have high Se requirements (see Granéli *et al.*, 1993; Harrison *et al.*, 1988). The "brown tide" organism *Aureococcus anophagefferens* grown

in citrate chelated artificial seawater "Aquil" showed crop limitation below 10^{-8} M Se (at low Fe) and 10^{-9} M Se (at 10^{-5}M Fe). Growth rate was markedly enhanced at high Se + high Fe (Cosper *et al.*, 1993). As a general rule it is prudent to keep added selenite at *ca.* 10^{-8} to 10^{-9} M even in enrichment and dilution cultures.

Undiluted "L1" medium contains molybdenum at about the average molar concentration in seawater - estimated as 1.1 x 10^{-7} M (Brand, 1986). Cobalt additions significantly increased the yields of *Chrysochromulina polylepis* grown in Atlantic seawater "spiked" with nitrate and phosphate (Granéli *et al.*, 1993); this occurred whether the cobalt addition was as the ion (0.2 - 3 x 10^{-9} M) or as vitamin B_{12}. Most seawater media have added cobalt as chelated Co^{2+} in the rather narrow range 10^{-7} - 10^{-8} M and vitamin B_{12} at concentrations varying from almost 10^{-5} M to 10^{-10} M. The requirements for B_{12} in culture are very small: half-saturation constants for growth range from 0.1 to 2.8 ngl^{-1} (10^{-13} - 10^{-12} M) (Swift, 1980). Insofar as dilution of the other vitamins is concerned, the only guides are, that for thiamine-limited *Pavlova lutheri* cell quotas and growth half saturation concentrations were 10^{-19} mol $cell^{-1}$ and 4 x 10^{-10} M. For biotin-limited growth of the dinoflagellate *Amphidinium carterae* these values were respectively 1.8 x 10^{-19} mol $cell^{-1}$ and 1.6 x 10^{-11} M (Carlucci and Silbernagel, 1969).

The nature and amount of chelator used in enrichment cultures can have extremely selective effects. The role played by the chelator is to maintain or create a favorable trace metal environment for the cells (Sunda, 1988/1989). In the case of most trace metals this is accomplished by the buffering action of the added chelator-trace metal mix on the concentrations of the free metal ions, or of inorganic ionic species proportional to them. The case of iron differs significantly in that the biologically active ionic iron species derive only in small part or indirectly from dissociation of the Fe (III)-chelator complex according to the thermodynamic equilibria. They must derive from photochemical reactions in which the chelator is oxidized and decomposed while the iron is reduced to Fe (II), from which usable Fe (III) species or colloids are rapidly formed (Price *et al.*, 1988/1989; Wells, 1988/1989). Chelators most often employed are EDTA (ethylenedinitrilotetraacetic acid), NTA (nitrilotriacetic acid), and citric acid, all usually specified as the sodium salts. EDTA is the strongest complexer and the one most commonly employed, NTA is less commonly used, while citric acid is the weakest and has a longer history of use than the other two chelators. Empirically it has been found that the addition of citrate to enrichment cultures (or even growth media) having other chelators favors some organisms. The experience with the difficult *Aureococcus anophagefferens* is relevant. It was obtained into unialgal culture in sterile filtered local seawater enriched as f/2 medium (Table 3.2) with glycerol phosphate replacing inorganic phosphate (Cosper *et al.*, 1987). Yet, in a commercial synthetic seawater base ("Instant Ocean") it was necessary to use either NTA or citrate rather than EDTA (at equal molar concentrations) to obtain reasonable growth even with glycerol phosphate replacing inorganic phosphate (Dzurica *et al.*, 1989). Further, Cosper *et al.* (1993) found that citrate was the most effective chelator when used in chemically defined variations of medium Aquil, most likely due to the greater availablity of iron which is much less well chelated by citrate.

Processing enrichment cultures. Observation of all enrichment cultures should begin as soon as 24 hours and continue daily because population changes can be very rapid. Direct isolation of cells by micropipette (Fig. 3.4.E) is the method of choice for large single-celled species whether motile or not and generally necessary for colonial forms such as *Phaeocystis, Trichodesmium* or chain forming diatoms or dinoflagellates. The general process has been described by Guillard (1973), Hoshaw and Rosowski (1973) and Guillard and Keller (1984). Glassware is best dry sterilized in an oven (2 hr at 160°C) to be particle-free. First, place a well or depression slide on the stereo microscope stage and add sterile particle-free "washing" fluid to the wells with a sterile Pasteur pipette; add a drop of culture to the first well. If there are too many cells, move a drop to the next well, and repeat until algal units are separated enough to facilitate capture of one or only a few cells. Locate the algal unit desired under the lowest usable magnification. Before attempting to isolate it, touch the end of the capillary into a nearby well (drop) of sterile medium to draw a little liquid (*ca.* 1 cm) into it as a "cushion". Flagellate cells that show a strong phototactic response can be concentrated and often freed of other species by

allowing them to swim in response to light across a Petri dish or other flat vessel containing sterile medium.

MANIPULATIONS - ANTIBIOTICS

Three somewhat different approaches of antibiotic treatment are described here (Fig. 3.4.F). The first "hunt and wash" method (see Guillard, 1973) makes antibiotics an adjunct to micropipette isolation. A moderate inoculum of healthy and rapidly growing algal culture is transfered to fresh medium (50 ml in 125 ml flasks) containing a tolerable level of antibiotics, and at several convenient times from 18 - 72 hours after exposure, one or a small number of cells is transfered by micropipette to each of several tubes of fresh medium (without antibiotics). There is a good chance that some tubes will then be bacteria-free (or at worst have fewer types of bacterial contaminants). The basic antibiotic solution recommended for this purpose is made as follows: dissolve 100 mg penicillin G (Na or K salt), 25 mg dihydrostreptomycin sulfate, and 25 mg gentamycin in 10 ml distilled (or deionized) water and sterilize by membrane filtration. Keep frozen until used (polycarbonate tubes are best).The "standard" dose is 0.5 ml per 50 ml of medium; try 0.25, 0.5, 1, 2 ml per flask.

The basic idea of the second method (Droop, 1967) is to dilute a strong antibiotic mix (made up in algal growth medium) using a dense but still rapidly growing algal culture as diluent. Do this in two-fold dilution steps so that the concentration of antibiotics is halved at each step while the concentration of algae is constant. Very small transfers are made from each tube of the series at 24 and 48 hr into separate tubes of medium without antibiotics, and these are incubated. At some point in the decreasing series of antibiotics the algae are (hopefully) still alive while the bacteria are dead. Droop (1967) used mixes of four antibiotics. Berland and Maestrini (1969) provide excellent quantitative data on the effects of 25 antibiotics on five diatoms.

A third approach is sequential transfer of the algal culture through a series of flasks of media containing different antibiotics at levels permitting algal growth. The aim is to lose the bacteria by attrition. Cottrell and Suttle (1993) thus purified the tiny flagellate species *Micromonas pusilla* using the following sequence of antibiotics: penicillin, 1 gl^{-1}; neomycin, 250 mg l^{-1}; gentamycin, 1 gl^{-1}; kanamycin, 0.5 or 1 gl^{-1}. The timing of exposure was critical.

No realistic recommendations can presently be given regarding choice of antibiotic treatments for purifying marine nuisance algal species. If forced to make a choice among available antibiotics for single or multiple use, a reasonable combination at present might include: 1. penicillin G, ampicillin, or the much more expensive carbenicillin.; 2. cephaloridine, which is moderately expensive, or cefotaxime; 3. gentamycin, about the same cost; 4. kanamycin, streptomycin, or neomycin - all inexpensive; 5. bacitracin, likewise inexpensive.

An axenic culture in usual practice means "without demonstrable unwanted prokaryotes or eukaryotes". Demonstrations ordinarily include microscopic examination and inoculation from the culture under test into enrichment media. The basic sterility test medium now suggested, enrichment L1pm (derived from its predecessor f/2 p), is given in Table 3.1. Tubes (or plates of medium solidified with agar, if used) should be examined frequently beginning *ca.* 48 hr. after inoculation and kept for about 21 days, or a month for cold water organisms.

MAINTENANCE OF STOCKS AND PRODUCTION

Success in maintaining stock cultures demands an unfailing year-round routine of preparing fresh medium and making transfers to it from the initial "parent" culture, using an inoculum of appropriate size. It is advantageous and usually possible to schedule rapidly growing species for transfer once a week, slower growing ones at two week intervals, and many larger and slow growing algae (some dinoflagellates or chloromonads) at three week intervals. The most

important point is to transfer before half the total life span of a culture has passed. A culture transferred on a weekly basis should last one, or preferably two more weeks if moved to dim light. It is often possible to keep both "parent" and "grandparent" cultures for security in case of equipment failure or other accidents (see Guillard (1975 or 1983) for details. Mass culturing techniques are not treated here.

MEDIA FOR MAINTENANCE OF CULTURES

Enriched natural seawater. Hundreds of recipes have been published for enriched natural or artificial seawater. Examination of these shows two things: first, remarkable similarity, suggesting convergence on a generally acceptable formulation, and second, one difference that can be taken as significant - an order of magnitude difference in the level of EDTA. Enrichments GPM (Loeblich, 1975) and K (Keller *et al.*, 1987) have EDTA at 1.3 x 10^{-4} and 10^{-4} respectively, while IMR (Eppley *et al.*, 1967) f/2 (Guillard, 1975) ESNW of (Harrison *et al.*, 1980) and BWM (Brand, 1986) all employ EDTA at about 10^{-5} M. The media with higher EDTA were developed for small oceanic species (K) or the dinoflagellate *Heterocapsa (Cachonina) niei* (GPM), and both have proven effective for oceanic species and a number of dinoflagellates. Medium K is not well suited to many coastal or estuarine species, though some can be adapted to it. Media with the lower EDTA concentration (10^{-5} M) have been laboratory standards for decades.

Enrichment L1 (Table 3.2) is suggested as a general purpose enrichment with 10^{-5} M EDTA; it is an improved version of f/2, and like f/2 has too much iron (equimolar with EDTA), but works very well in spite of this, perhaps indeed because of it. The resulting precipitate (especially in autoclaved media) may adsorb other metals or contaminants and serve as a "solid phase buffer". Table 3.3 gives additional directions for making vitamin primary and working stock solutions. Quantities for major element working stocks (liter lots) and for trace element primary stocks (in 100 ml lots) and trace metal working stocks (liter lots) are included in Table 3.2.

A more highly chelated medium that can easily be used as the basis for a K-like medium is enrichment L2, presented in Table 3.4; it is L1 with the EDTA raised to 10^{-4} M. It has been used successfully for *Prorocentrum lima* (Sohet *et al.*, 1993) and other epiphytic dinoflagellates (Bomber and Aikman, 1988/1989). Medium K substitutes Na glycero PO_4 (at 10^{-5} M) for the 3.63 x 10^{-5} M $NaH_2PO_4.H_2O$ of media f/2 or L1. Table 3.5 contains directions for a stock solution of Na_2 glycero PO_4 that can be used at concentrations in this range or for enriching natural collections. Medium K also adds 5 x 10^{-5} M NH_4Cl; Table 3.5 gives stock solutions of NH_4Cl suitable for K or other enrichments. Medium h/2 (Guillard, 1975;1983), which has been used for certain cryptomonads or estuarine green algae that cannot use nitrate, contains 5 x 10^{-4} NH_4Cl. Note that most coastal and oceanic algae show inhibition at around 10^{-4} M. Medium h/2 is f/2 with added NH_4Cl, hence the algae referred to above can be kept in L1 + NH_4Cl. Enrichments like L1 or L2 may be too concentrated for some species when they are first brought into culture; some adaptation may be necessary. It is usually best to make dilutions by adding sterile L1 or L2 to seawater autoclaved separately. *Phaeocystis* will only remain in colonial form if subcultured in L1 (or f/2) diluted at least 10-fold (20-fold is better). *Ceratium ranipes* loses the characteristic "fingers" at the ends of its horns if kept in concentrated media, but keeps them in f/50 (Brand and Guillard, unpublished).

Artificial seawaters. Recipes for artificial seawaters - sometimes referred to as "defined marine media" have also been produced in great numbers. Synthetic seawaters of two types have developed. Two of the first type - imitations of natural seawater - are Aquil and its few modifications (Price *et al.*, 1988/1989) and ESAW (Harrison *et al.*, 1980). The contents of both are compared by Brand (1990, Table 4). (Note that both are now enriched with selenite also, at *ca.* 10^{-8} M.). The other type of synthetic seawater medium, having ionic ratios differing from that of natural seawater, include the ASM and ASP series developed by Provasoli and co-

Table 3.2. Composition of L1, an enrichment of seawater (Guillard and Hargraves, 1993)[1]

Major Nutrients

Compound	Working Stock[2] gm l^{-1}	Final Concentration mol l^{-1}	Final Concentration in seawater medium[3] mol l^{-1}
$NaNO_3$	75	8.83×10^{-1}	8.83×10^{-4}
$NaH_2PO_4 \cdot H_2O$	5	3.63×10^{-2}	3.63×10^{-5}
$Na_2SiO_3 \cdot 9H_2O$[4]	30	1.07×10^{-1}	1.07×10^{-4}

Trace Elements

	gm/100ml	Primary Stock[2] mol l^{-1}	Working[2][5] Stock	Final conc. in medium (mol l^{-1})[3]
$FeCl_3 \cdot 6H_2O$[5]	- -	- -	3.15 gl^{-1}	1.17×10^{-5}
$Na_2EDTA \cdot 2H_2O$[5]	- -	- -	4.36 gl^{-1}	1.17×10^{-5}
$MnCl_2 \cdot 4H_2O$	18.0	9×10^{-1}	9.0×10^{-4} mol l^{-1}	9.0×10^{-7}
$ZnSO_4 \cdot 7H_2O$	2.2	8×10^{-2}	8.0×10^{-5} mol l^{-1}	8.0×10^{-8}
$CoCl_2 \cdot 6H_2O$	1.0	5×10^{-2}	5.0×10^{-5} mol l^{-1}	5.0×10^{-8}
$CuSO_4 \cdot 5H_2O$[6]	0.245	4×10^{-2}	1.0×10^{-5} mol l^{-1}	1×10^{-8}
$Na_2MoO4 \cdot 2H_2O$[6]	1.99	3×10^{-2}	9×10^{-5} mol l^{-1}	9×10^{-8}
H_2SeO_3[6][7]	0.13	10^{-2}	10^{-5} mol l^{-1}	10^{-8}
$NiSO_4 \cdot 6H_2O$	0.27	10^{-2}	10^{-5} mol l^{-1}	10^{-8}
Na_3VO_4	0.184	10^{-2}	10^{-5} mol l^{-1}	10^{-8}
K_2CrO_4	0.194	10^{-3}	10^{-6} mol l^{-1}	10^{-9}

Vitamins[8]

Thiamine. HCl		100 mgl^{-1}	3×10^{-7}
Biotin		500 mgl^{-1}	2.1×10^{-9}
B_{12}		500 mgl^{-1}	3.7×10^{-10}

(1) This version of the f enrichment has additional trace elements and changed levels of Cu and Mo. Complete directions for preparing f/2 and comments on other modifications are in Guillard (1975, 1983).

(2) Make stocks in distilled or deionized water.

(3) Natural seawater of appropriate salinity or suitable artificial seawater base. Use 1 ml of all working stocks per liter of final enriched sewater.

(4) Add silicate only for diatoms or silicoflagellates.

(5) Separate iron and EDTA additions can be replaced by 5 g ml^{-1} of ferric EDTA (13% Fe) as described in the f/2 formulation.

(6) Working stock of trace elements is made by adding EDTA and iron (or ferric EDTA) first, then using 1 ml l^{-1} of all the L1 primary stock trace element solutions. (If the f/2 primary stock solutions are available, use the f/2 Cu primary stock at 0.25 ml per liter of L1 working stock and the f/2 Mo primary stock at 3 ml per liter of L1 working stock. The Fe, EDTA, Mn Zn and Co levels in f/2 and L1 are the same.) Working stocks of the complete L1 trace element solution should be made in small batches or, if made in large volumes, be dispensed in smaller lots and kept sterile until opened for use in order to prevent bacterial decomposition of the selenite.

(7) Equivalent concentrations of anhydrous sodium selenite (Formula Weight 172.94) or sodium selenite pentahydrate (FW 263.02) may be used in the primary stocks instead of selenious acid.

(8) Directions for making vitamin stocks are given in Table 3.3.

liter of L1 working stock. The Fe, EDTA, Mn Zn and Co levels in f/2 and L1 are the same.) Working stocks of the complete L1 trace element solution should be made in small batches or, if made in large volumes, be dispensed in smaller lots and kept sterile until opened for use in order to prevent bacterial decomposition of the selenite.

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(8) Directions for making vitamin stocks are given in Table 3.3.

Table 3.3. Vitamin Primary Stock Solutions and Vitamin Working Stock Solutions (1)

Primary stock solutions: Biotin is obtained in crystalline form; allow for about 4% water of crystallizaton. A primary stock solution is made containing 0.1 mg/ml by weighing about 10mg and adding distilled water, 9.6 ml for each mg of biotin. Make the solution slightly acid if it is to be autoclaved. Keep the solution sterile and frozen.

Vitamin B_{12} in similarly obtained as crystals, and 11% should be allowed for water of crystallization. Weigh (or buy in weighed amounts) and make a primary stock solution having 1 mg/ml. Acidify the solution if it is to be autoclaved, and keep sterile and frozen.

Primary stocks can be put up in ampules, or screw-capped test tubes or flasks.

Vitamin working stock solution: Bring 1.0 ml of biotin primary stock and 0.1 ml of B_{12} primary stock to 200 ml and add 20 mg of thiamine HCl. No primary stock of thiamine is needed.

The vitamin stock solution is dispensed in 1, 2, or 5 ml lots in ampules or in 10 ml lots in screw-capped test tubes, autoclaved, and then stored in a refrigerator. Use 1 ml per liter of final medium. It is usually added before autoclaving. Keep opened containers sterile or refrigerated.

(1) Made according to the f/2 directions (Guillard, 1983) except at half that concentration so that all L1 working stocks are used at 1 ml l^{-1} (The f/2 vitamin stock was used at 1/2 ml l^{-1}).

Table 3.4. Enrichment L2

Algal enrichment L1(1)	1 liter
$Na_2.EDTA.2H_2O$(2)	32.86 mg

(1) Seawater algal medium L1 according to Table 4. 2.

(2) Prepare a stock solution of 3.286 g $Na_2.EDTA.2H_2O$ per 100 ml of distilled or de-ionized water and use 1 ml l^{-1} of final L2 medium to yield 10^{-4}M EDTA.

Alternatively, a separate L2 trace metal stock solution can be made containing the 10^{-4} M (EDTA (as disodium EDTA). The iron remains at 1.17×10^{-5} M.

Table 3.5. Stock solutions of sodium glycerophosphate and ammonium chloride.

Compound	Primary stock, mg/100 ml in distilled water
Na_2.glycero phosphate.H_2O(1)	234.1 mg
NH_4Cl(2)(3)	267.5 mg

(1) 1 ml of this stock per liter of final medium yields 10^{-5}M phosphorus.
(2) 1 ml of this stock per liter of final medium yields $5x10^{-5}$M nitrogen.
(3) If NH4Cl is added to seawater and autoclaved in 50 ml lots in 125 ml flasks, 25-30% of the ammonia is lost. If the amount of ammonium ion is critical, add NH_4Cl aseptically from a stock solution autoclaved separately.

Table 3.6. Artificial seawater base AK, modified, from Keller *et al.* (1987).

Compound	Concentration (mol l^{-1})	Weight l^{-1} (rounded)
NaCl	4.1×10^{-1}	24 g
KCl	8×10^{-3}	0.6 g
$MgCl_2 \cdot 6H_2O$(1)	1.48×10^{-2}	3.0 g
$MgSO_4 \cdot 7H_2O$(1)	2.03×10^{-2}	5.0 g
$CaCl_2 \cdot 2H_2O$(1)	2.7×10^{-3}	0.4 g
Na Br	10^{-5}	1.03 mg
$SrCl_2 \cdot 6H_2O$(1)	10^{-4}	27 mg
H_3BO_3(2)	9.7×10^{-5}	6 mg
NaF	10^{-5}	0.42 mg
KI	2×10^{-7}	33 mg
$NaHCO_3$(3)	2.38×10^{-3}	0.2 g

(1) Dissolve the hydrated salts separately in distilled or de-ionized water and add to the other compounds (except for $NaHCO_3$) already mixed.
(2) Ten times the concentration in AK.
(3) Keller *et al.* (1987) added the $NaHCO_3$ just before autoclaving and adjusted the pH to 7.0 with HCl. After autoclaving and equilibrating with air, the pH returned to *ca.* 8.0. This lowers the alkalinity by some indeterminate amount. If this is undesirable for some reason, the $NaHCO_3$ can be added and the pH lowered with bubbled CO_2 as in the preparation of Aquil, or by adding additional equimolar amounts of $NaHCO_3$ and HCl, as in the preparation of ESAW.

workers (Provasoli *et al.*,1957), as well as several formulations by Droop and others (Droop, 1969). Guillard and Keller (1984, Table VI) compare Aquil and ESAW with $ASP8_a$, ASP_{12} and NH-15. For all of them, the important differences lie in the minor elements and organic additions. For example, it may be significant that of media in Guillard and Keller's (1984) Table 4, only NH-15 listed selenite in its contents; it was designed for the dinoflagellate *Alexandrium monilatum,* which had been difficult to culture-but which grows very well in L1 medium.

The following approach is suggested. First, make an appropriate mix of the major and minor conservative elements of seawater, for example as described by Harrison *et al.* (1980, Table 2) or Keller *et al.* (1987, Table 4, corrected). Next, enrich this as if it were natural seawater, adding sources of N, P, Si, vitamins and one of the trace element solutions L1 or L2 (Tables 3.2 or 3.3) as deemed appropriate. Then sterilize by microwave or by using TRIS or other options, when medium is autoclaved (see Harrison *et al.*, 1980; Price *et al.*, 1988/1989). Table 3.6 is a suggested recipe based on medium AK referred to above.

Commercial seawater preparations. A number of useful preparations are commercially available. Some companies now supplying materials available on a world wide basis are listed (alphabetically) here. There are doubtless more:

Aquarium Systems Inc., 8141 Tyler Blvd., Mentor, Ohio 44060 U.S.A., Tel.: 216-255-1997.
"Instant Ocean" is a mix designed to be added to fresh water (tap or purified). For growing algae it should be treated as natural seawater and enriched. Its largest use is probably for maintaining animals in commercial aquaria though it is also used for growing algae as food.

Argent Chemical Laboratories, 8702 152nd Avenue N.E., Redmond, Washington 98052 U.S.A., Tel.: 206-855-3777, Telex: 269161 ACLI UR, Fax: 206-855-2112.
Argent sells "Nusalts" in three forms: Type I (basic); Type II (plus silicate) and Type III (plus ammonium chloride). All are designed to be added to seawater. Argent also sells component chemicals separately in both laboratory quantities and in bulk.

Fritz Industries, P.O. Drawer 17040, Dallas, Texas 75217 U.S.A., Tel.: 214-285-5471.
Two preparations are available as "Fritz salts". One is a nutrient mix (essentially f/2) to be added to seawater; the other is a complete artificial seawater mix (including the algal nutrients) to be added to tap or purified water. Ammonium or silicate additions can be provided.

Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178-9916 U.S.A., Tel.: 1-800-325-3010 (U.S. and Canada), Fax: 1-800-325-5052 (U.S. and Canada), Tel.: 314-771-5750 (Abroad, call collect), Fax: 314-771-5757.
Sigma supplies several freshwater and marine media as well as components. Among them are dry salt mixes for enrichment f/2 with or without silicate, and liquid concentrates of the same (to be used 20ml per liter of seawater). Enrichment K and complete artificial medium AK are similarly supplied, also offered are two of Provasoli's artificial seawaters (ASP_6 and ASP_{12}). Other synthetic seawaters, sterile seawater, charcoal-treated Gulf Stream seawater, vitamin mixes and antibiotic mixes are also available through the Sigma Plant Culture catalog (1994 edition).

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4. Estimating Cell Numbers

J. Throndsen

SERIAL DILUTION CULTURE (SDC) METHOD

The present method has proven convenient especially for the isolation of bloom-forming species. The principle of the method is to dilute the sample until only one specimen is left in the subsample (which is used as inoculum for cultures). Keeping track of the dilution, e.g. by doing it stepwise, also offers the opportunity to make estimates of the original cell number. By using 3 to 10 parallel series of dilutions and applying statistics to the obtained pattern of presence and absence in each dilution, the probability of the estimate may be calculated. Presence and absence is revealed by growth or lack of growth in the tubes. To facilitate growth, the whole dilution series is conducted with a suitable medium (see Chapter 3; Enrichment Media).

For toxic algal blooms, quantitative estimates are often more easily made by direct counts (see Counting), whereas it may be important to bring the species into culture for further studies. Among the harmful algae which may be brought into culture by SDC are *Prymnesium, Chrysochromulina, Heterosigma, Aureococcus, Nannochloropsis* and single celled stages of *Phaeocystis.* The SDC method can easily be combined with different media to find the most suitable one for a particular species under the defined laboratory conditions.

The syringe SDC technique may be adapted for inoculation into larger volumes of media and/or increasing the number of tubes at each step (up to 8 with the 10 ml syringe used as standard). Changing the volumes of the inocula or the size of the syringe used for the dilution, will offer a variety of dilution possibilities. However, the cell distribution achieved in each type of syringe used, has to be checked. Alternatively dilutions can be made by pipetting, with thorough mixing between the extraction of the inoculum for the next step.

The following procedure for making a dilution culture series is carried out with simple equipment, and the initial steps (inoculation) can be performed in 5-10 minutes, on location. The important precautions to be taken are: to use clean equipment for culturing, to keep temperature and salinity well within the tolerance limits of the species, and to choose the appropriate medium. The fulfillment of the latter condition may be a matter of trial and error, but some media are more universal than other (e.g. Erd-Schreiber). Before deciding upon the medium to be used, consult chapter 3 on culturing methods. Another important measure to be taken to provide optimal culturing conditions is to prepare the medium from water sampled together with the inoculum. The rationale is that the organisms in question are adapted to this milieu, that possible hostile fellow organisms will be killed during the heating of the medium, and that nutrient depletion which may be substantial at least under late bloom conditions, will be compensated for by the addition of nutrients for the growth medium.

Standard setup

For a standard SDC series, use a test tube rack which holds 30 (or more conveniently 40-50) test tubes in rows of five. Fill 25 test tubes each with 9-10 ml of growth medium, and mark the tubes for five dilution steps of five parallels, e.g. 1I, 1II, 1III, -- (meaning first dilution step, parallel one, two and three, --), 2I, 2II, 2III --, 3I, -----, to 5I, -----, at which step the inoculum will be 0.1 µl. Five tubes for performing the dilutions (Fig. 4.1D) are filled completely (15-20 ml). A disposable 10 ml sterile syringe, preferably with an eccentric opening, and graduated in divisions of 1 ml, is also necessary. The amount of medium required for each SDC series is

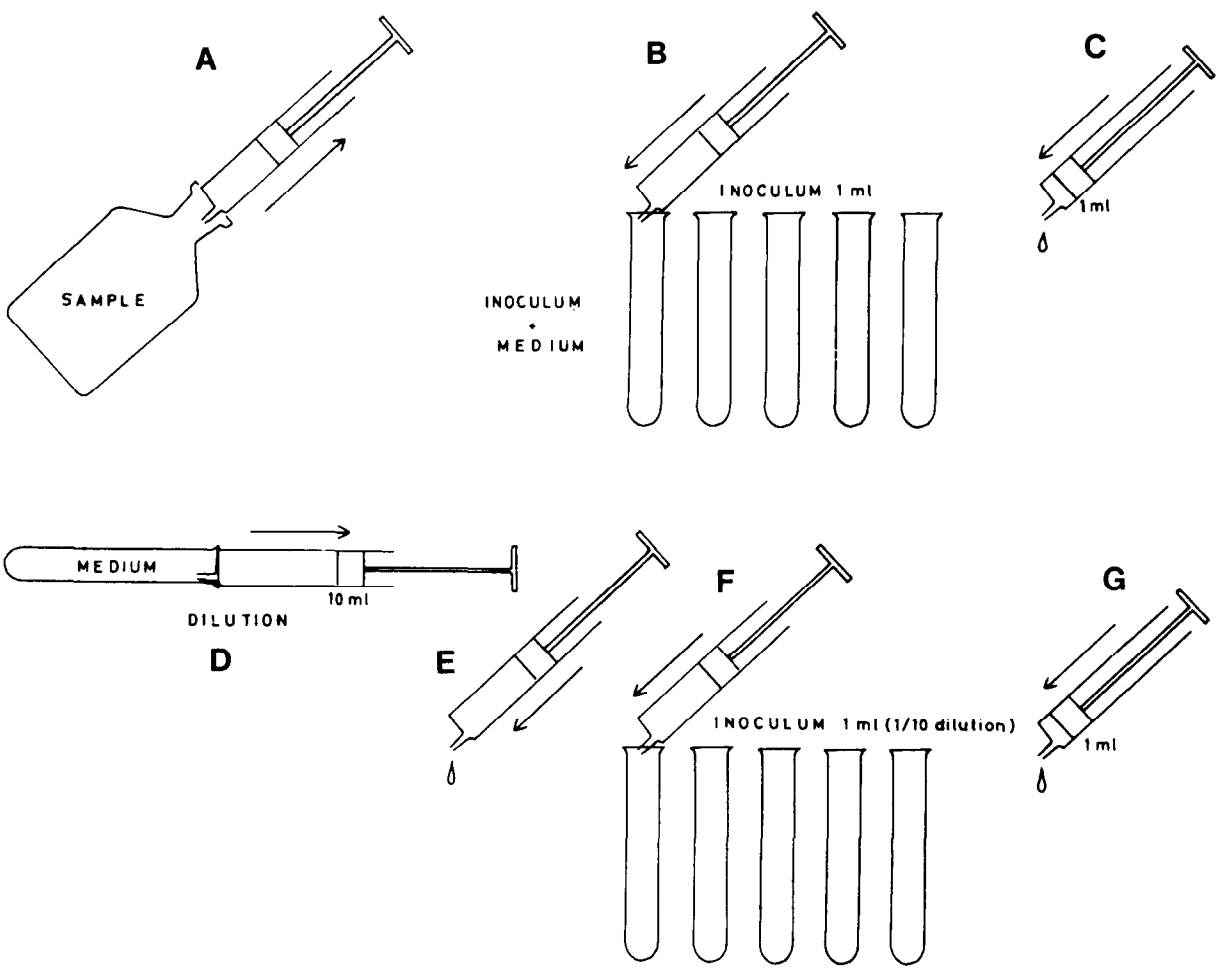

Figure 4.1. Serial dilution culture method, dilutions by syringe. A - subsampling from original water sample, B - inoculation of 1 ml into five test tubes/growth tubes each with 9-10 ml of medium, C - expelling all but one ml to prepare for the next dilution, D - making 1/10 dilution by sucking in fresh medium from a test tube filled with medium, E - expelling one ml to ensure the first inoculum to be equal to the rest in F - inoculation of 1 ml (diluted sample) into five test tubes/growth tubes each with 9-10 ml of medium, G - expelling all but one ml to prepare for the next dilution. Repeat steps D - G for as many dilution steps as wanted. Illustration rearranged from Throndsen (1978).

approximately 300 ml, but it may be wise to prepare excess in order to have a suitable medium for possible subculturing of interesting species which grow in the SDC.

Material to be used for initiating SDC should be collected with a non-toxic water sampler and handled carefully to avoid temperature, salinity and light shocks.

Procedure

Before starting inoculation, ensure that the temperature and salinity conditions are the same in the sample and the medium, and that all equipment/accessories needed are at hand to facilitate a quick accomplishment of the procedure.

Start by rinsing the syringe with sample water, and then (Fig. 4.1):
A) draw in 10 ml of the water sample into the syringe,
B) dispense 1 ml into 5 of the first tubes,
C) dispose of all but the last 1 ml from the syringe,
D) draw in 9 ml of medium from one of the full tubes,
E) dispose of 1 ml.

Repeat steps B-E (Figs 4.1F, G) for the next 4 dilution steps.

Incubation

The culture series should be placed under fluorescent tubes or in daylight at an irradiance of about 10 % of full daylight; lower light intensities may be better for deep-sea plankton or benthic species. More detailed information on incubation light intensities can be found in the previous chapter 3 on culturing methods. Take care to keep the temperature variation within the tolerance limits of the phytoplankton species which are under consideration. Examination (by optical microscopy) of the cultures after 4 and 6 weeks will often be sufficient for routine work in temperate areas. For microalgae from tropical and subtropical areas, the first examination ought to take place after 2 weeks, whereas Arctic and Antarctic dilution cultures may need up to 2 month (at 2-3 ^{o}C) before cell densities sufficient for further examination under the microscope are established.

Results

The expected result of a dilution culture series is growth of a variety of organisms present in the original sample, with the most abundant species established as unialgal cultures at higher dilutions within the series. Also, in tubes with more than one species present, a particular species may dominate, and by further dilution can give rise to unialgal cultures. For qualitative and quantitative purposes, the presence or absence of different species is noted for each of the tubes, starting with the most diluted. These presence and absence data can then be referred to tables for the most probable number - MPN, Table 4.1. When estimating cell numbers from Table 4.1, use the set of three successive dilution steps which gives the highest MPN, but make sure that growth has been recorded in at least two of the steps. Note that the SDC method only records cells viable under the culture conditions offered, thus MPN will be minimum values, with a standard deviation of +/- 20-50 % of the mean estimate.

Table 4.1. Most probable number (MPN) in original sample. To use the table compare the presence and absence of the species in question with the values below. Choose the three most diluted steps in which growths occur, for the estimate. See, however, also the text for advice. (Table based on data from Anonymous, 1955).

Growth in inoculum			MPN cells·ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
5	5	5	>24000	>2400	>240
5	5	4	16000	1600	160
5	5	3	9200	920	92
5	5	2	5400	540	54
5	5	1	3500	350	35
5	5	0	2400	240	24
5	4	5	4300	430	43
5	4	4	3500	350	35
5	4	3	2800	280	28
5	4	2	2200	220	22
5	4	1	1700	170	17
5	4	0	1300	130	13
5	3	5	2500	250	25
5	3	4	2100	210	21
5	3	3	1800	180	18
5	3	2	1400	140	14
5	3	1	1100	110	11
5	3	0	790	79	7.9
5	2	5	1800	180	18
5	2	4	1500	150	15
5	2	3	1200	120	12
5	2	2	950	95	9.5
5	2	1	700	70	7
5	2	0	490	49	4.9
5	1	5	1300	130	13
5	1	4	1100	110	11
5	1	3	840	84	8.4
5	1	2	640	64	6.4
5	1	1	460	46	4.6
5	1	0	330	33	3.3
5	0	5	950	95	9.5
5	0	4	760	76	7.6
5	0	3	580	58	5.8
5	0	2	430	43	4.3
5	0	1	310	31	3.1
5	0	0	230	23	2.3

Growth in inoculum			MPN cells·ml -1		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
4	5	5	810	81	8.1
4	5	4	720	72	7.2
4	5	3	640	64	6.4
4	5	2	560	56	5.6
4	5	1	480	48	4.8
4	5	0	410	41	4.1
4	4	5	690	69	6.9
4	4	4	620	62	6.2
4	4	3	540	54	5.4
4	4	2	470	47	4.7
4	4	1	400	40	4.0
4	4	0	340	34	3.4
4	3	5	590	59	5.9
4	3	4	520	52	5.2
4	3	3	450	45	4.5
4	3	2	390	39	3.9
4	3	1	330	33	3.3
4	3	0	270	27	2.7
4	2	5	500	50	5.0
4	2	4	440	44	4.4
4	2	3	380	38	3.8
4	2	2	320	32	3.2
4	2	1	260	26	2.6
4	2	0	220	22	2.2
4	1	5	420	42	4.2
4	1	4	360	36	3.6
4	1	3	310	31	3.1
4	1	2	260	26	2.6
4	1	1	210	21	2.1
4	1	0	170	17	1.7
4	0	5	360	36	3.6
4	0	4	300	30	3.0
4	0	3	250	25	2.5
4	0	2	210	21	2.1
4	0	1	170	17	1.7
4	0	0	130	13	1.3

Growth from inoculum			MPN cells·ml -1		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
3	5	5	450	45	4.5
3	5	4	410	41	4.1
3	5	3	370	37	3.7
3	5	2	320	32	3.2
3	5	1	290	29	2.9
3	5	0	250	25	2.5
3	4	5	400	40	4
3	4	4	360	36	3.6
3	4	3	320	32	3.2
3	4	2	280	28	2.8
3	4	1	240	24	2.4
3	4	0	210	21	2.1
3	3	5	350	35	3.5
3	3	4	310	31	3.1
3	3	3	280	28	2.8
3	3	2	240	24	2.4
3	3	1	210	21	2.1
3	3	0	170	17	1.7
3	2	5	310	31	3.1
3	2	4	270	27	2.7
3	2	3	240	24	2.4
3	2	2	200	20	2
3	2	1	170	17	1.7
3	2	0	140	14	1.4
3	1	5	270	27	2.7
3	1	4	230	23	2.3
3	1	3	200	20	2
3	1	2	170	17	1.7
3	1	1	140	14	1.4
3	1	0	110	11	1.1
3	0	5	230	23	2.3
3	0	4	200	20	2
3	0	3	160	16	1.6
3	0	2	130	13	1.3
3	0	1	110	11	1.1
3	0	0	78	7.8	0.78

Growth in inoculum			MPN cells·ml -1		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
2	5	5	320	32	3.2
2	5	4	290	29	2.9
2	5	3	260	26	2.6
2	5	2	230	23	2.3
2	5	1	200	20	2
2	5	0	170	17	1.7
2	4	5	280	28	2.8
2	4	4	250	25	2.5
2	4	3	230	23	2.3
2	4	2	200	20	2
2	4	1	170	17	1.7
2	4	0	150	15	1.5
2	3	5	250	25	2.5
2	3	4	220	22	2.2
2	3	3	200	20	2
2	3	2	170	17	1.7
2	3	1	140	14	1.4
2	3	0	120	12	1.2
2	2	5	220	22	2.2
2	2	4	190	19	1.9
2	2	3	170	17	1.7
2	2	2	140	14	1.4
2	2	1	120	12	1.2
2	2	0	93	9.3	0.93
2	1	5	190	19	1.9
2	1	4	170	17	1.7
2	1	3	140	14	1.4
2	1	2	120	12	1.2
2	1	1	92	9.2	0.92
2	1	0	68	6.8	0.68
2	0	5	160	16	1.6
2	0	4	140	14	1.4
2	0	3	120	12	1.2
2	0	2	91	9.1	0.91
2	0	1	68	6.8	0.68
2	0	0	45	4.5	0.45

Growth in inoculum			MPN cells·ml -1		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
1	5	5	240	24	2.4
1	5	4	220	22	2.2
1	5	3	190	19	1.9
1	5	2	170	17	1.7
1	5	1	150	15	1.5
1	5	0	130	13	1.3
1	4	5	220	22	2.2
1	4	4	190	19	1.9
1	4	3	170	17	1.7
1	4	2	150	15	1.5
1	4	1	130	13	1.3
1	4	0	110	11	1.1
1	3	5	190	19	1.9
1	3	4	170	17	1.7
1	3	3	150	15	1.5
1	3	2	130	13	1.3
1	3	1	100	10	1
1	3	0	83	8.3	0.83
1	2	5	170	17	1.7
1	2	4	150	15	1.5
1	2	3	120	12	1.2
1	2	2	100	10	1
1	2	1	82	8.2	0.82
1	2	0	61	6.1	0.61
1	1	5	140	14	1.4
1	1	4	120	12	1.2
1	1	3	100	10	1
1	1	2	81	8.1	0.81
1	1	1	61	6.1	0.61
1	1	0	40	4	0.4
1	0	5	120	12	1.2
1	0	4	100	10	1
1	0	3	80	8	0.8
1	0	2	60	6	0.6
1	0	1	40	4	0.4
1	0	0	20	2	0.2

Growth in inoculum			MPN cells·ml -1		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
0	5	5	190	19	1.9
0	5	4	170	17	1.7
0	5	3	150	15	1.5
0	5	2	130	13	1.3
0	5	1	110	11	1.1
0	5	0	94	9.4	0.94
0	4	5	170	17	1.7
0	4	4	150	15	1.5
0	4	3	130	13	1.3
0	4	2	110	11	1.1
0	4	1	94	9.4	0.94
0	4	0	75	7.5	0.75
0	3	5	150	15	1.5
0	3	4	130	13	1.3
0	3	3	110	11	1.1
0	3	2	93	9.3	0.93
0	3	1	74	7.4	0.74
0	3	0	56	6.6	0.66
0	2	5	130	13	1.3
0	2	4	110	11	1.1
0	2	3	92	9.2	0.92
0	2	2	74	7.4	0.74
0	2	1	55	5.5	0.55
0	2	0	37	3.7	0.37
0	1	5	110	11	1.1
0	1	4	91	9.1	0.91
0	1	3	73	7.3	0.73
0	1	2	55	5.5	0.55
0	1	1	36	3.6	0.36
0	1	0	18	1.8	0.18
0	0	5	90	9	0.9
0	0	4	72	7.2	0.72
0	0	3	54	5.4	0.54
0	0	2	36	3.6	0.36
0	0	1	18	1.8	0.18
0	0	0	0	0	0

Comments

The procedure may be adapted for different purposes and the setup can be varied (see above). For example, when cell concentrations are high (> 10^6 cells·litre^{-1}) more dilution steps are added in order to obtain unialgal cultures. If the aim is to clean up or isolate an organism already in culture, the original cell density will determine which dilution steps are critical; dilute so that the inoculum contains 1-2 cells, use many parallel dilutions, and add one or two further dilution steps. Provided growth occurs only in some of the tubes, the most diluted culture is likely to be clonal, unless cells in the inoculum are clumping.

The quality of the inoculum, the type of medium, and the growth conditions offered will determine the success of the method. This selectivity may be used deliberately by choosing a medium which facilitates the growth of particular organisms. For general purposes a modified Erd-Schreiber medium (Throndsen, 1978b, 1993) appears to be suitable for coastal waters, whereas diluted Guillard 'f' medium (Guillard & Ryther, 1962) as ('f/2'-'f/50') may prove better for more oligotrophic areas.

The disadvantage of the SDC method is its obvious selectivity and dependence on the growth conditions, whereas the advantage is that it yields material sufficient for identification. Since quantitation is determined by observing the presence or absence of a species, it is not critical that every single specimen in the sample examined be identified. It should be noted, however, that the presence or absence of taxa at higher levels has to be deduced from the species (level) in order to obtain a reliable estimate.

COUNTING METHODS

The enumeration of cells is still the most satisfactory way to obtain basic information on the toxic potential of an algal population, though variation in toxicity within a species has to be considered. Often the cell numbers combined with the known toxicity of a species, is as relevant as biomass, particulate carbon or nitrogen. In the following discussion the emphasis will thus be only upon estimating cell numbers.

Natural samples

Cell counts (with the identification of taxa) of natural samples, may be desirable both for monitoring water samples for toxic species, or during blooms when the concentration of one (or a few species) may be needed in order map the distribution of the toxic event. For many species living material is still necessary for a reliable identification by light microscopy, and for counting, a gentle "fixing" agent such as glutaraldehyde may be used if swimming disturbs the counting. A stronger fixative such as formaldehyde or iodine (Lugol's solution) may be used if the identification is possible with these preserving agents (Throndsen, 1993). Iodine will obscure the inner cell structures and formaldehyde may bleach photosynthetic pigments which may be important for identification of some species.

Counts of live cells may be made with electronic equipments such as a Coulter counter or a flow cytometer, but the taxonomic information obtained is then limited. If living cells are to be counted under the light microscope a shallow chamber is preferable, e.g. a Palmer-Maloney counting slide for nano- and microplankton and blood-cell counting chambers (haemacytometers) for smaller nanoplankton and picoplankton. For preserved samples larger chambers are also available for light microscopy. Those based on the use of an inverted microscope (Utermohl method) offer the best opportunities for detailed observations, and the most reliable cell counts, as larger sample volumes can be examined. However, this method (see Hasle, 1978) usually requires sedimentation overnight (minimum 6 hours for small chambers, 2 ml), and is only to be applied when detailed information on the species

composition (of preservable plankton) is needed. For routine surveys or mapping the distribution of a single bloom event, simple chambers such as the Palmer-Maloney counting slide are recommended, for both live and preserved samples (e.g. Throndsen, 1978a, Booth *et al.*, 1993). Fixation in glutaraldehyde and storage up to one year (epifluorescence microscopy) did not reduce estimates of population numbers when compared with counts of living cells immediately after collection (Booth *et al.*, 1993).

Laboratory cultures

For different reasons cell numbers are also important when working with cultures. In contrast to counting natural samples where identification requires that the specimens maintain their characteristic features, counting unialgal cultures only require that the cells do not disintegrate, and preferably do not aggregate into clumps. Cultured cells may thus be counted either live or preserved. The choice is often a practical one, and depends mostly on the equipment used; flagellates may be difficult to count under the microscope if they are actively moving, whereas motility does not affect a Coulter counter, or a flow cytometer. To follow growth in culture chlorophyll fluorescence measurements may also be conveniently applied.

Counting live material may be an advantage for non-motile cells as differences in cell colour may reveal the condition of the culture, whereas fixed material may be more convenient for motile cells. True preservation is usually not necessary unless there is a time lag of more than a few minutes between sampling and counting. A convenient short-term fixative is uranyl acetate (stock solution: 1 g per 20 ml seawater, use as little as possible) which effectively stops cell motion while leaving cell shape fairly intact (depending upon the species and physical state of the cells). Precipitates may occasionally form in sea water, and disturb the counting. Lugol's solution which kills cells instantaneously, and also stains the cells, is convenient when counting unialgal cultures under the light microscope.

Requisites/Equipment for microscopical counts

Sophisticated microscopical methods for enumeration of planktonic algae e.g.by image analysis, are less useful for routine counts, and only direct visual counting with microscope and simple counting chambers are included below.

Microscope. The type of microscope selected depends on the counting chambers used. For most methods a standard binocular microscope fitted with 10x (20x) and 40x objectives/lenses and preferably with phase-contrast optics, will be adequate. (A high power objective will be useful only for identification in ordinary preparations.) Be sure that the working distance of the objectives allows for focusing on the bottom of the chamber, and that the condenser also provides adequate light conditions for thick bottomed chambers. The high resolution, large aperture lenses usually have less working distance. For chambers holding large volumes e.g. 1-2 ml or more, an inverted microscope is needed, unless the cells are recognisable at low power, e.g. objectives 2.5-4x.

Oculars /Eyepieces. When chambers without a marked/etched grid in the bottom are used, a grid (e.g. a Whipple disc) must be inserted into the ocular to facilitate counting of fields of known size. This grid must be calibrated for the optics by comparison with a ruled microscopic slide (stage micrometer): measure the side length of the squares in the grid and calculate the area it represents for each of the objectives to be used. Then combine with depth of the chamber, and calculate the volume represented. The ratio between this volume and 1 L is the factor by which the cell count has to be multiplied to obtain cell numbers per litre.

Plates with etched grids such as Whipple discs (Fig. 4.2A), may be ordered through microscope dealers. Make sure that the eyepiece /ocular will accept the insertion of the plate, and that it can be focused on the grid.

When the entire volume of a chamber is to be counted, a double grid of lines (Fig. 4.2B) in the ocular will facilitate the counting of discrete transects of the bottom (see Hasle,

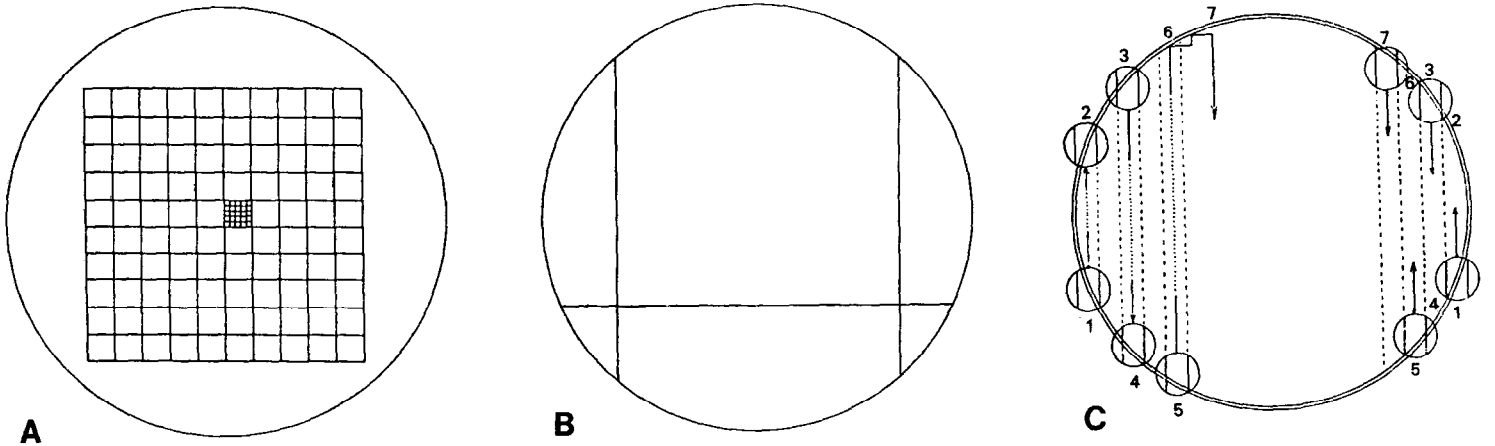

Figure 4.2. Ocular fields, A - with Whipple disc, B - double cross pattern. C - scanning the field with a double cross ocular, from Utermöhl (1958) as reproduced by Hasle (1978).

1978, Fig. 37 p. 195, or Fig. 4.2C. reproduced with reference to Hasle, 1978). The double grid may be introduced on a glass plate, or even made by glueing thin glass fibres in the inner focal plane of the ocular.

Counting chambers. Counting chambers and slides are all based on settling of the sample, so unless very low magnifications are used, settling time has to be considered. As the chambers have different depths, the time required before counting varies from a few minutes to several hours. Fixation agents such as I_2·KI (Lugol's solution) will shorten the settling time. The magnification used will determine the probability for detecting cells not properly settled i.e. out of focus. Counting chambers are characteristically of two types: those with a fixed counting grid on the bottom (blood counting slides, haemacytometers) and those which require a counting grid in the microscope eyepiece/ocular (Palmer-Maloney and Sedgewick-Rafter counting slides, and larger sedimentation chambers).

Two dimensions of the counting chambers are critical for observation: the depth of the chamber with cover glass versus the working distance of the objectives, and the thickness of the basal part of the chamber versus the working distance of the condenser. The latter is especially to be considered when haemacytometers are used with phase contrast optics.

A wide variety of chambers have been produced, only the most versatile are recommended here:

Blood counting chambers, haemacytometers, (Fig. 4.3A) e.g. with Fuchs-Rosenthal or Improved-Neubauer ruling, are selected because they combine a self-contained counting grid with a reasonable sample volume (3.2 - 0.9 µl) for high phytoplankton concentrations.

Palmer-Maloney chamber (Fig. 4.3B) useful for its convenient size for prebloom counts; holds 0.1 ml.

Sedgewick-Rafter chamber (Fig. 4.3C) included for its larger (1 ml) size when large and less abundant species are important in the plankton.

A 2 ml sedimentation chamber is required when phytoplankton concentrations are low in coastal areas, but usually this chamber will not be adequate for oligotrophic areas.

Workshop made chambers require a skilled technician, but simple and inexpensive chambers (Fig. 4.3D) to be used on conventional compound as well as inverted microscopes may be made. A chamber adequate for counting nano- and microplankton species, can be made from a 2 mm Plexiglass (Perspex) sheet cut to the size of an object glass, with a 16 mm hole in the center. The wall of the hole must be polished in order to prevent cells from sticking, and to facilitate the cleaning of the chamber, see below. Then carefully mount (with water resistant permanent glue!) a 20 mm circular cover glass to form the bottom of the chamber, taking care not to spill glue on the free cover glass surface. It is important to know the exact thickness of the plexiglass sheet to calculate the volume of the chamber. Provided that the thickness is exactly 2 mm, a 16 mm hole will yield a 0.4 ml chamber when covered by a loose cover glass on the top, the bottom area being 200 mm^2.

Note! Temperature variations during the cleaning procedure may loosen the glass as the expansion coefficient of glass is different from that of plexiglass. Also, avoid using acetone as a cleaning agent at any time, as it attacks the Plexiglass.

The main characteristics of selected counting chambers are given in Table 4.2.

Procedures

First judge the density of the sample by applying one drop (approx. 1/20 ml) to a microscope slide, put on a cover glass (20x20 mm), and observe the preparation under the microscope. If all the sample stays within the limits of the cover glass, this preparation is approximately 0.125 mm thick i.e. 1µl sample is covered in 8 mm^2. If there are only a few cells in the entire preparation, use a sedimentation chamber holding 2 ml or more, (if cell numbers exceed five use a Sedgewick-Rafter chamber, if more than 10 cells are counted the workshop- made chamber may be used,) if the distance between the cells is 100-1000 µm use a Palmer-Maloney chamber, and if less than 100 µm use a blood-cell counting chamber (haemacytometer), e.g.

Table 4.2. Characteristic dimensions of different types of counting chambers.

Chamber type	depth mm	area mm^2	volume µl	detectionlimit cells/l	reliable counts cells/l	diameter or size of ruling
Improved Neubauer	0.1	9	0.9	10^{6-7}	10^{8-9}	3x3 mm square
Fuchs-Rosenthal	0.2	16	3.2	10^{6}	10^{8}	4x4 mm square
Palmer- Maloney	0.4	250	100	10^{4}	10^{6}	17.9 mm diameter
Workshop type*	1 2	200 200	200 400	10^{4} 10^{4}	10^{5-6} 10^{5-6}	16 mm diameter
Sedgewick-Rafter	1	1000	1000	10^{4}	10^{5}	50x20mm rectangle
2 ml sedimentation cylinder**	10	200	2000	10^{3}	10^{4}	16 mm diameter

* May be used on standard or inverted microscopes
** To be used on inverted microscopes

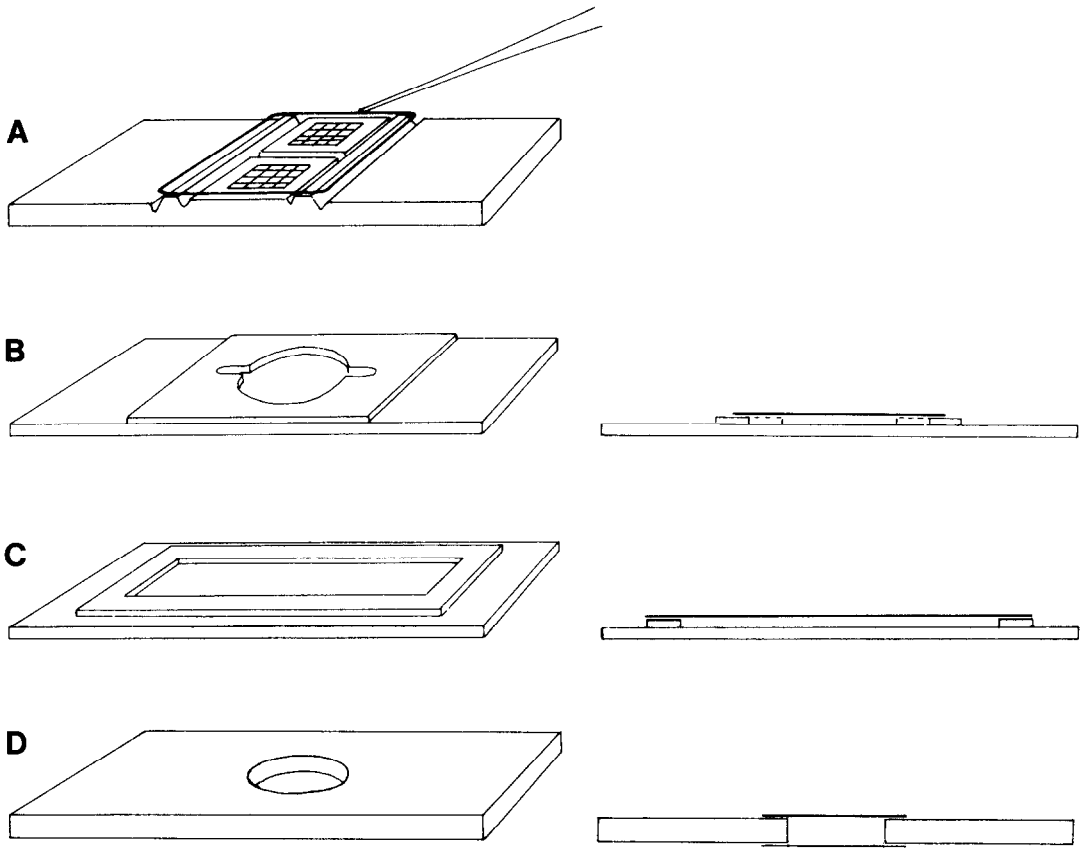

Figure 4.3. Counting chambers. A - Haemacytometer or blood counting cell, filling procedure indicated, B - Palmer-Maloney counting chamber, C - Sedgewick-Rafter counting chamber, and D - workshop-made chamber. Appearance on the left, optical section through the chamber with cover glass on the right. Not drawn to scale.

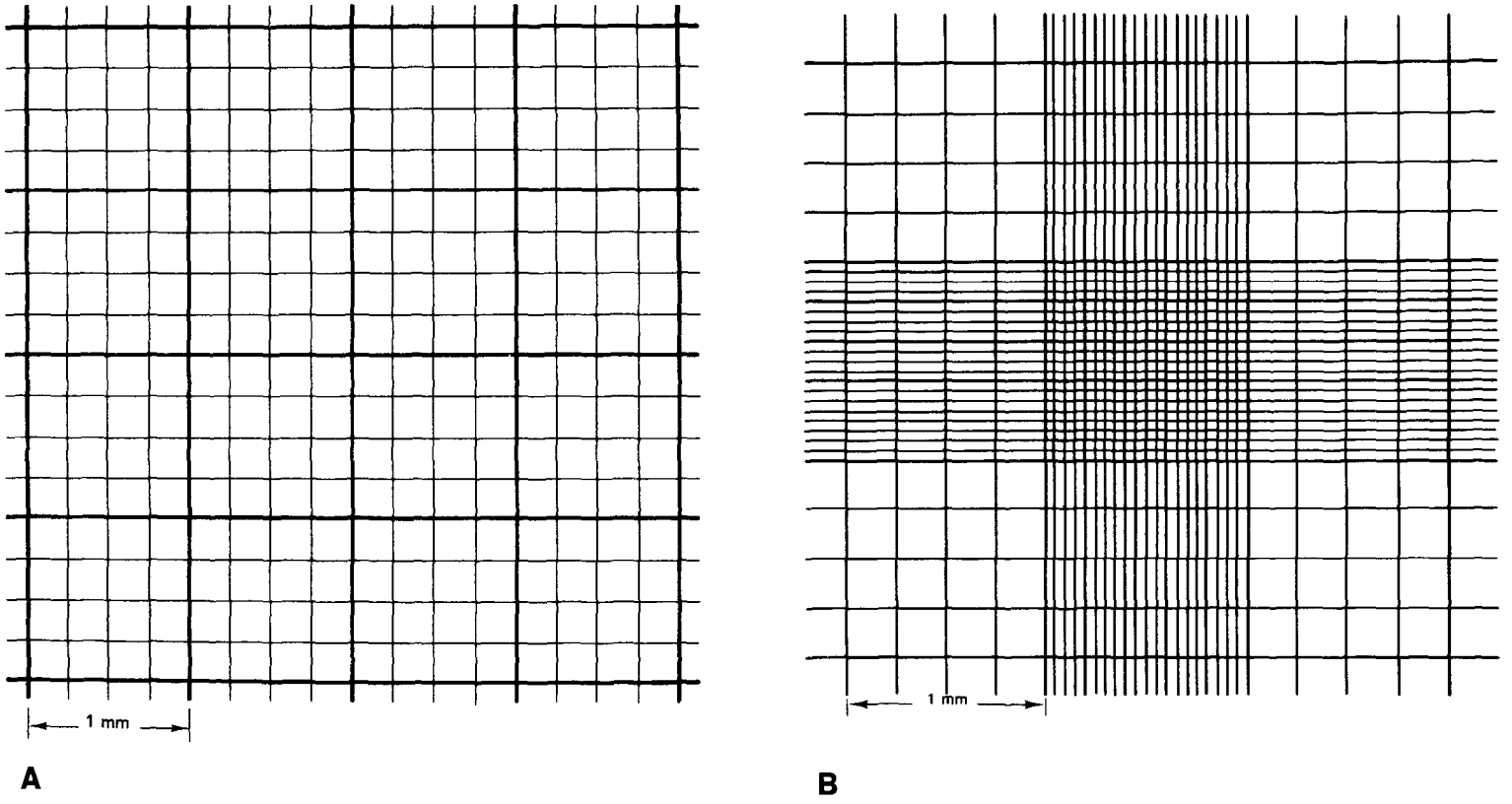

Figure 4.4. Grid pattern of A - Fuchs-Rosenthal and B - Improved-Neubauer ruling, from Guillard (1978).

with Fuchs-Rosenthal ruling, or the Improved Neubauer. If the preparation is very dense, dilute the sample.

Cleaning the chambers is an essential part of the procedure. Dirty chamber surfaces make it difficult to count cells properly, and specimens left from the previous count may ruin the counts. Oily, hydrophobic surfaces make it difficult to fill the chamber adequately (check the randomness of distribution upon filling). Cleaning is conveniently carried out by the use of a pure detergent like soap, it should not be a type leaving a film of oil, wax or silicon. Gently rub the surfaces with cotton wool tips, be sure to reach the corners in the closed chambers.

Filling open chambers such as the blood-cell counting slides where the sample is held between two horizontal glass surfaces, is critical: Mount the chamber dry, and add the sample quickly from a pipette taking care that the chamber does not overflow. If this happens, clean the chamber and repeat the procedure. Filling closed chambers such as the Palmer-Maloney chamber is carried out with the cover glass on, but in a position so that it leaves the two channels open; Add the sample by a pipette through one channel, let the air out through the other. When chamber and channels are filled turn the cover glass to close it. The Sedgewick-Rafter chamber is filled with the cover glass displaced to leave one corner open for filling, another for air escape. Filling larger chambers and the workshop made type is usually accomplished with the cover glass off, taking care to remove the overflow when the coverslip is put on. It is important to reduce the amount of water left to dry on chamber surfaces as the salt may optically disturb the counting; it is also corrosive if allowed to enter the metal parts of the microscope.

Reliability of cell number estimates

Cells may escape detection due to damage or inadequate fixation, thus the statistical accuracy of cell number estimates is important. There is a combined effect of subsampling and number of cells counted. To discuss this matter in detail is beyond the scope of this section, but some practical hints may be useful. First, the sample must be thoroughly mixed before drawing the subsample; second, the chamber should be checked visually for even distribution after filling; third, the desirable precision of the count should be decided upon in advance.

It is commonly assumed that at least 100 cells should be counted to give a fairly reliable cell number estimate i.e. to give a 95 per cent confidence interval of the estimate within +/-20% of the mean value, and 400 cells for a precision of +/- 10% of the mean (Venrick, 1978 with reference to Lund *et al.*, 1958).

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5. Post-Column derivatization HPLC methods for Paralytic Shellfish Poisons

Y. Oshima

Paralytic shellfish poisoning (PSP) is a serious toxicological problem affecting shellfish industries in many countries of the world. More than twenty analogues of saxitoxin (Fig. 5.1) have been reported to occur naturally. Traditionally, the presence of PSP toxins has been determined using the mouse bioassay (AOAC, 1990). However, due to the drawbacks related to the bioassay, *e.g.* lack of specificity and precision and inconvenience of maintaining a large stock of animals, alternative methods have been explored by many researchers. For the detection of PSP toxins, derivatisation of the toxins to fluorescent compounds by oxidation under alkaline conditions has a great advantage in yielding high sensitivity and fairly good toxin specificity. The oxidation reaction was incorporated with high-performance liquid chromatography (HPLC) as a post column reaction system. Sullivan *et al.* (1983a, b) used ion-interaction chromatography on a polystyrene divinylbenzene column with gradient elution of a mobile phase containing alkylsulfonates as counter-ion and periodate oxidation. The system was designed for determining the principal toxins from the dinoflagellate *Alexandrium* spp., namely gonyautoxins 1-6 (GTX 1-6), saxitoxin (STX) and neosaxitoxin (neoSTX) and was utilised most commonly for toxin analysis in shellfish. However, recent investigations on shellfish toxicity related to *Gymnodinium catenatum* from various countries (Oshima *et al.*, 1989, 1993; Rodriguez-Vazquez *et al.*, 1989) indicated the occurrence of large amounts of N-sulfocarbamoyl-11-hydroxysulfate toxins (C1-C4), which were inseparable by Sullivan's system. Moreover, in certain shellfish decarbamoyl toxins were also found either as enzymatic degradation products (Sullivan *et al.*, 1983) or originating from the dinoflagellates (Oshima *et al.*, 1993; Rodriguez-Vazquez *et al.*, 1989). Thus an accurate analytical method applicable to all the toxins is required. Previously, we proposed a post-column derivatisation HPLC method which determined toxin groups separately by isocratic elution of three different mobile phases to accomplish the resolution of most of the toxins (Oshima *et al.*, 1987, 1989), Further improvements to the system, together with a clean-up procedure for the extract are described here. As an example of its application, a comparative study against the mouse bioassay on low toxicity scallops is given.

METHODS

Apparatus and chromatographic conditions

The HPLC system is composed of a high pressure pump (Hitachi L-6000) with a syringe loading sample injector (Rheodyne 7125), a silica-based C8 reversed-phase column (Develosil C8, Nomura Chemical or Inertsil C8, GL Science; 4.6 x 150 mm), a double head reaction pump (Hitachi 655A-13) for delivering both oxidizing reagent and acid, 10 m Teflon tubing of 0.5 mm id immersed in a water bath or an aluminum block dry oven, a fluoromonitor (Hitachi F-1050) equipped with 150 W xenon lamp and a chromato-integrator (Hitachi D-2500) for calculation of peak area. Comparable equipment from other manufacturers with similar specifications is also acceptable.

Toxins are determined under the conditions described previously with slight modification, as summarized in Table 5.1. Major modifications to the previously published

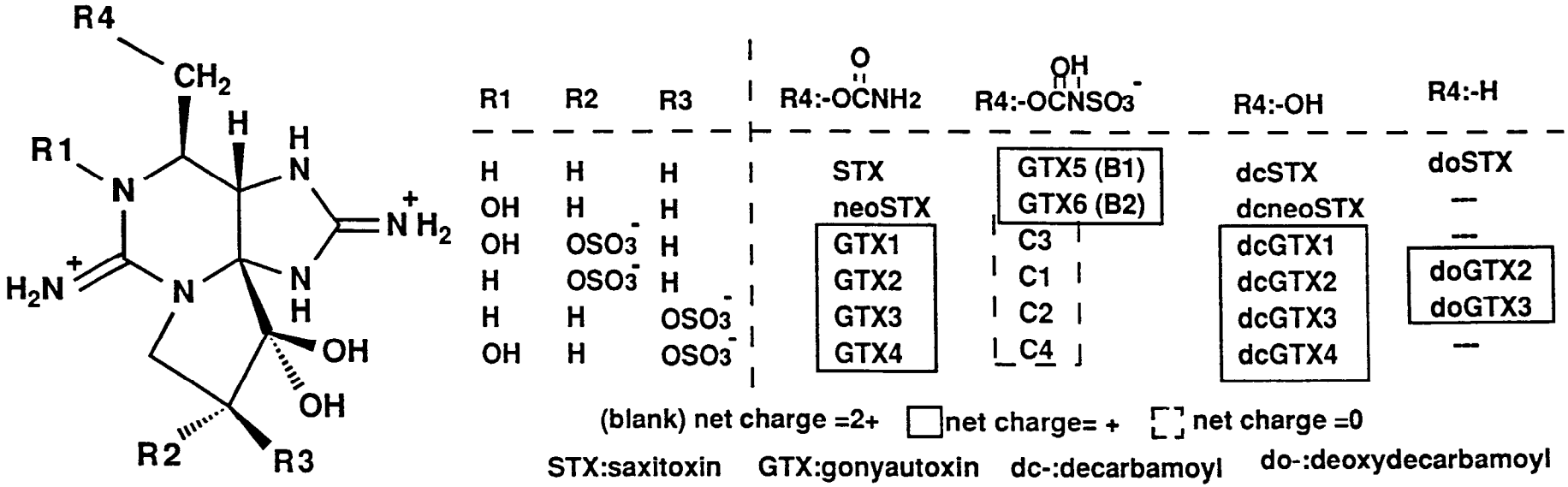

Figure 5.1. Structures of paralytic shellfish toxins.

Table 5.1. Operating conditions for HPLC analysis of PSP toxins.

1. Column:

Reversed phase, C8-bonded Silicagel column
Develosil C8-5 (Nomura Chemicals) or Inertsil C8-5 (GL Science), 4.6x150mm

2. Mobile phases: flow rate at 0.8 ml/min

(a) for C1-C4: 1 mM tetrabutyl ammonium phosphate adjusted to pH 5.8 with acetic acid
(b) for GTX1-GTX6,dcGTX2 and dcGTX 3:
2 mM sodium 1-heptanesulfonate in 10 mM ammonium phosphate, pH 7.1
(c) for STX, neoSTX and dcSTX:
2 mM sodium 1-heptanesulfonate in 30 mM ammonium phosphate,
pH 7.1:acetonitrile = 10 : 5

3. Oxidizing reagent: flow rate 0.4 ml/min

7mM periodic acid in 50mM potassium phosphate buffer, pH 9.0

4. Reaction: in 10 m Teflon tubing (0.5mm id) at 65°C in water bath and at 85°C in dry oven

5. Acidifying reagent: 0.5 M acetic acid, flow rate 0.4 ml/min

6. Detection: excitation wavelength 330 nm, emission wavelength 390 nm

method are pH and counter ion concentration in the mobile phases to suit a 15cm column. The buffer in the oxidizing reagent has been changed to potassium from sodium phosphate. Concentration of the toxin standards prepared by column chromatography (Oshima *et al.*,1989) is deduced from nitrogen content examined by combustion analysis (Genenah and Shimizu, 1981). For routine analysis of shellfish toxins, mixed solutions at concentrations ranging from 0.3 to 4 μM are prepared in 0.05 N acetic acid, and kept at -40°C until use. For the calculation of toxicity from HPLC chromatograms, the following values of specific toxicity (MU/ μmole) were determined: GTX1 (2468), GTX2 (892), GTX3 (1584), GTX4 (1803), GTX5 (160), dcGTX2 (1617), dcGTX3 (1872), C1 (15), C2 (239), C3 (33), C4 (143), STX (2483), neoSTX (2295) and dcSTX (1274).

Resolution and detection limit

In this HPLC system, three different isocratic chromatographic conditions are used for the toxin groups categorized by their basicity. Chromatograms of low concentration toxin mixtures and detection limit of each toxin are shown in Fig. 5.2. For the C-toxin group, the use of tetrabutylammonium phosphate solution results in complete separation of four toxins. The same ion-pairing reagent has also been used with a polymeric column (PRP-1, Hamilton) by Boczar *et al.* (1988), for analysis of this toxin group. Combination of the C8-column and heptanesulfonate as counter ion gives good separation and sharp peak shapes for other toxin groups. Decarbamoylgonyautoxins (dcGTX) -3 and -2 elute before their carbamate counterparts and dcGTX3 is slightly overlapped with GTX5 (B1) on a 15 cm column (Fig. 5.3). Separation of dcSTX from STX can be accomplished by addition of acetonitrile to the mobile phase but

dcneoSTX is inseparable from neoSTX. The deoxydecarbamoyl toxin group can be analyzed with the same mobile phase and shows longer retention times than their carbamate counterparts (Oshima *et al.*, 1993).

To improve sensitivity, peak broadening is minimised using 0.25 mm id teflon tubing and dead volume free connectors in the post-column reaction system. By optimizing reaction conditions, high sensitivity with detection limits of 20-110 femtomole can be attained as shown in Fig. 5.2. Use of potassium phosphate as the buffer in the oxidizing reagent solves the problem of precipitation which often interfered with chromatography, and the modified reagent can be stored for nearly one week.

Clean-up procedure for shellfish extracts

In analyses of shellfish samples, fluorescent compounds sometimes interfere with the analysis. In scallop extracts, a huge peak of an unknown compound can appear near GTX6 (B2) and GTX4 (Fig. 5.4). Thus, clean-up methods for the extracts were explored using a reverse-phase cartridge column (Sep-Pak C18). Use of a dry cartridge column did not retain the interfering substance but when regenerated with methanol and equilibrated with water, the artifact peak can be removed from the toxic matrix (Fig. 5.4). The first fraction of the eluate was diluted with water remaining in the column and the interfering substance started to appear after 2.5 ml. Thus the 1.5 and 2.0 ml fraction was collected for further purification by ultrafiltration. The dilution factor by glycerol used as lubricant of the ultrafiltration membrane was negligible when tested with 50 µl of toxin solution. Treatment of the extract with a Sep-pak C18 cartridge column was also effective in removing peaks eluting after C1 to C4 and STX (data not shown), and analysis time could be shortened significantly. The clean-up procedure was also effective in prolonging the lifetime of the column.

Analysis of low toxicity scallops

As an illustration of the application of this method to low toxicity shellfish samples, the following example is presented. Scallops, *Patinopecten yessoensis*, were collected from Funka Bay, Hokkaido, Japan, in January and February, 1990. The extracts were prepared and tested according to the mouse bioassay (AOAC, 1990). The same extract was treated by a cartridge column (Sep-Pak C18, Waters) and an ultrafiltration membrane (Ultrafree C3GC, Millipore) as indicated in the results. For the HPLC analysis, 10 µl of each extract was injected into the chromatograph. To determine the recovery of toxin by both HPLC and mouse bioassay, extracts of nontoxic scallops collected from Mutsu Bay, Aomori, Japan, was mixed with purified toxins in various concentrations and tested as well.

Scallops from Hokkaido contained GTX1-4, neoSTX and STX as major components, and dcGTX2, dcGTX3, C1 and C2 as minor fractions. The correlation between the HPLC and the mouse bioassay results on 20 specimens of toxicities ranging from 2.0 to 12 MU/g is summarized in Fig. 5.5. A fairly good correlation (r=0.88) was observed between the two methods, but HPLC values exceeded those of the mouse bioassay in most of the samples. To investigate the cause of the discrepancy, nontoxic extract was spiked with purified toxins and tested as well (Fig. 5.6). While almost 100% of the toxin was recovered by HPLC, the mouse bioassay showed significantly lower values. No mouse died with the toxin extract added at the concentration equivalent of 2.4 MU/g. These results clearly indicate that the mouse bioassay underestimates toxin content in low toxicity shellfish, as reported in the older literature (Schantz *et al.*, 1958).

DISCUSSION

The post-column derivatization HPLC method for paralytic shellfish toxins has a great advantage over other methods in its ability to quantitate each toxin in a crude sample of small size. Simple clean-up procedures can avoid toxin transformations which easily occurs during purification and concentration procedures. It is a powerful tool in research, especially for studies on toxin production by dinoflagellates. The high sensitivity of the system enabled us to elucidate the complete toxin profile of *Alexandrium* cysts with samples as small as 100 cells (Oshima *et al.*, 1992).

Using this method, a better correlation was observed between the HPLC and mouse bioassay than the result reported on low toxicity mussels and clams from the east coast of the United States (Salter *et al.*, 1990). A clean-up procedure with a reverse-phase cartridge column minimizes the errors involved in HPLC analysis. Use of carefully prepared standards is also essential for accurate evaluation of toxin content. The major cause of discrepancy between the HPLC and the bioassay methods is inaccuracy of the mouse bioassay. The mouse test is known to involve significant errors (McFarren, 1959; Park *et al.*, 1986), especially when testing samples of low toxicity, whereas the replicated HPLC analyses showed less than 5% error. In conclusion, the mouse bioassay can be complemented by HPLC as a regulatory measure for seafood safety.

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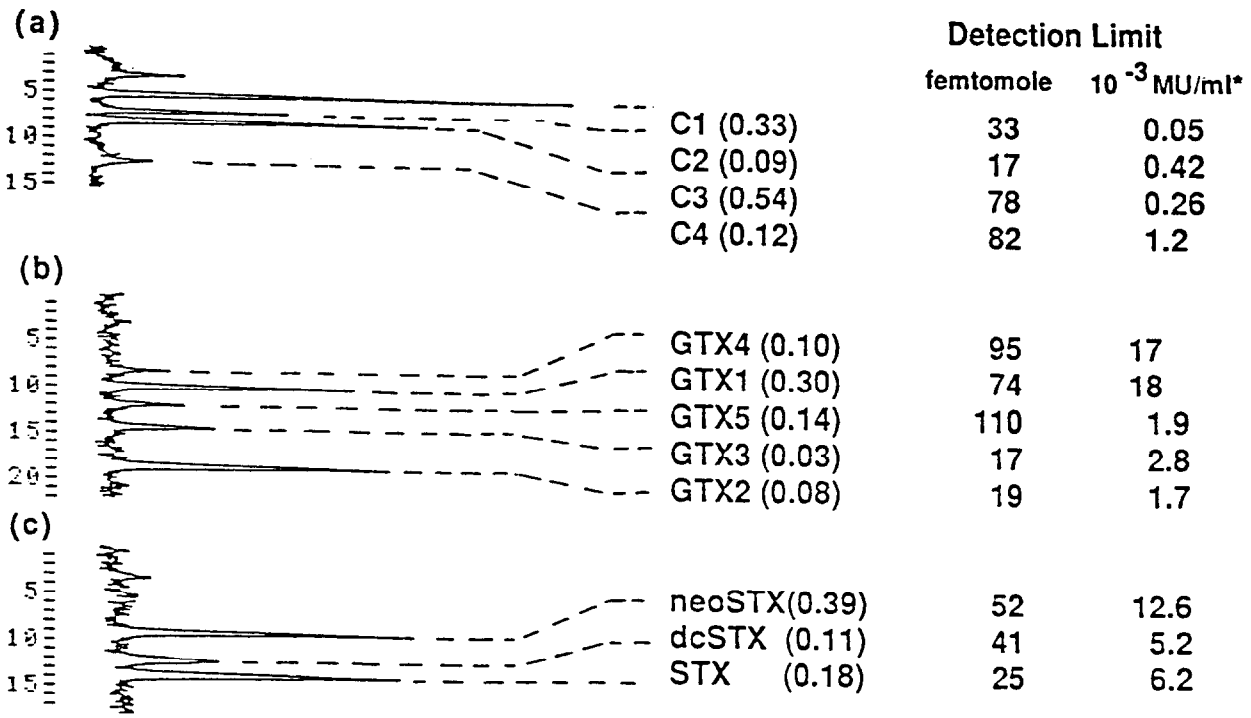


Figure 5.2. Chromatograms of toxin mixtures in low concentration and detection limit. (a), (b) and (c) denote mobile phases as in Table 5.1. Numbers in parentheses are the amounts of toxin (picomole) injected. Detection limits are expressed by toxin required for S/N=2 peak height (in femtomoles) and toxicity level (in MU/ml) at 10 ml injection.

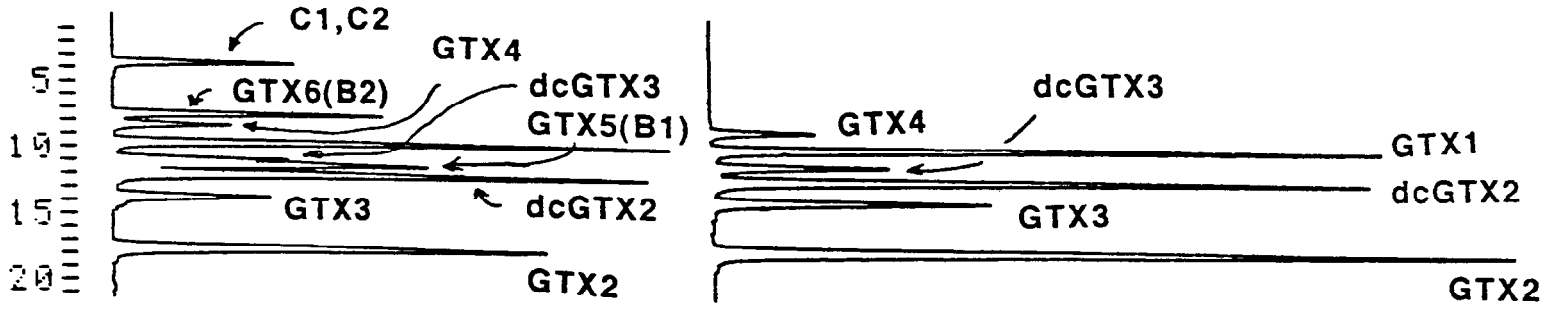


Figure 5.3. Chromatograms of toxin mixtures containing decarbamoyl-gonyautoxins 2 and 3. Mobile phase (b).

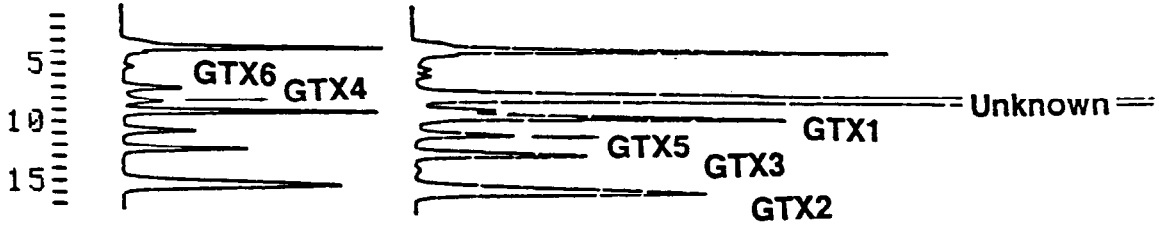


Figure 5.4. Chromatograms of a scallop extract spiked with toxin mixture before (right) and after (left) treatment with Sep-Pak C18 cartridge column.

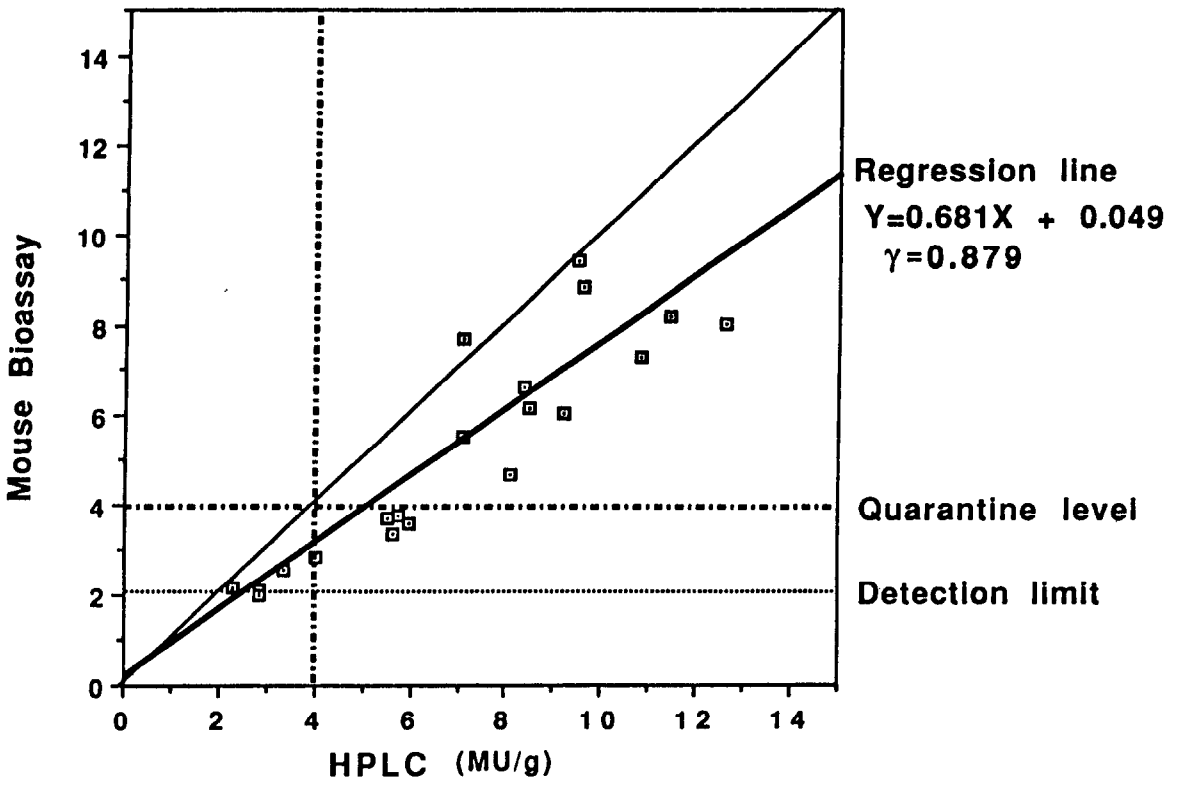


Figure 5.5. Correlation between the HPLC and mouse bioassay methods on scallops of low toxicity.

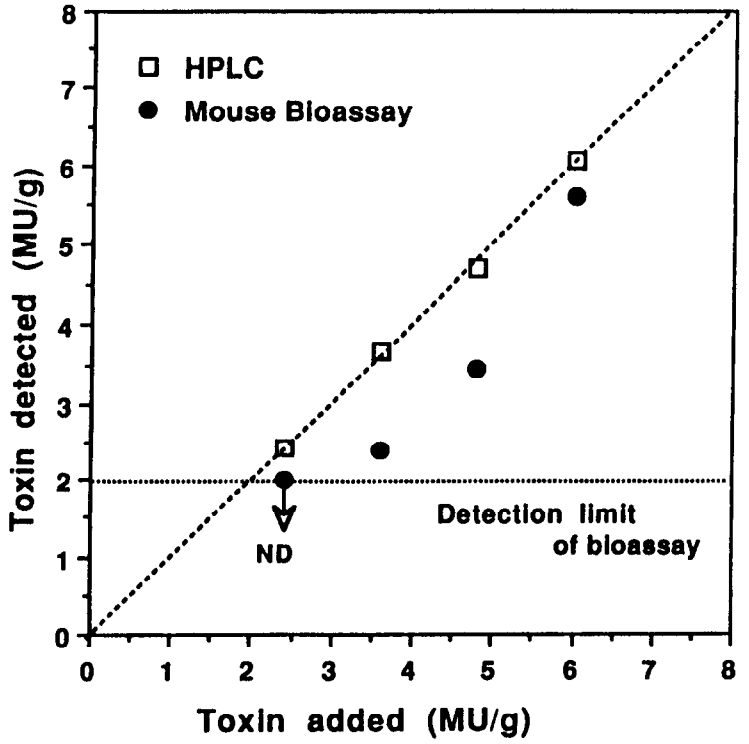


Figure 5.6. Comparison of the HPLC and mouse bioassay methods on spiked samples.

APPENDIX

1. Apparatus

The system is composed of the following apparatus (Fig. 5.7). and the specifications are the minimum required to build a reliable HPLC system. Instrument brand names in parentheses are those tested at Tohoku University.

(a) *High Pressure Pump for Mobile Phase* (Hitachi L-600, Hitachi L-6200, Shimadzu LC6A, Waters): Any model capable of mobile phase flow at more than 1.0 ml/min or higher than 200 kg/cm2 with reliable flow control. Programmable or dual head pumps for gradient elution are not necessary.

(b) *Injector*: Loop or variable volume (syringe loading) injector (10-100 ml) (Rheodyne 7125 or equivalent)

(c) *Chromatographic Column*:: Well- endcapped chemically bonded C8 silica gel column of column size 4.6 (id) x 150 mm (length) or 4.6 x 250 mm (Develosil C8-5, Nomura Chemical, Inertsil C8, GL Science)

(d) *Post Column Reaction System*:

d-1: Pump for oxidizing reagent; preferably low pulsation with acid resistant (inert) pump head (Hitachi 655A 13, Nihon Seimitsu mini-chemical pump SP-D2502U, Seishin Pharmaceutical Co. PSD-3.2, Altex). If possible, insert a pressure gauge (50 kg/cm2) and 10 m Teflon tubing of 0.25 mm id between the pump and T bar, to monitor reagent flow and diminish pulse.

d-2: Reaction coil of Teflon tubing: 0.5 mm id and 10m length.

d-3: Water bath or dry oven (aluminum block type not hot air type) to keep (d-2) up to 100°C. Temperature fluctuation should be within ± 1°C (Taiyo Thermominder Jr100 in 2L water bath; Shimamura Instruments Dry Reaction Bath DB-5).

d-4: Pump for acid to acidify reaction mixture; same as d-1. One dual head pump can be used as d-1 and d-4.

(e) *Detector*: Highly sensitive fluorescence detector (Hitachi F-1000, Hitachi F-1050, Shimadzu PF-535) equipped with small volume flow cell and xenon lamp (50W) as light source. It should be capable of selecting excitation and emission wavelengths of 330 and 390 nm by dual monochromators. Slit widths at both sides should be 10 nm or less.

(f) *Recorder*: Conventional one-pen recorder fitted to detector output (usually 10 mV) can be used. However, an electronic integrator is more convenient in covering a wider range of the fluorescent signals and being capable of calculating peak area (Hitachi D-2000, Hitachi D-2500, Shimadzu Chromatopak C-R6A).

(g) *Tubing and fitting*: All the tubing connecting individual apparatus after the LC-column should be 0.25 mm id Teflon, except for reaction coil. Use T-joints or fittings of small bore size to minimize peak broadening.

2. Mobile Phases and Oxidizing Reagent

The following procedures are recommended to prepare the mobile phases and oxidizing reagent shown in Table 5.1 (Summary of operating conditions). The volume of each solution can be increased or decreased proportionally at the user's convenience.

(a) *Stock solutions*

(1) Tetrabutyl ammonium phosphate solution: 500 mM or 1.0M solution (HPLC grade) is commercially available from Tokyo Kasei Kogo (IPA-TBA-Ph, cat.no.1-365). A 1.0M solution from Aldrich Chemical Company (cat.no.26, 810-0) can also be used.

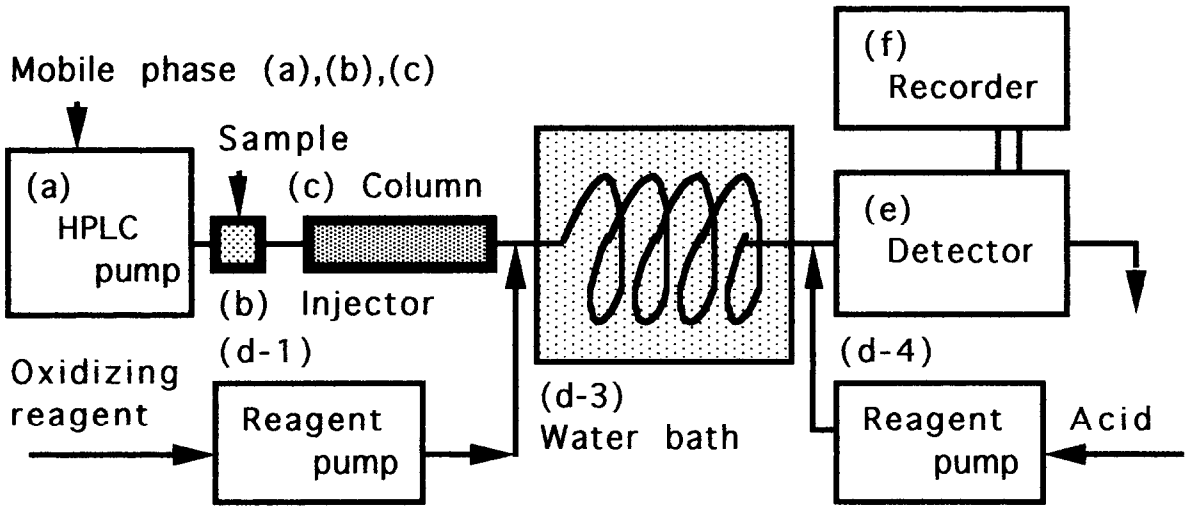


Figure 5.7.Flow diagram of the HPLC system.

(2) Sodium 1-heptanesulfonate solution: 100 mM. Prepare by dissolving 2.02g of HPLC grade reagent (Tokyo Kasei Kogyo IPC-ALKS-7 cat.no.1-345, MW 202.25) in 100 ml of distilled water. Keep in a refrigerator.

(3) Acetic acid 50 mM. Prepare by dissolving glacial acetic acid, analytical grade, in distilled water.

(4) Phosphoric acid 500 mM. Dissolve 28.8 g of concentrated phosphoric acid of analytical grade (85%, MW 98.0) in distilled water and make up to 500 ml.

(5) Ammonium hydroxide solution ca 1N. Dilute analytical grade ammonia water (25%) with 13 volumes of distilled water.

(6) Periodic acid 350 mM. Dissolve 7.98 g of periodic acid dihydrate ($HIO_4.2H_2O$, MW 227.94) of analytical grade in 100 ml of distilled water.

(7) Dipotassium phosphate solution 250 mM. Dissolve 21.77 g of K_2HPO_4 (analytical grade, MW 174.18) in distilled water and make up to 500 ml.

(8) Potassium hydroxide ca.1 N. Dissolve 5.6 g of KOH in 100 ml of distilled water.

(b) *Mobile phases*

Mobile phase (a) for the analysis of C1-C4: 2mM tetrabutyl ammonium in acetate buffer pH 6.0. Dissolve 2 ml (500 mM) or 1 ml (1.0 M) of stock solution (1) in 450 ml of distilled water. Adjust pH to 5.8 by carefully adding 0.05 N acetic acid (3) or dilute ammonium hydroxide solution. Make up to 500 ml with distilled water.

Mobile phase (b) for the analysis of gonyautoxins 1-6: 2mM heptanesulfonate in 10 mM ammonium phosphate buffer pH 7.1. Dissolve 10 ml each of stock solution (2) and (4) in 450 ml of distilled water. Raise pH to 7.1 by adding 1N NH_4OH (5). Make up to 500 ml with distilled water.

Mobile phase (c) for the analysis of saxitoxin-neosaxitoxin: 2 mM heptanesulfonate in 30 mM ammonium phosphate buffer pH 7.1 : acetonitrile 100:5 for 150 mm column, 100:6 for 250 mm column. Dissolve 10 ml of stock solution (2) and 30 ml of (4) in 440 ml of distilled water, adjust to pH 7.1 by adding NH_4OH solution (5) and make up to 500 ml. Add acetonitrile (25 ml for 15 cm column and 30 ml for 25 cm column), mix well and degas by sonication or gentle aspiration.

(c) *Oxidizing reagent.*: 7.0 mM periodic acid in 10 mM sodium phosphate buffer pH 9.0. Add 10 ml of stock solution (6) and 100 ml of stock solution (7) to 300 ml of distilled water. Titrate to pH 9.0 with stock sodium hydroxide solution (8) and make up to 500 ml with distilled water.

(d) *Acidifier*: 500 mM acetic acid. Prepare with glacial acetic acid of analytical grade and distilled water.

3. Operation

(a) Start-up procedure

(1) Run 25% acetonitrile through column for 5 min and then prime the HPLC pump with the mobile phase and establish column flow at 0.8 ml/min. Run HPLC pump for at least 15 min.

(2) Prime post column reaction pumps with oxidizing reagent and acid. Set each reagent flow at 0.4 ml/min.

(3) Heat up reaction coil in water bath at 65°C.In case of a dry oven, set temperature at 85°C.

(4) Operate fluorescent detector at wavelengths Ex 330 nm and Em 390 nm.

(5) When the baseline becomes stable, inject standard solution repeatedly until retention times of toxins become constant, indicating the column has been equilibrated with the mobile phase. Check reaction and detection systems are working properly by constant peak heights or areas of standard toxins.

(b) Sample analysis

(1) Inject 5-10 µl of shellfish extract and record peak heights or peak areas.

(2) Inject standard solution after every 2-4 samples to ensure the system is working properly.

(3) Use the nearest standard chromatogram for calculation of toxin content in the sample.

(c) Shut-down procedure

(1) Shut down recorder, monitor and water bath.

(2) Prime reaction pump for oxidizing reagent with distilled water. Flow water for at least 15 min and stop.

(3) After running reaction pumps for 5 min with distilled water, prime HPLC pump with 25% acetonitrile and keep running for 30 min.

(4) To analyze a different toxin group, change the mobile phase after flushing the column with 25% acetonitrile for 10 min.

(5) Be careful not to run the column dry. Before storing the column, flush well with 50% acetonitrile and cap tightly.

4. Preparation of extract for HPLC analysis

(a) From shellfish

(1) Prepare the shellfish extract according to the standard mouse bioassay (heat homogenate with equal volume of 0.1 N HCl for 5 min and centrifuge or filter)

(2) If the extract is too turbid, centrifuge it again at 10,000 g for 5-10 min.

(3) Wash Sep-Pak C-18 cartridge column (Waters) with 10 ml of methanol and drain by flushing with air, using a glass syringe. Equilibrate the column with 10 ml of distilled water and drain well (use another syringe). Put ca. 3 ml of extract in another dry syringe and pass it through the column. Discard the first 1.5 ml of eluate and collect the next 0.5 ml in the reservoir of an ultrafiltration kit (Waters Ultrafree C3GC, 10,000 dalton cut-off).

(4) Centrifuge the cartridge at 5,000 xg for 5 min. If the filtrate on the bottom of the tube is not sufficient, centrifuge it again.

(b) From cultured or natural plankton sample

(1) Count dinoflagellate number in the sample (culture or suspension of plankton collected with a plankton net).

(2) Weigh a plastic centrifuge tube (1.5 - 15 ml size depending on sample size and plankton density).

(3) Transfer the sample of exact volume to the tube and centrifuge gently (1500 xg). Remove supernatant by pipetting carefully so as not to disturb the pellet.

(4) Add 0.5 N acetic acid, approximately 10 times the volume of the pellet.

(5) Weigh the centrifuge tube again to estimate the volume of sample and acid considering density =1.

(6) Rupture the cells using a sonicator equipped with rod-shaped oscillator (three times 30 sec sonication). Avoid high sample temperature by putting the sample in an ice bath. Examine a small portion of sample under the microscope to check whether or not all the cells are broken. Repeat sonication if necessary.

(7) Centrifuge the tube for 10 min at > 3,000 xg.

(8) Transfer the supernatant to an ultrafiltration cartridge (as in (a.3)) and centrifuge at 5,000 xg for 5 min. If filtrate on the bottom of the tube is <100 µl, centrifuge again.

5. Calculation of shellfish toxicity

Calculate the concentration of each toxin using the conventional method for an external standard and calculate total concentration as the summation. The following is an example.Concentration of each toxin (Ct per ml) in the extract is given by the following equation:

$$Ct = \frac{ToxSt}{ArSt} \times ArSm \times \frac{1000}{VSm}$$

ToxSt = the amount of standard toxin injected (unit: picomole, MU or ngST eq.)
ArSt = area or peak height of standard toxin in the nearest chromatogram
ArSm = area or peak height of the toxin in sample
VSm = volume of extract injected (in ml)

Add all Ct values for each toxin to obtain the total toxin concentration of the extract (Cts). The toxicity of the sample (Tox) is given as follows:

Tox (nanomole/g) = Cts (picomole/ml) x 0.002 xDF
Tox (MU/g) = Cts (MU/ml) x 2 x DF
Tox (μgSTX eq./100g) = Cts (ngSTXeq./ml) x 0.2x DF

DF = dilution factor of the extract before injection. Usually no dilution is necessary when using an integrator (maximum recording range 1V).

The above calculation can be done more easily using a programmable integrator or by preparing a worksheet using computer spreadsheet software.

6. Toxin standards

Pure toxins of known concentration are essential for this analysis. Mixtures of each group of toxins are available from selected laboratories.

6. Methods for Diarrhetic Shellfish Poisons

M.A. Quilliam and J.L.C. Wright

The term "diarrhetic shellfish poisoning" has been associated with a number of different groups of toxic compounds (Yasumoto *et al.*, 1989, 1990). These groups include: the polyether compounds, okadaic acid (OA), dinophysistoxin-1 (DTX-1) (Murata *et al.*, 1982), DTX-2 (Hu *et al.*, 1992a), and DTX-3 (Yasumoto *et al.*, 1985); the macrocyclic polyether lactones, the pectenotoxins (Yasumoto *et al.*, 1985; Murata *et al.*, 1986) and prorocentrolide (Torigoe *et al.*, 1988); and the fused polyether yessotoxin (Murata *et al.*, 1987). The okadaic acid/dinophysistoxin compounds have been shown to be potent phosphatase inhibitors (Bialojan and Takai, 1988) and this property is linked to inflammation of the intestinal tract and diarrhea (Hamano *et al.*, 1986; Terao *et al.*, 1986; Cohen *et al.*, 1990). The mechanism of action of the other compounds has not been fully established though they have been described as hepatotoxins and do not cause diarrhea (Yasumoto *et al.*, 1989). Thus in this chapter, only those compounds belonging to the okadaic acid/dinophysistoxin group will be considered as diarrhetic shellfish poisoning (DSP) toxins.

The first recorded incident of gastrointestinal distress and diarrhea in humans, associated with the consumption of mussels that had ingested dinoflagellates, is reported to have occurred in the Netherlands in the 1960s (Kat, 1979, 1985). In Japan, where most of the early chemical studies on DSP toxins were performed, a similar occurrence of DSP poisoning was reported in the late 1970s (Yasumoto *et al.*, 1979, 1980), and because the outbreaks were correlated with the appearance of the dinoflagellate *Dinophysis fortii*, the toxin(s) responsible was named dinophysistoxin (DTX-1), and the syndrome was first described as diarrhetic shellfish poisoning (DSP) (Yasumoto *et al.*, 1980).

The chemical structure of these toxins emerged following the isolation of a new polyether toxin named okadaic acid from the black sponge *Halichondria okadai* (Tachibana *et al.*, 1981) found along the Pacific coast of Japan, as well as from the sponge *H. melanodocia* found in the Florida Keys (Schmitz *et al.*, 1981). The similarity between okadaic acid and DTX-1 isolated from toxic Japanese scallops was quickly recognised and led to the structural characterization of DTX-1 (Murata *et al.*, 1982).

Although it is believed that there were similar occurrences of DSP in Scandanavia during the 1960s, it was not until the mid-1980s, after the chemical structures of the toxins were known, that an episode of DSP was unambiguosly confirmed (Kumagai *et al.*, 1986). Once again, dinoflagellates belonging to the genus *Dinophysis* were implicated in these outbreaks as they were in other major outbreaks of DSP in France and Spain in the 1980s. Such toxin-producing dinoflagellates were also implicated in less serious DSP incidents in Germany, Portugal and Ireland where the new DSP toxin DTX-2 was isolated (Hu *et al.*, 1992a).

In 1990, an episode of DSP in eastern Canada was confirmed (Quilliam *et al.*, 1993), and represents the first documented report of DSP in North America. In this case the toxicity could not be clearly associated with the presence of any *Dinophysis* species, though another dinoflagellate *Prorocentrum lima*, isolated from the toxic area, was found to be a producer of DSP toxins in unialgal culture (Marr *et al.*, 1992a). More recently, another DSP incident in eastern Canada has been confirmed, this time in Newfoundland (Gilgan *et al.*, 1994).

These confirmed incidents, together with other scattered literature reports, suggest that DSP is common in nature, occurring throughout the Pacific, Indian, and Atlantic oceans, though often the culprit toxin is not unambiguously identified.

CHEMISTRY

Chemical and Physical Properties

The DSP toxin group is composed of three compounds, OA, DTX-1, and DTX-2 (Fig. 6.1), and any one or all of these compounds may occur together during a DSP incident. In Japan, DTX-1 is usually the major toxin as it is in the Canadian incidents, whereas in European episodes, with the odd exception, okadaic acid is the major toxin. In DSP outbreaks in Ireland, DTX-2 is usually the major toxin. However, despite these broad generalities, considerable regional and yearly variation in the toxin profile can be observed (Yasumoto *et al.*, 1989).

The DSP toxins are lipid-soluble long chain compounds containing trans-fused or spiro-linked cyclic polyether rings. They are soluble in acetone, chloroform, methylene chloride, methanol and DMSO. Physical-chemical data are shown in Table 6.1.

Naturally Occurring DSP Derivatives

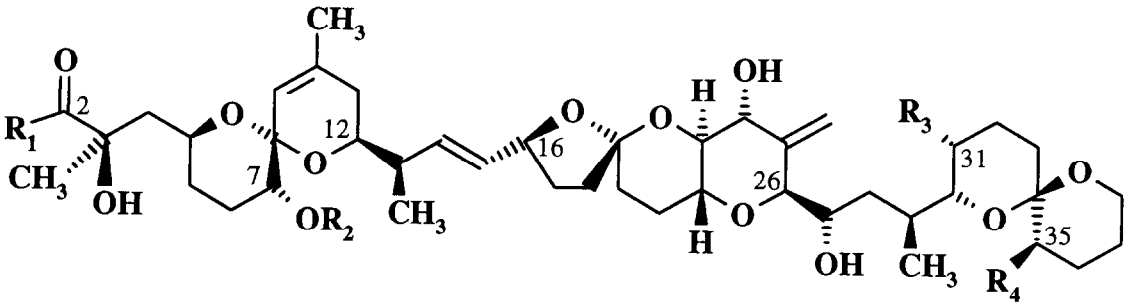
The term DTX-3 was originally coined to describe a group of DSP toxin derivatives in which saturated or unsaturated fatty acyl groups are attached through the 7-OH group of DTX-1 (Fig. 6.1) (Yasumoto *et al.*, 1985). These acyl derivatives also possess toxic activity (Yanagi *et al.*, 1989). Since these compounds have only been observed in shellfish tissue it has been suggested that they are probably metabolic products (Yasumoto *et al.*, 1989) and not *de novo* products of toxin-producing microalgae. More recently, using HPLC-MS analysis to directly examine chromatographic fractions obtained from digestive gland extracts of toxic mussels, it has been shown that any of the parent toxins, okadaic acid, DTX-1 or DTX-2, can be acylated with a range of saturated and unsaturated fatty acids from C_{14}-C_{18} (Marr *et al.*, 1992b).

Two naturally occurring ester derivatives named diol esters were originally isolated from a strain of *P. lima* (Yasumoto *et al.*, 1989). Later, new diol esters were isolated from a Caribbean strain of *P. maculosum* (originally described as *P. concavum*), and one of these was also found in another eastern Canadian strain of *P. lima* (Hu *et al.*, 1992b). Identical diol esters have been reported for a Spanish strain of *P. lima* (Norte *et al.*, 1994). These diol esters do not inhibit the phosphatases PP1 and PP2A *in vitro* (Hu *et al.*, 1992b), perhaps not unexpectedly in light of the earlier observation that the synthetically prepared methyl ester is also inactive in the assay (Holmes *et al.*, 1990; Nishiwaki *et al.*, 1990). Nevertheless, it should be noted that these allylic diol esters may be somewhat labile and could be hydrolysed to yield the active parent DSP toxin. Thus it is recommended that during toxin analysis the diol esters be included in the total toxin concentration.

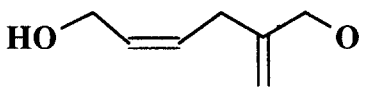
TOXICOLOGY

Action Levels

Compared with other shellfish toxins such as the PSP toxins or ASP toxin (domoic acid), the action level for DSP toxins set by regulatory authorities varies considerably from country to country perhaps reflecting the sensitivity of the recommended detection method (van Egmond *et al.*, 1992, 1993). This variation in action levels is further complicated by the fact that different sampling and detection techniques are also employed from country to country (see Table 6.4, Chapter 22). Generally speaking, when levels of DSP toxin in shellfish exceed 200 ng/g, closure of harvesting and marketing operations is recommended.



R1	R2	R3	R4		
H	H	Me	H	1	(Okadaic acid)
H	H	H	Me	2	(DTX2)
H	H	Me	Me	3	(DTX1)
H	CH_3CO	Me	H	4	(AcOA)
H	Acyl	Me	H	5	
H	Acyl	H	Me	6	("DTX3")
H	Acyl	Me	Me	7	
Et	H	Me	H	8	



H	Me	H	9	
H	Me	H	10	
H	Me	H	11	
H	Me	H	12	
H	Me	H	13	

Figure 6.1. Structures of DSP toxins. Compound 4 is a synthetic internal standard.

Table 6.1 Physical-Chemical Properties of Okadaic Acid, DTX-1 and DTX-2.

	Okadaic Acid	**DTX-1**	**DTX-2**
Melting Point	164-166°C 163-164°C	not reported	128-130°C
Optical Rotation	$[\alpha]_D$ +23.0 ($CHCl_3$) $[\alpha]_D$ +21.0 ($CHCl_3$)	$[\alpha]_D$ +28.0 ($CHCl_3$)	$[\alpha]_D$ +15.49 ($CHCl_3$)
Molecular Wt.	$C_{44}H_{68}O_{13}$ 804.4661	$C_{45}H_{70}O_{13}$ 818.4817	$C_{44}H_{68}O_{13}$ 804.4661
U.V. (CH_3CN)	<220 nm	<220 nm	<220 nm
I.R.	3462(br), 2937, 1736, 1078, 1008, 999, 878 cm^{-1}	not reported	not reported

Toxic Effects

The toxic effects of okadaic acid on mice have been reported (Yasumoto *et al.*, 1979). Symptoms of lethargy, general weakness and diarrhea begin to appear about 30 mins following i.p. injection, and death can occur within a time span of 1.5-48 hrs depending upon the dose. The LD_{50} dose is 200 µg/kg for okadaic acid and 160 µg/kg for DTX-1, and the oral dose is about 16 times greater.

Experiments with suckling mice indicated swelling, congestion and degeneration of the intestines (Hamano *et al.*, 1986; Terao *et al.*, 1986) following i.p. injection of DSP toxins, and a later study with mice and rats confirmed these findings and also revealed additional liver damage (Terao *et al.*, 1993).

In humans, gastrointestinal distress including cramps, diarrhea, nausea and vomiting, are the principal symptoms reported, and these effects persist for about three days before recovery. It has been suggested that the primary symptom of diarrhea is caused by hyperphosphorylation of proteins that control sodium secretion by intestinal cells (Cohen *et al.*, 1990) or by increased phosphorylation of cytoskeletal or junctional moieties that regulate solute permeability, resulting in passive loss of fluids (Dho *et al.*, 1990).

DSP toxins bind to a particular class of receptors, in this case protein phosphatases, found in particulate and cytosolic fractions of mouse tissue (Suganuma *et al.*, 1989). Binding to these receptors results in a rapid build-up of phosphorylated proteins in the cell (Haystead *et al.*, 1989) and this phenomenon is linked to the fact that DSP toxins are powerful tumor promoters (Fujiki *et al.*, 1988; Suganuma *et al.*, 1988). Significantly, these same receptor sites are also present in the stomach, small intestine and colon, and it has been suggested (Suganuma *et al.*, 1988) that these toxins may also act as tumor promoters in the stomach.

Pharmacokinetics and Mechanism of Action

Little information exists on the pharmacokinetics of DSP toxins in mammalian systems. From their chemical nature it is assumed that they are easily absorbed through the cell wall, but no data has been published on their subsequent metabolism and excretion.

Originally, it was found that okadaic acid caused prolonged contraction of smooth muscle from human arteries (Shibata *et al.*, 1982). Since such contractions are activated by phosphorylation of a sub-unit of myosin, it was proposed that the effect of okadaic acid was due to inhibition of myosin light chain phosphatase (Takai *et al.*, 1987). Following this observation, okadaic acid was found to be a powerful inhibitor of the serine/threonine phosphatases PP1 and PP2A (Bialojan and Takai 1988, Haystead *et al.*, 1989). The protein

phosphatases are a critical group of enzymes linked closely with many crucial metabolic processes within a cell (Cohen, 1989), and consequently the perturbation of these phosphatases through hyperphosphorylation results in a wide variety of secondary effects.

Extensive structure-activity studies (Holmes *et al.*, 1990; Nishiwaki *et al.*, 1990) that measured the inhibition of protein phosphatase activity, have indicated that a free carboxyl group in the DSP molecule is essential for activity, since the methyl esters do not inhibit the phosphatases. This is also true for the naturally occurring diol esters which do not inhibit either PP1 or PP2A *in vitro* (Hu *et al.*, 1992b). However, the amide and reduced carboxyl (okadaol) derivatives, are about half as active as okadaic acid, as are the naturally occurring DTX-3 compounds (Yanagi *et al,* 1989; Nishiwaki *et al.*, 1990), and therefore these latter derivatives should be considered during any toxin analysis.

BIOASSAY METHODS

The most commonly used assay method for DSP toxins is the mouse bioassay procedure developed by the Japanese Ministry of Health and Welfare (1981). The digestive glands (weight H in grams) are removed from drained whole shellfish tissues (weight W in grams, where $W \geq 100$) and extracted with 3 x 3H mL acetone. After removal of the acetone by evaporation, the aqueous suspension is extracted with 3 x 3H mL diethyl ether. The combined ether solution is backwashed with 2 x H mL water and evaporated. The residue is suspended in 1.0 mL of 1% Tween-60 saline solution for each 20 g of whole tissue equivalent. Aliquots (0.5-1.0 mL) of this solution (equivalent to 10-20 g whole tissue) are injected (i.p.) into three male mice (ddY or ICR strain) weighing 16-20 g. For very toxic samples, 4-fold and 16-fold dilutions of the Tween-60 suspension (equivalent to 5 and 1.25 g tissue per mL) are also injected. One mouse unit (MU) is defined as the amount of toxin that kills a mouse in 24 h. The toxicity of the sample (MU/g whole tissue) is determined from the smallest dose at which two mice or more in a group of three die within 24 h. The regulatory level in several countries has been set at 0.05 MU/g whole tissue. Since 4 μg of okadaic acid equals approximately one MU, this level is equivalent to 200 ng/g. The assay is suitable for detecting OA, DTX-1, DTX-2 and DTX-3. The major problems associated with the mouse bioassay are long assay time, poor reproducibility and false positives. The latter appears to be due primarily to the presence of high levels of fatty acids in shellfish at certain times of the year (Takagi *et al.*, 1984; Kogawa *et al.*, 1988; Lawrence *et al.*, 1994).

A suckling mouse assay for DSP toxins has been reported by Hamano *et al.* (1985). In this procedure, an extract of shellfish tissue is administered intragastrically to 4-5 day old mice. The degree of fluid accumulation in the gastrointestinal tract is determined after a 4 h period by measuring the ratio of intestine mass to that of the remaining body. Ratio values above 0.8-0.9 indicate a postive reaction. The assay time is shorter than with the mouse bioassay, but quantitation of the results is much more difficult.

Several European countries use a rat bioassay first reported by Kat (1985). Shellfish digestive glands are mixed with normal rat feed and offered to rats that have been starved for 24 h. After a 16 h observation period, an estimate of DSP toxicity is made on the basis of signs of diarrhea, the consistency of the feces, and feed refusal.

An assay based on *Daphnia magna* has been reported recently (Vernoux *et al.*, 1993) to be advantageous because it is inexpensive, simple, and potentially more precise than the mouse bioassay. An intercomparison study indicated that the *Dapnia* assay performed very well compared with mouse bioassay, cytotoxicity tests and HPLC analysis (Marcaillou-LeBaut *et al.*, 1994).

CHEMICAL ANALYSIS

Extraction and Clean-up Methods

All the DSP toxins, being lipid soluble, are extracted from wet or frozen phytoplankton or shellfish tissue using organic solvents such as methanol or acetone. However, such lipid-soluble extracts are considerably more complex compared with aqueous extracts of the same organism and additional cleanup steps are often necessary before further analysis can be undertaken.

In a typical preparative extraction procedure the digestive glands are blended in methanol (ca 2-3 mL/g tissue) and allowed to stand for several hours. The extract is centrifuged, the supernatant collected and the residue extracted once or twice with fresh methanol and the combined methanol extracts then concentrated under vacuum to a thick sludge. This thick viscous material is resuspended in 70% methanol in water and washed thoroughly with hexane. This removes a large amount of fat and non-polar lipid material. The aqueous methanol solution is then adjusted to 25% methanol in water and further extracted with methylene chloride or chloroform. The chlorinated solvent is removed under vacuum and the oily residue dried in a stream of dry nitrogen. This extract contains DSP toxins, their diol esters, and DTX-3-type compounds if they are present.

Extraction of shellfish tissues for routine chemical analysis is best accomplished with a single-step homogenization of 4 mL of 80% methanol in water with one gram of tissue (Lee *et al.*, 1987). Usually only digestive glands are extracted since the toxins accumulate primarily in these organs and this provides an amplification factor of 5 to 10 to facilitate higher sensitivity. The crude methanol extract may be cleaned by partitioning first with petroleum ether or hexane and then with chloroform to extract the DSP toxins. A detailed procedure that has been fully validated is provided in Appendix 2 (Quilliam, 1995).

Thin Layer Chromatography

DSP toxins can be detected by thin layer chromatography (TLC) methods (Hu and Wright, unpublished results). Usually the extracts as prepared above are still too complex for detection on TLC plates and an additional clean-up step (e.g., silica gel column chromatography or gel permeation) is required. Once completed, fractions can be applied directly to a silica gel plate (Merck silica gel 60 F_{254}, 250mm) and eluted with a toluene-acetone-methanol (7.5:4:1) mixture. In this system, the inactive, non-polar diol esters elute rapidly and appear as a strong UV-quenching spot at around R_f 0.85. The acidic DSP toxins themselves appear as a weak UV-quenching spot at R_f 0.4. Both the diol esters and the free acid toxins give a characteristic pinkish-red stain after spraying the plate with a solution of vanillin (0.5 g) in concentrated sulfuric acid-ethanol (100mL, 4:1mixture) and allowing it to stand at room temperature for several minutes. The free acids produce a bright pinkish-red colour whereas the colour is duller with the diol esters. When clean material is applied to a TLC plate, 1 µg of the toxin can be detected; with cruder fractions, 2-3 µg of toxin is required before detection is possible by this method.

High Performance Liquid Chromatography

The DSP toxins may be separated by reversed phase HPLC using an octadecylsilica (ODS) stationary phase and an acidified aqueous acetonitrile or methanol mobile phase. Due to the lack of a good chromophore in the DSP structures (except for some of the diol esters, compounds 10-11 in Fig. 6.1), detection must be accomplished with UV absorbance at 205 to 215 nm or with a refractive index detector. The detection level is about 10 µg/mL in solution, but the low selectivity of the detector requires a high degree of cleanup prior to analysis.

DSP toxins bearing a carboxyl function may be converted to fluorescent ester derivatives for HPLC analysis with fluorescence detection. Early work by Lee *et al.* (1987, 1989) demonstrated the suitability of 9-anthryldiazomethane (ADAM) for this purpose and their method has been implemented in many laboratories. Problems with the implementation of this method on a routine basis have been noted, however. The low purity of the ADAM reagent (which results from its poor stability), and the presence of numerous other reactive co-extractives in shellfish tissues, necessitate a silica column cleanup following the derivatization step (Lee *et al.*, 1987). Other derivatization reagents have been studied for the HPLC analysis of DSP toxins (Allenmark *et al.*, 1990; Luckas, 1992; Dickey *et al.*, 1993; Marr *et al.*, 1994) but none have proven as selective and as sensitive as the ADAM reagent. An extensive study of the ADAM-HPLC procedure (Quilliam, 1995) has shown that it is necessary to pay particular attention to the silica cleanup following the derivatization step. The activities of both the silica gel and solvents must be carefully controlled. An internal standard, 7-O-acetylokadaic acid (compound 4 in Fig. 6.1), was also developed and shown to provide improvements in precision and accuracy by correcting for incomplete recovery in extraction, cleanup and derivatiztion steps and for volumetric errors and instrumental drift (Quilliam, 1995). A detailed procedure based on these developments is provided in Appendix 2. The ADAM method is very sensitive for DSP toxins being able to detect as little as 10 pg of the OA derivative injected on-column. The minimum detectable concentration in shellfish tissue, however, is limited not by detector sensitivity but by chemical background, which can vary considerably between samples. The practical quantitation limit is about 100ng/g tissue. If digestive glands only are used in the analysis this limit translates to 10 to 20ng/g for whole tissue for mussels. Typical ADAM-HPLC analyses of standards and some mussel samples are shown in Fig. 6.2.

Due to their high molecular weight and lipophilicity, the toxins comprising DTX-3 cannot be analyzed directly by HPLC as ADAM derivatives and must first be converted back to OA, DTX-1 or DTX-2 via alkaline hydrolysis (Yasumoto *et al.*, 1989). The diol esters of the DSP toxins cannot be analyzed by the ADAM-HPLC method, but can be analyzed by mass spectrometric techniques (see below).

Mass Spectrometry

Mass spectrometry is a powerful tool for the analysis of marine toxins. Besides high sensitivity and selectivity, mass spectrometry can provide structural information useful for the confirmation of toxin identity and for the identification of new toxins. Fast atom bombardment ionization has proven valuable for generating mass spectra of DSP toxins (Pleasance *et al.*, 1990; Bencsath and Dickey, 1991). Okadaic acid and its synthetic methyl, pentafluorobenzyl and trimethylsilyl ester and ether derivatives are also easily detected by electron ionization and chemical ionization mass spectrometry (Bencsath and Dickey, 1991).

The combination of HPLC with ion-spray mass spectrometry (LC-ISMS) appears to be one of the most sensitive and rapid methods of analysis for DSP toxins (Pleasance *et al.*, 1990, 1992; Quilliam, 1995). A detection limit of 1 ng/g in whole edible shellfish tissue is easily achieved. It is also possible to analyze the diol esters of OA and DTX-1, as well as the DTX-3 toxins, directly by this technique (Hu *et al.*, 1992b; Marr *et al.*, 1992a,b). Although LC-ISMS equipment is expensive in terms of the initial capital purchase, the cost per analysis can be much less than that with HPLC methods because of reduced labor costs, due to fast and simple sample preparation, as well as the possibility of very high sample throughput.

BIOCHEMICAL ANALYSIS

Sensitive immunological assays have been developed for okadaic acid (Levine *et al.*, 1988; Hokama, 1993; Usagawa *et al.*, 1989; Shestowsky *et al.*, 1992, 1993) but they cannot be used

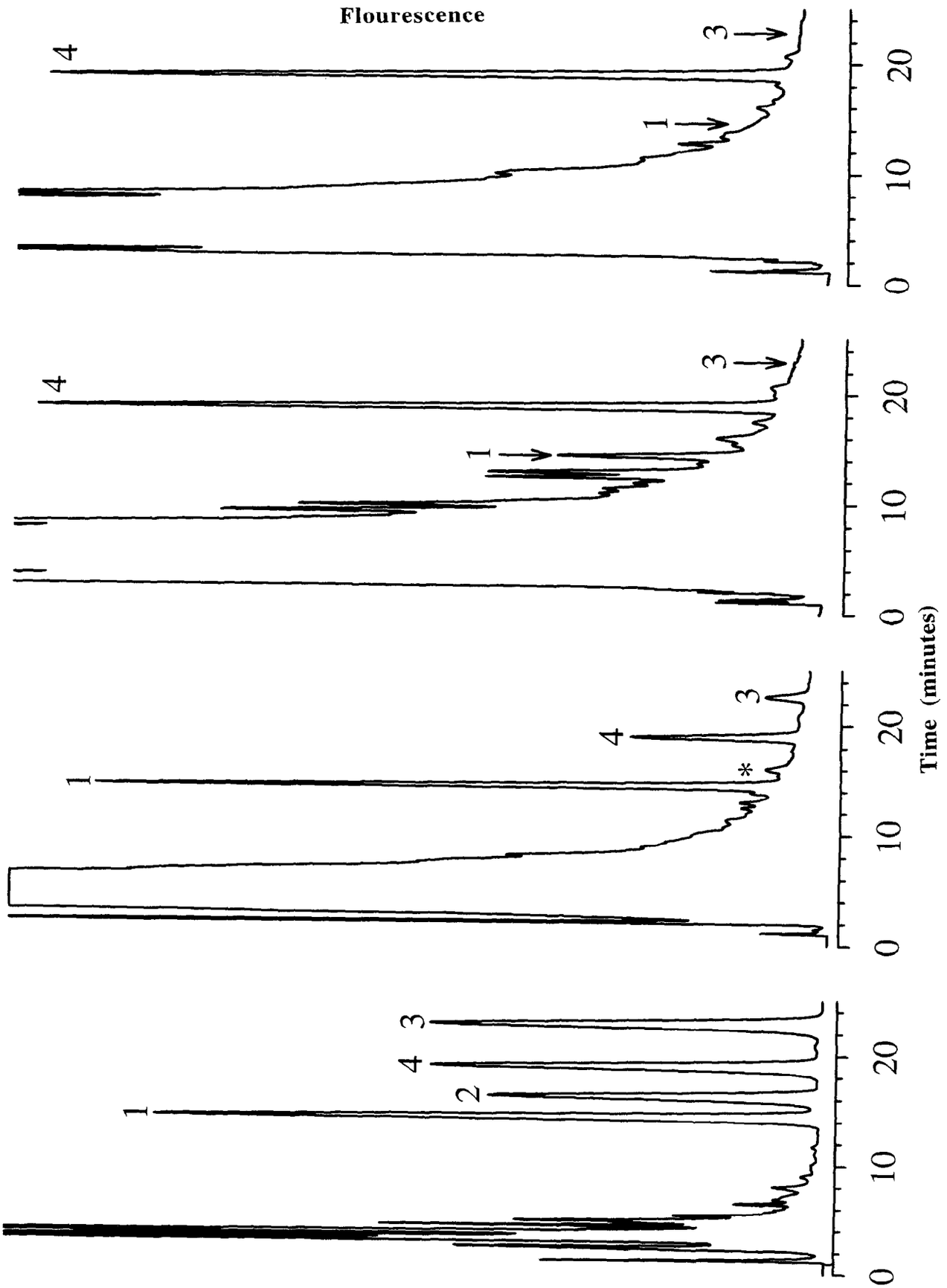


Figure 6.2. HPLC-FLD analysis of ADAM-derivatized calibration standard mixture (a) and extracts of MUS-2 certified mussel tissue reference material (11 mg/g OA, 0.9 mg/g DTX-1) (b), Danish mussels (0.94 mg/g OA) (c), and control mussels (d). Peak identities are given in Figure 6.1. Chromatographic conditions are given in Appendix 2.

for precise quantitative analysis of toxin mixtures because of differences in cross-reactivities of individual toxins with the antibodies. Holmes and co-workers (Holmes, 1991; Luu *et al.*, 1993; Boland *et al.*, 1993) have developed a liquid chromatography-linked phosphatase radioassay based on the inhibition of type 1 and type 2A protein phosphatases. Although this method is very sensitive, it requires the handling of radioactivity (^{32}P) and the use of special techniques and enzymes not usually available in routine analytical laboratories. A colorimetric phosphatase inhibition assay has recently been reported that may make this approach more amenable (Simon and Vernoux, 1994). A cytotoxicity assay has also been reported that has promise (Amzil *et al.*, 1992).

APPENDIX 1

Commercial Sources

Okadaic acid is commercially available from several sources:

(a) Diagnostic Chemicals Ltd., (West Royalty Industrial Park, Charlottetown, PEI, Canada, C1E 1B0; tel 902-566-1396; fax 902-566-2498; or DCL (USA), 160 Christian St., Oxford, CT 06478; tel 203-881-2020; fax 203-888-1143).

(b) Calbiochem (P.O. Box 12087, La Jolla, CA, 92039-2087, USA; tel. 619-450-9600; fax 619-453-3552) (Okadaic acid: Catalog No. 495604).

(c) LC Services Corporation (165 New Boston Street, Woburn, MA, 01801, USA) (Okadaic acid: Catalog No. O-2220).

(d) Sigma Chemical Company (P.O. Box 14508, St. Louis, MO, 63178, USA; tel. 314-771-5750; fax 314-771-5757) (Okadaic acid: Catalog No. O1506; Ammonium okadaate: Catalog No. O8010).

These companies also offer other derivatives including the less potent okadaol reduction product, the tetraacetate derivative, and the methyl ester.

Calibration Standards and Reference Materials

An important concern in any analytical method is the availability of accurate calibration standards and reference materials. Since okadaic acid is expensive and difficult to acquire in high purity, the NRC Marine Analytical Chemistry Standards Program (MACSP) has developed a calibration standard (OACS-1) with a certified level (25.3 µg/mL) of okadaic acid in methanol (package contains four 0.5 mL ampoules, stable for one year at -12°C in dark conditions). MACSP has also developed a mussel tissue reference material (MUS-2) which was made by blending contaminated mussel digestive glands with a small amount of cultured *Prorocentrum lima* biomass and water, to give an homogenate containing ca. 11 µg/g of okadaic acid and 1 µg/g of DTX-1 (package contains four 4g vials, stable in unopened form at -12°C for one year). The reference material is useful for testing the full implementation of an analytical method, such as the one detailed in Appendix 2, and for testing newly developed analytical methods. They may be ordered from: MACSP, National Research Council of Canada, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, NS, B3H 3Z1, Canada; telephone 902-426-8280; fax 902-426-9413.

APPENDIX 2

HPLC Analysis of DSP Toxins

This section presents a detailed procedure for the determination of the acidic DSP toxins OA, DTX-2 and DTX-1, in shellfish (Quilliam, 1995). Toxins are extracted from shellfish digestive glands by homogenization with methanol-water (8:2, v/v). After an initial cleanup with liquid-liquid partitioning, derivatization with 9-anthryl-diazomethane (ADAM), and further cleanup on a silica column, toxins are analyzed by high performance liquid chromatography with fluorescence detection.

Reagents and Equipment

(a) *Water:* Distilled and passed through a water purification system equipped with ion-exchange and carbon filters.

(b) *Solvents:* Distilled-in-glass grade acetonitrile, methanol and hexane; anhydrous ethanol; ACS grade diethyl ether; ACS grade chloroform with ethanol preservative.

(c) *Alumina:* Woelm basic alumina (activity grade 1), activated at 450°C overnight (Alupharm Chemicals, New Orleans, LA 70130).

(d) *Silica:* Analytichem Bondesil 40 μm (No. 1221-3001, Varian Sample Preparation Products, Harbour City, CA 90710). Individual 500 mg portions should be measured into small beakers, covered with foil and stored in an oven at 130°C (for at least 24 hr).

(f) *Chloroform (with 1.15% ethanol) for silica cleanup wash step:* Activated alumina (50 g) (c) is dry packed into an oven-dried glass column (35 cm x 21 mm i.d.) with glass frit and teflon stopcock. ACS grade chloroform is then passed through the column. The first 10 mL is discarded and the next 50 mL collected into an oven-dried 50-mL volumetric flask containing 575 μL of anhydrous (absolute) ethanol.

(g) *ADAM:* 9-Anthryldiazomethane, delivered on dry ice from the supplier (No. A-1400, Molecular Probes Inc., Eugene, OR 97404), should be split immediately into 2 mg portions and stored in amber vials at -80°C.

(h) *ADAM solution:* 2 mg ADAM dissolved in 1 mL methanol. The solution must be prepared daily, used as soon as possible, and handled under yellow or subdued lighting.

(i) *7-O-Acetylokadaic acid (AcOA, internal standard) (optional):* Synthesized by mixing 1 mg okadaic acid, dissolved in a mixture of 100 μL pyridine and 50 μL of 3.2 mg/mL acetic anhydride in pyridine (Aldrich Chemical Co., Milwaukee, WI 53233), and allowing the reaction to proceed at room temperature overnight. After evaporation and redissolution in methanol, the major product (AcOA) is isolated by preparative HPLC using a Vydac 201TP column (250 x 10 mm i.d.) with 4 mL/min aqueous 60% methanol and UV detection at 220 nm.

(j) *AcOA (internal standard) solution:* 100 μg AcOA dissolved in 1 mL methanol.

(k) *Deoxycholic acid (DCA):* 98% purity (No. D2510, Sigma Chemical Co., St. Louis, MO 63178).

(l) *DCA solution:* 3.5 mg deoxycholic acid dissolved in 100 mL methanol.

(m) *Okadaic acid (OA):* 99% purity (Diagnostic Chemicals Ltd., Charlottetown, PEI).

(n) *OA solution:* OACS-1 certified calibration solution (25.3 µg/mL, Marine Analytical Chemistry Standards Program, National Research Council Canada, 1411 Oxford St., Halifax, NS, Canada, B3H 3Z1). Perform accurate dilutions of OACS-1 in methanol to give solutions of concentrations 1.0, 2.5, 5.0 and 12.5 µg/mL.

(o) *Calibration solution:* Mix exactly 400 µL of OA solution (n) (either OACS-1 or one of the accurate dilutions of OACS-1 in methanol), 140 µL DCA solution (l), 50 µL AcOA solution (j) and 110 µL methanol in a 1.5-mL amber glass screw-cap vial (if AcOA solution is not available, the methanol should be increased to 160 µL).

(p) *Mussel tissue reference material:* MUS-2 (Marine Analytical Chemistry Standards Program).

(q) *Empty SPE tubes:* 7 mL glass barrels and teflon frits (No. 7999BJ, Baxter Healthcare Corp., Burdick & Jackson Division, Muskegon, MI 49442).

(r) *Derivatization vial:* Screw cap amber glass vial, 1.5 mL capacity, teflon-lined cap (No. C221200A, Chromatographic Specialties, Brockville, Ontario). Vials should be cleaned thoroughly with acetone and dried at 70°C overnight.

(s) *Liquid chromatograph:* Isocratic system able to generate >2500 psi and equipped with a dual monochromator fluorescence detector with 254 nm excitation and 412 nm emission protected by a 280 nm cut-off filter. A filter fluorescence detector with the appropriate bandpass filters can also be used.

(t) *HPLC column:* Stainless steel, 4.0 mm id x 25 cm, packed with 5 µm LiChrospher-100 RP18 octadecylsilica (Merck, Darmstadt, Germany).

Preparation of Samples

1. *Tissue preparation:* Tissues should be drained after removal from shellfish. Separate and pool the meats and digestive glands from several animals in a lot. Weigh the pooled tissues to determine the fraction of whole edible tissue represented by digestives glands. For representative sampling, approximately 20 g of digestive glands pooled from several animals should be homogenized in a blender. Subsampling from this homogenate is done immediately after blending while still well-mixed, or later after mixing again. If an analysis must be performed on a limited amount of sample, a portion of chopped or ground tissue can be weighed directly into the extraction tube.

2. *Extraction:* Weigh a 2.0 g portion of homogenized tissue sample accurately into a 50-mL plastic centrifuge tube. Add an accurate 100 µL aliquot of AcOA (internal standard) solution (j) and 7.9 mL of aqueous 80% methanol. If AcOA is not available use 8.0 mL extraction solvent instead. Homogenize the mixture using a Polytron mixer for 3 min at 6-10K rpm. Sample adhering to the probe may be shaken into the extraction tube but no additional solvent should be used to recover the sample residues; the probe should be washed separately before use on the next sample. Centrifuge the sample at 4000xg or higher for 10 min. Decant the supernatant into a screw-cap vial and store in the freezer until step 3 is executed.

3. *Liquid-liquid partitioning cleanup:* Transfer an accurate 5.0-mL aliquot of crude extract (supernatant from step 2) to a 15-mL glass centrifuge tube and extract twice with 5-mL aliquots of n-hexane by vortex mixing for 0.5 min. After discarding the hexane layers, add 1

mL of water and 6 mL chloroform to the tube and vortex mix the mixture for 0.5 min. Transfer the lower chloroform layer to a 50-mL glass test tube. Repeat the extraction of the aqueous layer with another 6 mL of chloroform. Evaporate the combined chloroform layers to dryness under a stream of nitrogen. Dissolve the residues after evaporation in exactly 200 µL DCA solution (l) plus 800 µL methanol by vortex mixing. Transfer this solution (without further addition of solvent) to a 1.5-mL amber glass screw-cap vial. The dissolution and transfer operations must be done quickly to avoid evaporation of the methanol. This extract contains 1 g digestive gland equivalent per mL of solution.

4. *ADAM derivatization:* Transfer 35.0-µL aliquots of cleaned sample extracts (from step 3), calibration solution (o), or methanol (reaction blank) into individual cleaned 1.5-mL amber vials. Working under yellow or subdued light, add 100-µL aliquots of 0.2% (w/v) ADAM solution (h) to each of the vials. After sealing tightly with a teflon-lined screw cap, sonicate the solutions for 10 min in warm water (37°C) and then heat at 37°C for 2 hours in the dark. Finally, use a vacuum centrifuge or nitrogen stream to evaporate all reaction solutions to dryness.

5. *Silica cleanup:* Place clean, dry glass SPE tubes (7 mL capacity) equipped with teflon frits on an SPE vacuum manifold and pack with 500 mg of activated silica (d) (this operation should be done immediately prior to use in order to avoid deactivation of the silica by atmospheric moisture). Condition the columns with 6 mL chloroform followed by 3 mL chloroform-hexane (1:1). Stop the flow when the meniscus reaches the top of the packing; the columns should not be allowed to go dry thereafter. Redissolve residues from the evaporated ADAM reactions (from step 4) and transfer to the columns using three 300 µL aliquots of chloroform-hexane (1:1) and pass slowly (1 drop/sec) through to waste. Wash the columns with 5 mL chloroform-hexane (1:1) and 5 mL alumina-cleaned chloroform containing 1.15% ethanol (f). After placing clean glass test tubes under each column, elute the ADAM derivatives with 5 µL methanol-chloroform (1:9). Evaporate the eluates to dryness under a nitrogen stream and then dissolve the residues in exactly 500 µL methanol. Transfer these solutions (without further addition of solvent) to amber vials for HPLC analysis.

HPLC Determination

Analyze derivatized, cleaned samples from step 5 by HPLC using isocratic conditions with mobile phase of 1.0 mL/min aqueous 80% acetonitrile, the HPLC column maintained at 40°C, and an injection volume of 10 µL. If a column heater is not available, ambient temperature may be used with aqueous 85-90% acetonitrile. The fluorescence detector should be set for 254 nm excitation and 412 nm emission protected by a 280 nm cut-off filter (wavelength accuracy should be checked). A full calibration curve experiment should be run at least once using the entire set of derivatized calibration solutions (o) to ensure linearity and zero intercept. Thereafter, a single point calibration may be performed by analyzing one calibration solution with a concentration similar to that of the samples. Replicate injections should have CVs <5%. The reaction blank should show no interfering peaks at the retention times of the derivatized toxins. Inject all samples in duplicate. Avoid carry-over between injections of different samples by washing the injector loop. Average peak areas for each sample. Repeat single injections of calibration standard every 2 hr and duplicate injections every 8 hr. In calculations, average the peak areas of standards immediately following and preceding a series of samples.

Calculate the concentration of OA, DTX-1 or DTX-2 (µg/g) in each sample according to equation 1 where A_{TS} is the average peak area for the toxin in the sample, A_{TC} is the average area for okadaic acid in the calibration standards (concentration C_{TC} µg/mL) bracketing the sample, A_{IS} is the average peak area for the AcOA (internal standard) in the sample, A_{IC} is the average area for the AcOA in the calibration standard, W is the weight in grams of digestive glands extracted (ca 2.0 g), and F is the fraction of the whole tissue represented by the digestive glands.

$$\text{Toxin concentration } (\mu g/g \text{ whole tissue}) = 2(A_{TS}/A_{TC})(A_{IS}/A_{IC})(C_{TC}/W)(F) \quad [1]$$

If AcOA is not available as an internal standard, equation 2 should be used.

$$\text{Toxin concentration } (\mu g/g \text{ whole tissue}) = 2(A_{TS}/A_{TC})(C_{TC}/W)\,(F) \quad [2]$$

The second internal standard (DCA) is used to determine the effectiveness of the derivatization reaction. Incomplete derivatization may occur due to an impure reagent and/or excessive matrix interference. The AcOA internal standard will correct for as much as 50% incomplete derivatization since it reacts at exactly the same rate as the toxins. DCA reacts more slowly than the toxins, however, and is therefore more sensitive to matrix effects. The peak areas for the DCA internal standard in both samples and standards should be within 10% of each other for quantitation by the second equation to be valid.

Safety

The DSP toxins cause severe gastrointestinal illness and are tumour promotors. Chloroform and methanol are toxic, volatile solvents. All of these substances are harmful if swallowed, inhaled or absorbed through the skin. The toxicity of ADAM has not been reported but it is a potential carcinogen and skin irritant.

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7. Methods for Domoic Acid, the Amnesic Shellfish Poisons

J.L.C. Wright and M.A. Quilliam

The amnesic shellfish poisoning toxin, domoic acid, was originally isolated from a red macroalga *Chondria armata* by Japanese researchers (Takemoto and Daigo, 1958) studying the insecticidal properties of algal extracts. The structure was later revised by Ohfune and Tomita (1982) (see Fig. 7.1). It belongs to a group of amino acids called the kainoids and these compounds are classed as neuroexcitants or excitotoxins that interfere with neurotransmission mechanisms in the brain. The original member of the group, kainic acid, was isolated earlier from another red macroalga *Digenea simplex* (Murakami *et al.*, 1953).

A serious outbreak of shellfish poisoning occurred in eastern Canada in 1987, following the consumption of contaminated mussels. None of the known shellfish toxins was implicated in this incident and eventually domoic acid was identified as the toxic agent (Wright *et al.*, 1989). It was present at levels as high as 1000 µg/g of edible tissue. This represented the first report of domoic acid as a shellfish toxin, and although known as an excitatory amino acid, until that time there had been no reports of human toxicity. In fact, extracts of *D. simplex* containing the related excitatory amino acid kainic acid, are used in parts of south east Asia for their anthelmintic properties. There is a report that domoic acid was used in a similar fashion (Daigo, 1959) though the amounts ingested are calculated to be much lower than experienced in the Canadian poisoning incident.

It was eventually shown (Subba Rao *et al.*, 1988; Bates *et al.*, 1989) that the source of the domoic acid in the eastern Canadian incident was the diatom *Pseudonitzschia pungens* f.*multiseries* (=*Nitzschia pungens* f.*multiseries*). It was shown that a laboratory culture of the organism produced domoic acid at a level of 1 to 20 pg/cell. Until this discovery, it was generally believed that phycotoxins were produced only by dinoflagellates, and diatoms were not regarded as sources of toxins.

In September 1991, the unexplained deaths of pelicans and cormorants in Monterey Bay, California were attributed to another outbreak of domoic acid poisoning (Fritz *et al.*, 1992; Work *et al.*, 1993). In this case, the toxic species was identified as a related diatom *Pseudonitzschia australis* (Fritz *et al.*, 1992; Buck *et al.*, 1992; Garrison *et al.*, 1992), which was consumed by anchovies that in turn were eaten by the birds. The anchovies were shown to contain domoic acid at concentrations as high as 100 µg/g with analyses by high performance liquid chromatography (HPLC) and mass spectrometry. Shortly after this episode, in October 1991, it was found that 0.1N HCl extracts of razor clams from the coast of Oregon induced domoic acid-like symptoms in mice. Analyses showed that the toxin was indeed present at levels over 100 µg/g (Loscutoff, 1992). The appearance of domoic acid on the west coast of North America prompted the regulatory authorities to conduct a massive survey of many marine species for the presence of domoic acid (Loscutoff, 1992; Wekell *et al.*, 1994). The toxin was found widely spread from California to Washington. Unexpectedly, domoic acid was found in the viscera of Dungeness crabs, the first time the toxin had been found in a crustacean, and this had a severe effect on the crab fishery during 1991 and 1992.

Since these incidents, global awareness to domoic acid has been raised, and toxin-producing strains of *Nitzschia* have also been reported from the Gulf of Mexico region (Dickey *et al.*, 1992), as well as Denmark (Lundholm *et al.*, 1994) while low levels of the toxin were reported in shellfish from New Zealand, though no cases of human toxicity were reported (Chang *et al.*, 1993).

CHEMISTRY

Domoic acid is a naturally occuring compound belonging to the kainoid class of compounds that has been isolated from a variety of marine sources including macro- and microalgae. As well as kainic acid, another marine product, the group also includes the acromelic acids A and B, isolated from mushrooms (Konno *et al.*, 1988).

Chemical and Physical Properties

A considerable amount of chemical data for domoic acid has been described (Takemoto and Daigo, 1958, 1960; Ohfune and Tomita, 1982; Wright *et al.*, 1989). The toxin is a crystalline water-soluble compound displaying properties typical of an acidic amino acid. The physical-chemical data for the toxin are shown in Table 7.1.

Table 7.1. Physical-Chemical Properties of Domoic Acid.

M.Pt.	Optical Rotation	Mol. Weight	UV (ethanol)	IR (film)
215-216°C (dihydrate)	$[\alpha]_D^{25}$ -120.5° (anhydrous) $[\alpha]_D^{25}$ -108° (dihydrate)	$C_{15}H_{21}NO_6$ 311.14	242 nm $\varepsilon = 2.43 \times 10^4$ (pH 2) $\varepsilon = 2.61 \times 10^4$ (pH 7)	3500-2500, 1715, 1400, 1215, 966 cm^{-1}

The UV (Falk *et al.*, 1989), IR (Falk 1988), and NMR data (Walter *et al.*, 1992) for domoic acid are pH-dependent, and five protonated forms of the toxin are possible. The carboxyl group at C2 is most acidic, followed by the one at C5' and finally the C7 group.

Secondary amino acids such as proline, kainic acid and domoic acid form a yellow derivative with ninhydrin. The colour is distinct enough for the qualitative detection of domoic acid in mixtures or hydrolysates separated by thin layer or paper chromatography. The utility of other colour reagents has been investigated, and although both isatin and vanillin give well developed coloured derivatives with domoic acid, the vanillin stain was found to be particularly characteristic for domoic as well as kainic acid (Dallinga-Hanneman *et al.*, 1993).

Naturally Occurring Isomers

Later investigations of the insecticidal kainoids present in the red alga *Chondria armata* resulted in the discovery, in minor amounts, of the isomers isodomoic acid A, B, and C (Maeda *et al.*, 1986) (see Fig. 7.1) as well as domoilactones A and B (Maeda *et al.*, 1987).

None of these isomers found in seaweed has been reported in extracts of plankton or shellfish tissue. Domoic acid is the major toxin present in plankton or contaminated shellfish, although three geometrical isomers (isodomoic acids D, E and F) and the C5' diastereomer (see Fig. 7.1) have been isolated in small amounts from both plankton cells and shellfish tissue (Wright *et al.*, 1990; Walter *et al.*, 1994). The geometrical isomers can be prepared in the laboratory by brief exposure of dilute solutions of domoic acid to UV light (254nm), and are not considered to be *de novo* products of the plankton. Pharmacological studies indicate that these photoisomers bind less strongly to the kainate receptor proteins than domoic acid itself, suggesting that they are not as toxic as the parent amino acid (Hampson *et al.*, 1992).

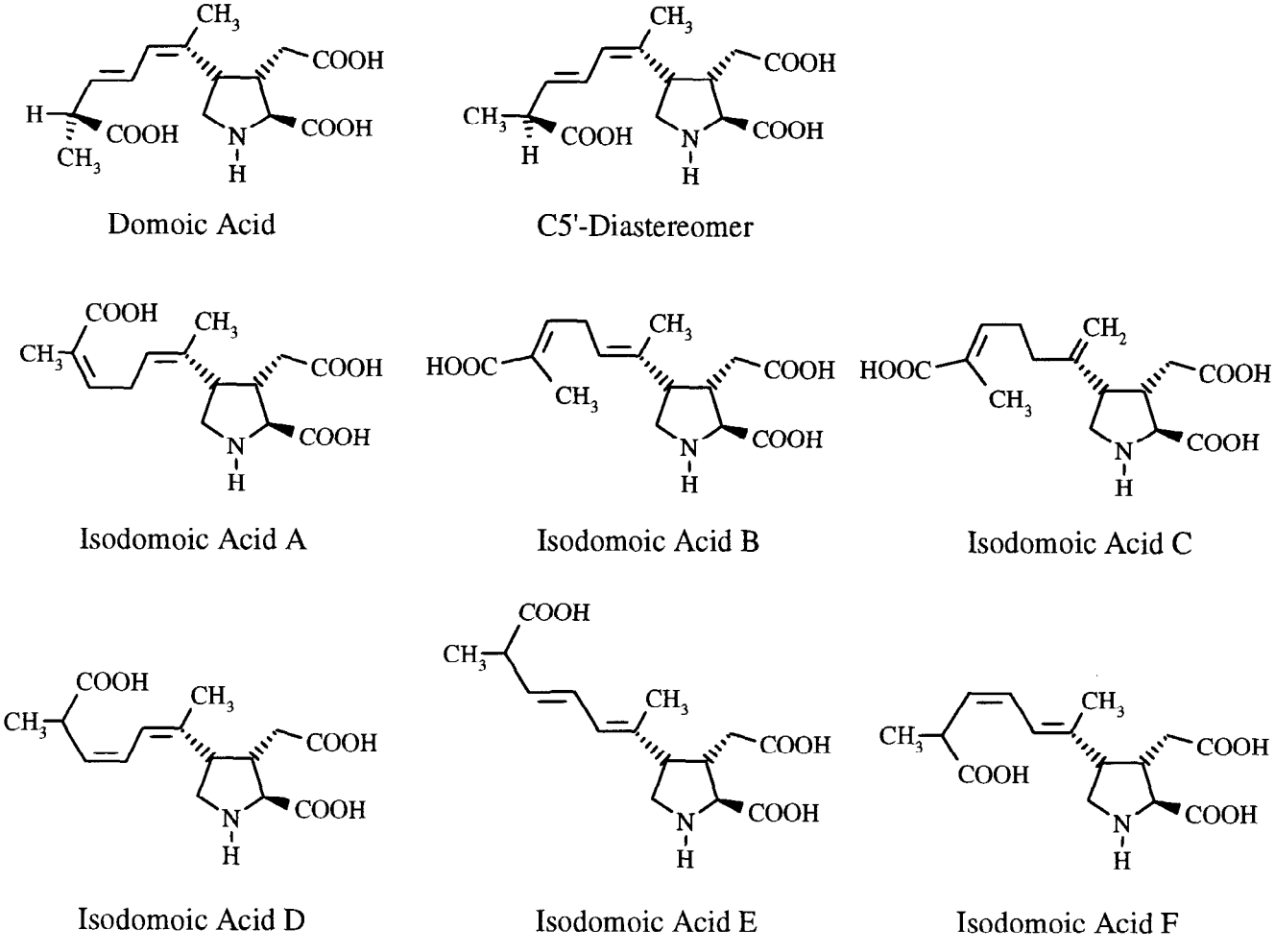


Figure 7.1. Structures of domoic acid and some of its isomers.

Formation of the C5' diastereomer is accelerated with warming (Quilliam, unpublished results) and importantly shows almost the same binding efficacy to the kainate receptor as domoic acid itself (Hampson *et al.*, 1992). All of these isomers can be separated from domoic acid by the HPLC method described later.

TOXICOLOGY

Action Levels

The safe limit or action level for domoic acid in Canada was set at 20 µg/g shellfish tissue (Iverson *et al.*, 1990; Iverson and Truelove, 1994), and for the moment at least this level has been adopted by other countries screening for the toxin. Recently the regulatory action level for domoic acid in crab viscera was raised to 80 µg/g. During the 1987 Canadian incident, toxic shellfish were estimated to contain domoic acid in the concentration range 300-1000 µg/g, and it is believed that individuals may have ingested as much as 1-2 mg/kg of the toxin.

Toxic Effects

The toxic effects of domoic acid have been studied using mice, rats and cynomolgus monkeys (Iverson *et al.*, 1990; Tryphonas *et al.*, 1990a,b,c,d), and a behavioural rating scale for mice was developed (Tasker *et al.*, 1991). The toxin induces very characteristic symptomology in mice following intraperitoneal injection (Tasker *et al.*, 1991). The most characteristic symptoms include a unique scratching of the shoulders by the hind leg, followed by convulsions and often death. More subtle effects include hypoactivity, sedation-akinesia, rigidity, sterotypy, loss of posteral control, and tremors.

Information on human toxicology comes from the 1987 Canadian incident, when 107 people were admitted to hospital. Fourteen individuals displayed severe neurological poisoning and four patients died (Teitelbaum *et al.*, 1990; Perl *et al.*, 1990). Symptoms included gastrointestinal effects such as nausea, vomiting, gastric distress, gastric bleeding and diarrhea. Neurological effects tended to follow and included dizziness, confusion, weakness, lethargy, somnolence, coma, seizures, and permanent short term memory deficit.

There is no information or knowledge of the effects of repeated exposure to low levels of domoic acid, whether this will manifest itself in later life, or whether previous exposure to the toxin results in a different pharmacodynamic response.

Pharmacokinetics and Mechanism of Action

Domoic acid in seafood is absorbed through the gastrointestinal mucosa, though the rate of absorption is very low (Iverson *et al.*, 1990). The rate of transfer to brain tissue as measured in rats is also low (Preston and Hynie, 1991) but it does eventually reach circumventricular central nervous system targets (Bruni *et al.*, 1991). Orally administered domoic acid is excreted almost entirely in the feces (Iverson *et al.*, 1990), and domoic acid in the blood stream is cleared rapidly by the kidneys (Suzuki and Hierlehy, 1993; Truelove and Iverson, 1994). In rats and monkeys, the half life for domoic acid in the blood stream is 65 min and it is fully cleared after 2h (Truelove and Iverson, 1994). Both kainic and domoic acids can be regarded as conformationally restricted forms of glutamic acid, and both act as glutamate agonists in the brain and central nervous system though domoic acid is several times more potent than kainic acid (Biscoe *et al.*, 1975, 1976; Coyle, 1983; Laycock *et al.*, 1989). Domoic acid binds strongly to glutamate receptors of the kainate sub-type, present throughout the central nervous

system (Stewart *et al.*, 1990; Hampson *et al.*, 1992) and studies of pathological tissues indicate damage to neurons in the hippocampal region of the brain (Tryphonas *et al.*, 1990a,b,c,d).

BIOASSAY METHODS

The mouse bioassay (intraperitoneal injection), as it was developed to monitor PSP toxins but with longer observation times (up to 4 hrs), was employed initially during the Canadian crisis in 1987 to trace and isolate domoic acid (Wright *et al.*, 1989). The success of this approach was due in part to the high levels of toxin present in contaminated shellfish (300-1000 μg/g tissue). However, the sensitivity of the bioassay method is inadequate for the action level of 20 μg/g tissue that was set following the crisis. Fig. 7.2 shows a dose response curve for the mouse bioassay of domoic acid. Nevertheless, the toxin does induce in mice the very characteristic symptomology discussed above (the scratching syndrome in particular), so from this viewpoint the method still has some merit. Such symptoms may be observed with extracts of shellfish containing >40 μg/g domoic acid, and are more definite when levels are around 100 μg/g.

CHEMICAL ANALYSIS

Extraction and Cleanup Methods

During the early days of the 1987 Canadian crisis, domoic acid was first extracted from contaminated shellfish using the standardized extraction procedure for mouse bioassay of paralytic shellfish poisoning (PSP) toxins (AOAC, 1984). This involves boiling drained shellfish tissue with an equal volume of 0.1 N HCl and then filtering a portion of the supernatant. Because this procedure provides an extract suitable for mouse bioassay and HPLC analysis of both PSP toxins and domoic acid, it was adopted with slight modifications for routine regulatory analysis (Lawrence *et al.*, 1989, 1991; AOAC, 1991). There are a number of problems with this procedure, however: (a) partial decomposition of domoic acid during the extraction, especially at trace levels; (b) difficulty with certain matrices (e.g., anchovy tissue tends to form a gel); (c) a long sample preparation time; (d) inability to store the extract (due to rapid decomposition of domoic acid in the acidic solution); and (e) short HPLC column lifetime due to the lack of a cleanup. Preparative isolation of domoic acid from mussel tissues for structural characterization in the 1987 incident was accomplished with an aqueous 50% methanol extraction (Wright *et al.*, 1989).

Alternative extraction solvents, such as aqueous methanol and boiling water, were investigated by Quilliam and coworkers (1989a) for quantitative determinations. While both solvents gave reproducible, high recoveries, the boiling water procedure was recommended at that time because it was compatible with a C18 solid phase extraction (SPE) cleanup. The latter was useful for extending the lifetimes of HPLC columns. More recently, a rapid extraction and cleanup procedure was developed for shellfish and finfish tissues that is based on an extraction with aqueous methanol (1:1) and cleanup by strong anion exchange (SAX) solid phase extraction (SPE) (Quilliam *et al.*, 1991, 1995). With this procedure the recovery of domoic acid is greater than 90% even at trace levels. The crude methanolic extract is cleaner than aqueous extracts, but must be diluted 5-fold with water before analysis by HPLC to avoid appreciable peak broadening due to the "solvent wash-out" effect. Under the conditions developed, the SAX cleanup provides unique selectivity towards the strongly acidic domoic acid, thus eliminating most interferences in the HPLC analysis. It also provides a preconcentration that facilitates trace level detection of domoic acid. Details of this procedure are provided in Appendix 2 at the end of this Chapter.

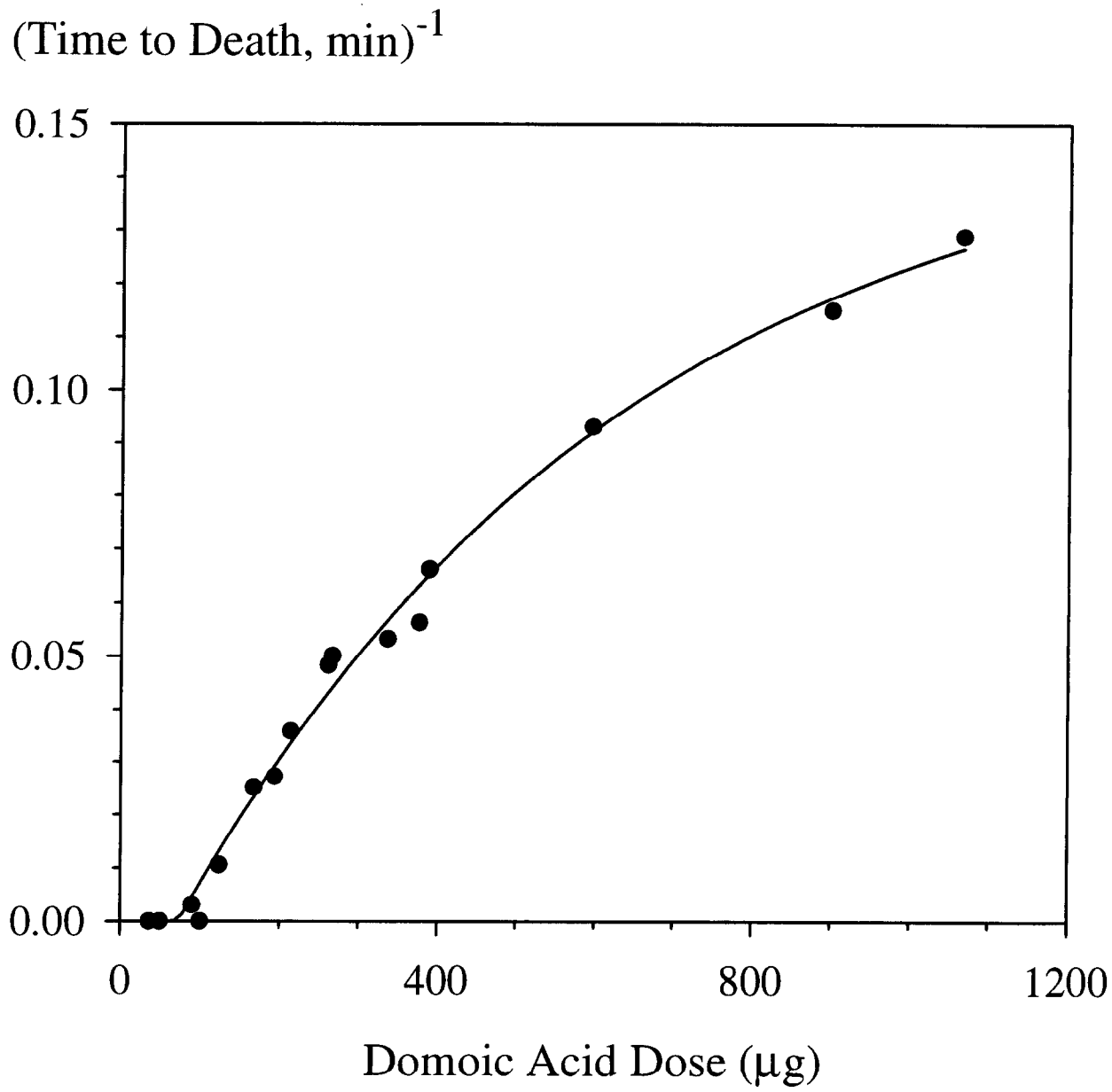


Figure 7.2. Dose response curve for the mouse bioassay of domoic acid.

Thin Layer Chromatography

Domoic acid can be analyzed semi-quantitatively by thin layer chromatography (TLC) methods (Thomas and Wright, unpublished results). This should be useful for those laboratories not equipped with an HPLC system. Domoic acid can be detected on TLC plates (silica gel 60 F_{254}) as a weak UV-quenching spot that stains yellow after spraying with a 1% solution of ninhydrin. Normal amino acids that are present in crude extracts will interfere and must be separated from domoic acid. This can be accomplished with plankton samples by two-dimensional TLC. Crude extracts of shellfish tissues cannot be analyzed directly, as they are too complex. The SAX-SPE cleanup method, with the minor modification described in Appendix 2 (step 6), yields fractions that can be used directly or concentrated *in vacuo* before applying to a silica gel plate. Only one-dimensional TLC is required when this cleanup is used as almost all interfering amino acids are removed by the cleanup. Details of the method are provided in Appendix 2. The detection limit for domoic acid is about 0.5 µg by this method, which permits detection in shellfish tissues at about 10 µg/g.It is also possible to detect domoic acid on the TLC plate using some of the other spray reagents mentioned above (Dallinga-Hanneman *et al.*, 1993). For example, after spraying a TLC plate with vanillin, a yellow colour with domoic (or kainic) acid forms first and changes to pink on standing.

Amino Acid Analysis

Crude aqueous extracts of plankton can be analyzed directly by an amino acid analyzer system (M.V. Laycock, personal communication). Using the buffer solutions and ion-exchange column normally used for the analysis of protein hydrolysates, domoic acid elutes close to methionine. Absorbance measurement at 440 nm provides detection of amino acids with primary amine groups, while absorbance at 570 nm selectively detects imino acids such as proline and domoic acid. The detection limit of this method for domoic acid is about 1 µg/mL, with about 50 µL of extract injected on-column. Although the sensitivity of the amino acid analysis method is close to that of the HPLC-UV method, it is not as effective for samples containing a high concentration of free amino acids and the analysis time is much longer. Shellfish extracts can be analyzed by this approach after the necessary cleanup and concentration of material as described above.

High Performance Liquid Chromatography

During the 1987 Canadian incident, it was found that domoic acid could be analyzed, as well as preparatively isolated, by either high-performance liquid chromatography or ion exchange chromatography using ultraviolet absorbance detection (Wright *et al.*, 1989). Reversed-phase HPLC-UV gives the fastest and most efficient separations. Use of an acidic mobile phase to suppress ionization of the carboxyl functions is recommended, and selective separation of domoic acid and its isomers is best achieved with "polymeric-like" octadecylsilica phases such as Vydac 201TP (Quilliam *et al.*, 1989a).

HPLC-UV is the preferred analytical technique for the determination of domoic acid in shellfish (Quilliam *et al.*, 1989a; Lawrence *et al.*, 1989, 1991; AOAC, 1991) and has been used since 1987 by Canadian regulatory agencies to prevent other incidents of shellfish poisoning. The detection of domoic acid is facilitated by its strong absorbance at 242 nm. The HPLC-UV detection limit for domoic acid is about 10-80 ng/mL, depending on the sensitivity of the UV detector that is used. The detection limit in tissue is dependent upon the method of extraction and cleanup. If crude extracts (either acidic or aqueous methanol) are analyzed without cleanup, the practical limit for quantitation is about 1 µg/g (ppm). This is suitable for most regulatory laboratories concerned with detecting contamination levels greater than 20 µg/g. However, interferences are commonly encountered that can give false positives with crude extracts. For example, it has been shown that tryptophan and some of its derivatives are often present in

substantial concentrations in shellfish and finfish tissues and that these compounds elute close to domoic acid (Quilliam *et al.*, 1989a). A photodiode array detector can be used to examine UV spectra in order to confirm domoic acid, but this option may not always be available. Fig. 7.3 shows the HPLC-UV chromatograms for crude aqueous methanol extracts of three mussel tissues with 0, 2 and 20 µg/g domoic acid. A large peak due to tryptophan (marked by T) is apparent in the chromatograms. Under these HPLC conditions it does not interfere with domoic acid but it does coelute with one of the domoic acid isomers. With the combined aqueous methanol extraction, SAX-SPE cleanup and HPLC-UV analysis procedure detailed in Appendix 2, the detection limit is 20-30 ng/g (ppb) and the chromatograms are free from such interferences. Fig. 7.4 shows the analysis of the same three samples shown in Fig. 7.3 after cleanup with the SAX-SPE procedure. The enhanced detectability due to preconcentration and the elimination of tryptophan due to selective cleanup are clearly apparent in these chromatograms when compared to Fig. 7.3.

A very sensitive procedure, based on reaction with 9-fluorenylmethylchloroformate to form the fluorenylmethoxycarbonyl (FMOC) derivative and HPLC analysis with fluorescence detection, has been developed for monitoring of domoic acid in marine matrices such as seawater and phytoplankton (Pocklington *et al.*, 1990). The detection limit is as low as 15 pg/mL for domoic acid in seawater. Appendix 3 presents a detailed procedure for performing such analyses. This procedure has recently been adapted to shellfish tissue extracts (Quilliam *et al.*, unpublished results).

Capillary Electrophoresis

A new technique that has tremendous potential for the analysis of marine toxins is capillary electrophoresis (CE). This relatively simple method allows rapid, high resolution separations of complex, polar compounds. A narrow bore (50-100 µm i.d.) fused silica capillary tube filled with buffer is connected between two liquid reservoirs. After a small volume of sample (typically 1-10 nL) is injected into the capillary, a differential voltage of 20-30 kV is applied at the ends of the capillary. Ionic substances migrate as narrow bands down the capillary, eventually passing by a detector (UV absorbance, fluorescence, etc.). Two reports on the application of CE-UV to domoic acid have appeared thus far (Nguyen *et al.*, 1990; Quilliam *et al.*, 1992). More recent work has shown that the SAX-SPE cleanup method is compatible with CE (Thibault *et al.*, manuscript in preparation) and also that the FMOC derivatization method can be applied to CE with fluorescence detection (Zhao *et al.*, manuscript in preparation). Detection limits are similar to those of HPLC methods.

Mass Spectrometry

During 1987 investigation, fast atom bombardment (FAB) mass spectrometry using a direct probe inlet was found to be useful for the qualitative confirmation of domoic acid in concentrated LC isolates (Wright *et al.*, 1989; Thibault *et al.*, 1989). The development of an analytical procedure based on combined gas chromatography-mass spectrometry for the analysis of domoic acid and related compounds in shellfish tissue samples has since been reported (Pleasance *et al.*, 1990). Although this method is applicable to concentrations of domoic acid in contaminated shellfish ranging from 1 to 500 µg/g wet tissue, its implementation required the development of an extensive cleanup procedure to facilitate derivatization to the *N*-trifluoroacetyl-*O*-silyl derivatives. Since HPLC-UV is the method most commonly used for routine regulatory purposes, a combined liquid chromatography-mass spectrometry (HPLC-MS) method would be most desirable for confirmation of peak identity. Various HPLC-MS interfaces for the analysis of ASP toxins have been investigated (Quilliam *et al.*, manuscript in preparation); these include continuous-flow FAB, thermospray, and ion-spray interfaces. In a preliminary communication (Quilliam *et al.*, 1989b), it was shown that ion-spray HPLC-MS is

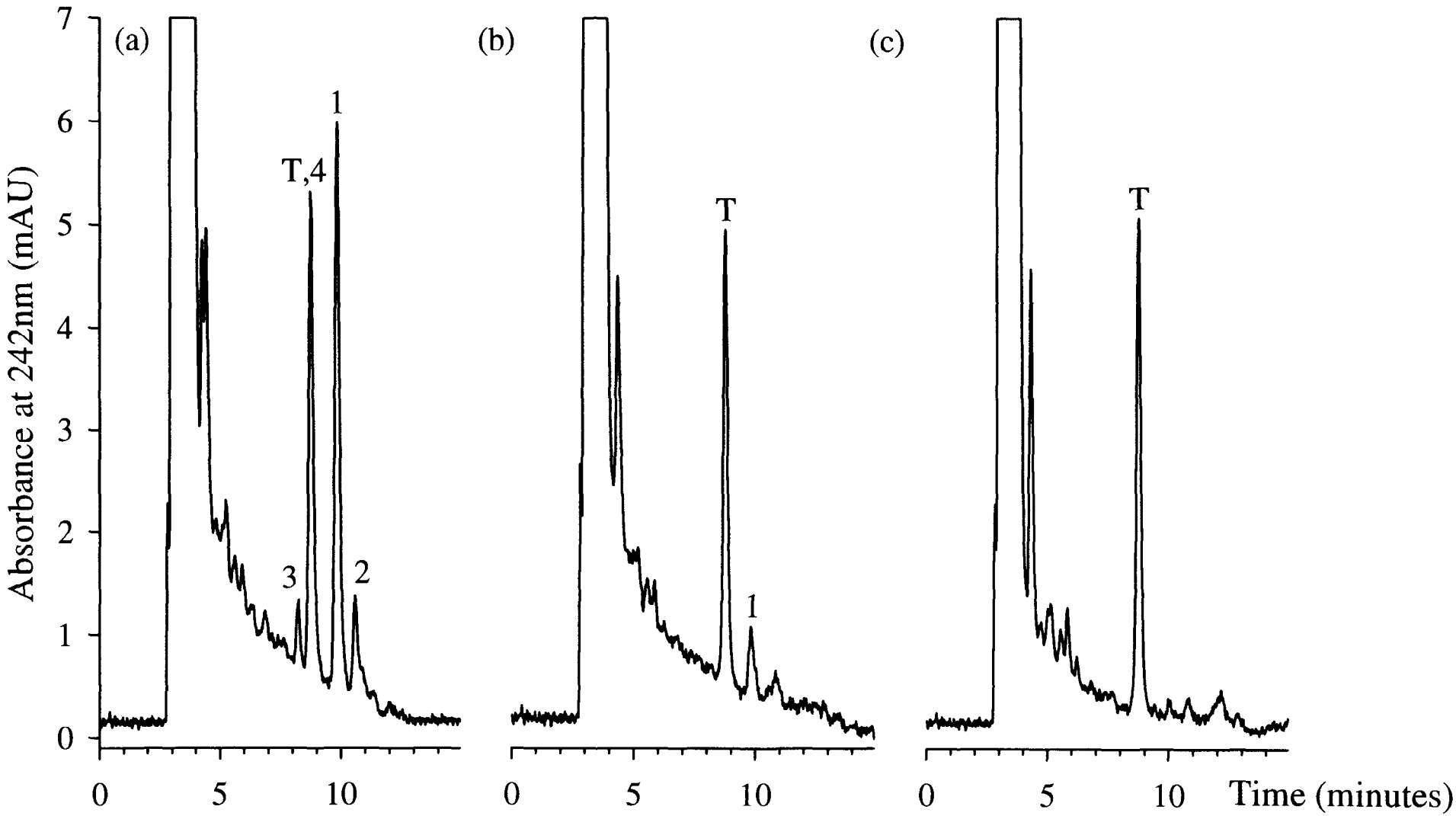
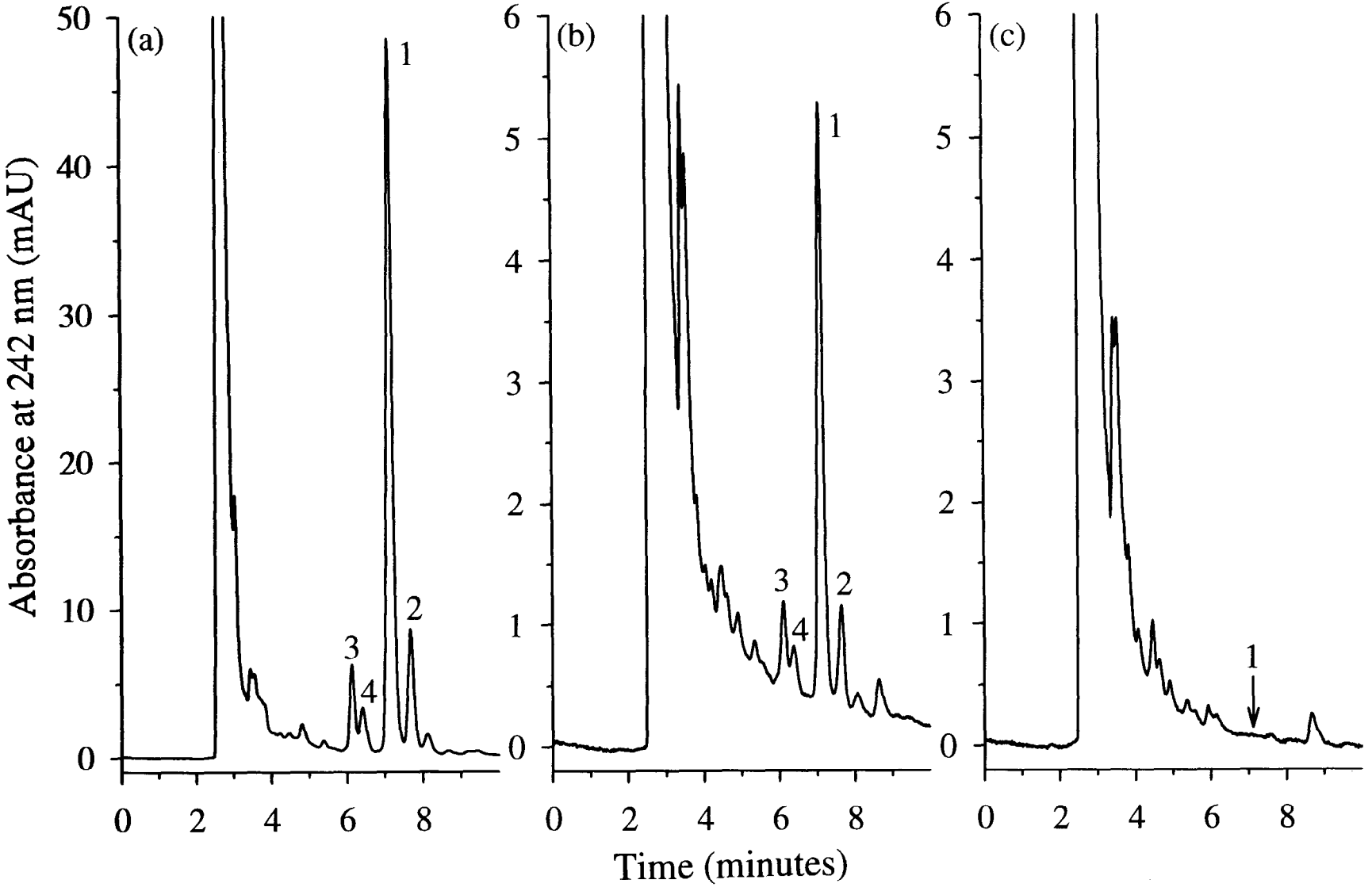


Figure 7.3. HPLC-UV chromatograms of the crude aqueous methanol extracts of three mussel tissues with (a) 20, (b) 2 and (c) 0 mg/g domoic acid. These were produced by blending the MUS-1 mussel tissue reference material with control mussel tissues. Peak identities: 1 = domoic acid; 2 = C5'-diastereomer of domoic acid; 3 = isodomoic acid D; 4 = isodomoic acid E; 5 = isodomoic acid F; T = tryptophan. Conditions are reported in Appendix 2.

Figure 7.4. HPLC-UV chromatograms of the SAX-SPE-cleaned extracts of the same mussel tissues shown in Fig. 7.3, with (a) 20, (b) 2 and (c) 0 mg/g domoic acid. Peak identities as in Fig. 7.3. Conditions are reported in Appendix 2. These analyses were performed on a different instrument than those in Fig. 7.3, thus explaining the different retention times.



a very promising method for the analysis of ASP toxins, as well as of other marine toxins. Ion-spray HPLC-MS has a detection limit similar to that of HPLC-UV.

BIOCHEMICAL METHODS

A receptor binding assay, using commercially available ^{3}H-labelled kainic acid and freshly prepared or cloned kainate receptors has been reported (van Dolah *et al.*, 1994). The method, which is extremely sensitive and has the potential to rapidly screen many samples, has been used successfully to assay shellfish extracts, crab viscera, and algal extracts, as well as the serum of laboratory animals. A radioimmunoassay has also been developed for domoic acid (Newsome *et al.*, 1991) and was used to detect the toxin in shellfish extracts and the serum of laboratory animals. Both these biochemical methods are still at the research stage and assay kits are not yet commercially available. These assays are reviewed in more detail in another chapter of this book.

APPENDIX 1

Commercial Sources

Pure domoic acid is commercially available through:

(a) Diagnostic Chemicals Ltd. (West Royalty Industrial Park, Charlottetown, PEI, Canada, C1E 1B0; tel. 902-566-1396; fax 902-566-2498; or DCL (USA), 160 Christian St., Oxford, CT 06478; tel. 203-881-2020; fax 203-888-1143).

(b) Sigma Chemical Company (P. O. Box 14508, St. Louis, Missouri, USA 63178; tel. (314) 771-5750; fax (314) 771-5757; Catalog No. D6152).

Calibration Standards and Reference Materials

Accurate calibration standards and certified reference materials for domoic acid are available through the Marine Analytical Chemistry Standards Program (MACSP), Institute for Marine Biosciences, 1411 Oxford St., Halifax, N.S., Canada B3H 3Z1; telephone (902) 426-8280; fax (902) 426-9413. The mussel tissue reference material (Catalog No. MUS-1, 4 x 15g units) is certified to contain 100 µg domoic acid/g tissue and is stable for one year at 4°C. This material is useful for testing the full implementation of an analytical method, such as the one detailed in Appendix 2, and for testing newly developed analytical methods (Hardstaff *et al.*, 1990). The certified calibration solution (Catalog No. DACS-1B, 4 x 0.5 mL units) contains 99.5 µg/mL in aqueous 10% acetonitrile and is stable for one year at -12°C in dark conditions.

APPENDIX 2

HPLC-UV Determination of Domoic Acid in Shellfish Tissues

This section presents a detailed procedure for the determination of domoic acid in shellfish (Quilliam *et al.*, 1995). Domoic acid is extracted from shellfish tissues by homogenization with methanol-water (1:1, v/v). The concentration of domoic acid is determined by HPLC with ultraviolet absorbance detection. Sample extracts are injected following dilution and filtration of the crude extract or after cleanup on strong anion exchange (SAX) solid phase extraction (SPE) cartridges. The latter provides selective isolation of domoic acid and related compounds from interfering substances, as well as preconcentration to facilitate analysis of trace levels. The cleanup step is suitable only for unsalted product. Methodology for TLC analysis has been included for those labs not equipped with HPLC systems.

The procedures below can also be used to analyze plankton biomass isolated in the field by net tows or from cultures by centrifugation. However, the procedure presented in Appendix 3 has been designed specifically for samples of seawater containing plankton at low cell concentrations.

Reagents and Equipment

(a) *Water:* Distilled and passed through a water purification system equipped with ion-exchange and carbon filters.

(b) *Solvents:* LC or distilled-in-glass grade acetonitrile and methanol, analytical grade formic acid, and spectrophotometric grade (>99% purity) trifluoroacetic acid (TFA).

(c) *HPLC mobile phase:* Mix 100 mL acetonitrile with ca 400 mL water, add 1.0 mL TFA, and dilute to 1 L with water. Degas with ultrasonication and gentle vacuum.

(d) *Extraction solvent:* Mix equal volumes of methanol and water.

(e) *Cartridge wash solution and injection diluent:* Mix 1 volume acetonitrile with 9 volumes water.

(f) *Citric acid:* Analytical grade citric acid monohydrate.

(g) *Tri-ammonium citrate:* Analytical grade tri-ammonium citrate.

(h) *Citrate buffer eluent (0.5 M, pH 3.2):* Dissolve 40.4 g of citric acid monohydrate and 14.0 g of tri-ammonium citrate in 400 mL distilled water, add 50 mL acetonitrile and dilute to 500 mL with distilled water. (Tri-sodium citrate dihydrate (17.0 g) can be substituted for the ammonium salt. It is also possible to prepare this buffer by dissolving 52.55 g of citric acid monohydrate in 400 mL distilled water, adjusting the pH to 3.2 with concentrated ammonium hydroxide (ca 13 mL), adding 50 mL acetonitrile and diluting to 500 mL with distilled water).

(i) *Domoic acid calibration solutions:* DACS-1B (certified 99.5 µg/mL, see Appendix 1) and accurate dilutions of DACS-1B in injection diluent (e) to give 1.0, 2.5, 10.0, 25.0 mg/mL. Refrigerate solutions when not in use. Warm to room temperature before use. A small peak for the domoic acid diastereomer is present in the HPLC trace of most standards. The compound separates from domoic acid on some columns but not others; the area for that peak should generally be included with that of domoic acid for puposes of calibration. The issue of whether to quantify the diastereomer in samples has not yet been addressed by regulatory authoritories.

(j) *Mussel tissue reference material:* MUS-1 (100 µg/g, see Appendix 1).

(k) *Liquid Chromatograph:* Isocratic system equipped with UV detector capable of measuring absorbance at 242 nm and providing a S/N of 10:1 on injection of a 0.2 µg/mL domoic acid solution using conditions given below.

(l) *HPLC Column:* 25 cm long x 4.6 mm id packed with 5-10 µm C18 bonded silica gel (Vydac 201TP, Supelco LC-PAH, or equivalent); use of a guard column is recommended. Operating conditions: column temp. 40°C; mobile phase flow rate in the range 1.0-1.5 mL/min; injection volume 20 µL. If the HPLC is millibore compatible, a 25 cm long x 2.1 mm id column can be substituted, using a 5 µL injection volume and mobile phase flow rate in the range 0.2-0.3 mL/min. Retention time of domoic acid should be 7-15 min (k'= 3 is best).

(m) *Strong Anion Exchange (SAX) Cartridges:* 3 mL capacity, containing 500 mg of silica derivatized with a quaternary ammonium silane (JT Baker Scientific, Supelco, or equivalent).

(n) *Thin Layer Chromatography Plates:* Activated silica gel 60 plates (Merck, F_{254}-250 µm).

Preparation of Samples

1. *Tissue preparation:* After removal from the shell, drain tissues to remove saltwater. For representative sampling it is advisable to homogenize 100 g of pooled tissue in a blender. Subsampling from this homogenate can be done immediately after blending while still well-mixed, or later after mixing again. If an analysis must be performed on a limited amount of sample, a portion of chopped or ground tissue can be weighed directly into the extraction tube. Tissue homogenates may be stored for several weeks at -10°C or lower if tightly sealed.

2. *Extraction:* Accurately weigh 4.0 g tissue homogenate (as prepared in step 1) into a graduated centrifuge tube. Add 16.0 mL extraction solvent (d) (1:1 methanol-water) and homogenize the sample extensively (3 min at 10,000 rpm). Do not try to recover all the tissue remaining on the homogenizer probe but do wash it thoroughly afterwards to prevent contamination of the next sample. (Or, if a blender must be used, weigh the homogenate into a tared stainless-steel micro blender cup, add 16.0 mL of extraction solvent (d) and blend at medium speed for 4 min. Pour ca 15 mL of the resulting slurry into a centrifuge tube. The weight of tissue and volume of solvent may be scaled up if a micro blender cup is not available.). Centrifuge at 3000xg or higher for 10 min. Filter 10-15 mL of the supernatant through a dry methanol-compatible 0.45 µm filter into a screw-capped storage vessel and seal tightly. Extracts should be analyzed as soon as possible, otherwise store tightly sealed at -10°C or lower.

3. *Diluted crude extract:* For screening samples for a high level of contamination and for salted samples, deliver 1.0 mL of filtered supernatant from step 2 into a 5-mL volumetric flask or graduated cylinder, dilute to 5.0 mL with water, mix, and analyze without the SAX cartridge cleanup (omitting steps 4 and 5 below).

4. *SAX cartridge conditioning:* Pass first 6 mL of methanol, then 3 mL water, and finally 3 mL extraction solvent (d) through the SAX cartridges prior to use or testing. Do not allow the cartridges to go dry at any point in the procedure.

5. *SAX cartridge cleanup (for HPLC):* Load 5.0 mL filtered supernatant from step 2 onto the cartridge and allow to flow slowly (about 1 drop per second). Stop flow just as the sample meniscus reaches the top of the cartridge packing. Discard the effluent. Wash the cartridge (at about 1 drop per second) with 5 mL of the cartridge wash solution (e). Stop the flow just as the solvent meniscus reaches the top of the cartridge packing. Discard the effluent. Add 0.5 mL

citrate buffer eluent (h) and carefully allow to flow just until the solvent meniscus reaches the top of the cartridge packing. Discard the effluent. Place a 2-mL volumetric tube or flask under the cartridge. Elute the domoic acid with 2 mL citrate buffer eluent (h) (slowly, 1 drop per second) just until the mark is reached on the volumetric tube. Mix the solution before withdrawing an aliquot for HPLC analysis. Samples should be analyzed as soon as possible; otherwise store crude extracts tightly sealed in a screw-capped glass vial in the refrigerator (do not store for more than one week). The SAX-cleaned extracts should not be frozen, as domoic acid can decompose under such conditions.

6. *SAX cartridge cleanup (for TLC):* The same procedure as in step 5 is used, except that the citrate buffer eluent (h) is replaced with 2% (v/v) formic acid in acetonitrile-water (1:9).

HPLC Determination

Inject domoic acid calibration solutions (i) over the range of 1 to 100 µg/mL. If good linearity of response and a zero intercept are evident, single point calibration (e.g., 10 µg/mL) may then be used routinely. Replicate injections should have CVs <5%. Inject sample extracts (diluted and filtered crude methanol-water extracts from step 3 or SAX-cleaned extract from step 5 under *Preparation of Samples*) in duplicate. Avoid carry-over between injections of different samples by washing the injector loop with injection diluent (e). Average peak areas for each sample. Repeat single injections of domoic acid calibration solution every 2 hr and duplicate injections every 8 hr. In calculations, average the peak areas of standards immediately following and preceding a series of samples.

Calculate the concentration of domoic acid (µg/g) in each sample according to the following formula, where A_S is the average peak area for the sample, A_C is the average area for the calibration standard (concentration C_C µg/mL) bracketing the sample, W is the weight in grams of tissue homogenate extracted (ca 4.0 g), and F is the dilution factor (F = 8 for the SAX-cleaned extract; F = 100 for the diluted crude methanol-water extract).

$$\text{Concentration of domoic acid (µg/g tissue)} = (A_S/A_C)(C_C/W)(F)$$

Thin Layer Chromatography

Apply 50-100 µL of domoic acid calibration solution (i) or SAX-cleaned extract of mussel tissue or plankton from step 6 under *Preparation of Samples* to an activated silica gel plate (Merck, silica gel 60 F_{254} 250 µm) and elute with butanol-acetic acid-water (3:1:1). After drying the plate, it can be examined under short wavelength UV light (254 nm). Domoic acid appears as a UV-quenching spot at approximately $R_f = 0.5$. After this, spray the dried plate with a 1% solution of ninhydrin. Domoic acid appears as a yellow spot. The detection limit for domoic acid is about 0.5 µg by this method, which permits detection in shellfish tissues at about 10 µg/g.

Blanks and Recoveries

(a) *Extraction Blank:* Perform step 2 under *Preparation of Samples*, substituting 4 g water in place of sample tissue. Analyze a portion of the diluted and filtered methanol-water extract from step 3, and then carry the remaining methanol-water extract through steps 4 and 5 and analyze again. Chromatograms should be free of peaks eluting near domoic acid or causing excessive baseline slope. As needed, replace methanol, water, modify between-sample rinsing procedures, use alternative SAX column source, etc.

(b) *SAX-column recoveries:* Perform an HPLC determination in duplicate of a filtered solution containing domoic acid in extraction solvent (d) in the range 10-30 µg/mL (ideally this should be an extract prepared from the tissue of interest, either naturally contaminated or spiked with domoic acid or a domoic acid-containing tissue). Using three cartridges from the lot to be used, take the same solution through the SAX cleanup (steps (d) and (e) under *Preparation of Sample*). Perform the determination in duplicate for each of the three eluates. Calculate the percent recovery for each eluate using the appropriate dilution factors and the average domoic acid level determined in the crude extract. All three recoveries determined should fall in the range 85% to 115%, with an average recovery >90%. If the recoveries determined do not satisfy these criteria, try another source of SAX cartridges.

APPENDIX 3

HPLC Determination of Domoic Acid in Seawater and Plankton

This section presents a detailed procedure for the determination of domoic acid in seawater and plankton samples (Pocklington *et al.*, 1990). The method involves pre-column derivatization with 9-fluorenylmethylchloroformate to form the FMOC derivative followed by reversed-phase HPLC with fluorescence detection. The detection limit for domoic acid in seawater with an isocratic HPLC system is about 0.5 ng/mL, while a gradient elution system can detect as low as 15 pg/mL. These detection limits will permit the analysis of *Pseudonitzschia* culture samples with cell densities in the range of 1000-10,000 cells/mL or 20-10,000 cells/mL, respectively, assuming production of domoic acid at 1-20 pg/cell.

Reagents and Equipment

(a) *Water:* Distilled and passed through a water purification system equipped with ion-exchange and carbon filters.

(b) *Seawater:* Filtered through a glass fibre filter (Type A/E, Gelman Sciences Inc., Ann Arbor, MI) prior to use.

(c) *Solvents:* LC or distilled-in-glass grade acetonitrile, LC-grade ethyl acetate, and spectrophotometric grade (>99% purity) trifluoroacetic acid (TFA).

(d) *9-Fluorenylmethylchloroformate (FMOC-Cl):* Available from Aldrich Chemical Co. (Milwaukee, WI).

(e) *FMOC-Cl Reagent Solution (15 mM):* Dissolve 38.7 mg FMOC-Cl in 10.0 mL acetonitrile. Store in 2-mL glass screw-cap vials with teflon-lined septa in a dessicator at -20°C. All vials should be purged with nitrogen prior to storage. Once the septum has been pierced, any unused reagent should be discarded.

(f) *Borate buffer (1M, pH 6.2):* Dissolve 6.18 g orthoboric acid (BH_3O_3) in 95 mL deionized water, adjust the pH to 6.2 with 2 M sodium hydroxide, and dilute with water to 100 mL.

(g) *Domoic acid calibration solutions:* DACS-1B (certified 99.5 µg/mL, see Appendix 1). Prepare working solutions daily by accurately diluting DACS-1B in seawater to give 5, 10, 25, 50, 100, 200 and 500 ng/mL.

(h) *Dihydrokainic acid(DHKA):* Available from Sigma Chemical Company (St. Louis, MO).

(i) *DHKA internal standard solution:* Prepare a stock solution (100 µg/mL) by dissolving 1.0 mg DHKA in 10.0 mL acetonitrile-water (1:9). Prepare a working solution (2 mg/mL) by diluting the stock solution 50-fold in seawater.

(j) *HPLC mobile phase: (i) Isocratic system:* 40% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in water. *(ii) Gradient system:* Solvent A = 0.1% TFA in water; B = 0.1% TFA in acetonitrile. Degas with ultrasonication and gentle vacuum.

(k) *Liquid Chromatograph:* Isocratic or preferably gradient system equipped with a dual monochromator fluorescence detector set for excitation at 264 nm and emission at 313 nm protected by a 280 nm cut-off filter. A filter fluorimeter equipped with the appropriate filters may be substituted.

(l) *HPLC Column:* 25 cm long x 4.6 mm id packed with 5 µm C18 bonded silica gel (Vydac 201TP, Supelco LC-PAH, or equivalent); use of a guard column is recommended. Operating conditions: ambient column temp. for isocratic, 55°C for gradient; 1.0 mL/min mobile phase flow rate; 10 µL injection volume for isocratic, up to 100 µL for gradient. If the HPLC is millibore compatible, a 25 cm long x 2.1 mm id column can be substituted, using a 2 µL injection volume for isocratic or 20 µL for gradient, and a mobile phase flow rate of 0.2 mL/min. Gradient elution: programmed linearly from 30% to 50% B over 15 min, followed by an increase to 100% B over 2 min which is maintained for 5 min before programming back to initial conditions over 2 min. Maintain initial conditions for a further 12 min before the next injection.

Preparation of Samples

1. For determination of domoic acid dissolved in seawater or culture media, filter a 1-mL subsample through a 0.22-µm disposable filter (Millex-GS, Millipore Corp., Bedford, MA). Take the filtrate through the derivatization and HPLC procedures below.

2. For determination of overall concentrations of domoic acid in plankton culture samples or suspensions of plankton in seawater, first determine the cell density in representative sub-samples. Sonicate an homogenous subsample (10 mL) for 1 min at 100 W using a 1-cm diameter probe to disrupt the cells. Then filter the 1 mL of sample through a Millex-GS 0.22-µm disposable filter.Take the filtrate through the derivatization and HPLC procedures below.

3. For determination of concentrations of domoic acid in phytoplankton cells as well as in the medium, first determine the cell density in representative sub-samples. Centrifuge a 10-mL aliquot of sample in a conical tube for 5 min at approximately 900 x g. Remove 5 mL of supernatant and treat as in step 1. Centrifuge the remaining material for another 5 min at 900 x g. Remove all but approximately 0.2 mL supernatant, make the volume to 10 mL with seawater. Sonicate for 1 min at 100 W using a 1-cm diameter probe to disrupt the cells. Then filter the solution through a Millex-GS 0.22-µm disposable filter. Take the filtrate through the derivatization and HPLC procedures below.

4. For other samples, such as plankton biomass (fresh or freeze-dried) or plankton cells isolated on filters, first suspend the cells in seawater and then proceed with step 3 above.

Derivatization

Mix the following in a glass test tube (10 x 75 mm) using a vortex mixer for 10 sec: 200 µL sample, calibration solution (g) or seawater blank; 50 µL borate buffer (f); and 10 µL DHKA internal standard solution (i). Then add 250 µL FMOC-Cl reagent solution (e) and mix. After exactly 45 sec, add 500 µL ethyl acetate and mix for an additional 45 sec.After the mixture has

settled, two distinct phases will be visible. Remove the upper organic layer with a disposable glass pipette and discard. This is repeated with two more 500 µL portions of ethyl acetate and 20 sec of mixing. Transfer most of the aqueous bottom layer to a vial for HPLC analysis.

The control of contamination is important when working with samples having low domoic acid concentrations. All glassware, syringes and vials should be rigorously washed (water, methanol, acetone). Deliver all reagents using glass syringes dedicated to each solution, and deliver the sample with an air displacement pipette with disposable tips.

HPLC Analysis

Inject derivatized calibration solutions (g) over the concentration range 10-300 ng/mL. If good linearity of response and a zero intercept are evident, single point calibration may then be used routinely. Replicate injections should have CVs <5%. Inject derivatized blank seawater samples. There should be no interference at the retention time for domoic acid. Avoid carry-over between injections of different samples by washing the injector loop with acetonitrile and water. Inject derivatized sample extracts. Repeat single injections of calibration solution every 2 hr. In calculations, average the peak areas of standards immediately following and preceding a series of samples.

Calculate the concentration of domoic acid (ng/mL) in each sample according to the following formula, where A_S and I_S are the average peak areas of domoic acid and internal standard (DHKA) for the sample, respectively, and A_C and I_C are the corresponding average peak areas for the calibration standard of concentration C_C (ng/mL) bracketing the sample.

$$\text{Concentration of domoic acid (ng/mL)} = (A_S/A_C)(I_C/I_S)(C_C)$$

Calculate the concentration in plankton cells (pg/cell) according to the following formula, where D is the cell density (cells per mL).

$$\text{Concentration of domoic acid (pg/cell)} = (1000)(A_S/A_C)(I_C/I_S)(C_C/D)$$

Safety

Domoic acid is a neurotoxin and must be handled with caution. Acetonitrile and methanol are toxic, volatile solvents. Trifluoroacetic and formic acids are toxic, volatile and corrosive and should only be handled in a fume hood. 9-Fluorenylmethylchloroformate is toxic and a skin irritant. All these substances are harmful if swallowed, inhaled or absorbed through the skin.

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8. Detection of Ciguatoxins and related Benthic Dinoflagellate Toxins: *in vivo* and *in vitro* Methods

R.J. Lewis

Ciguatera is a disease caused by heat stable sodium channel activator toxins and is characterised by a wide array of gastrointestinal and neurological symptoms (Gillespie *et al.*, 1986; Lewis and Ruff, 1992). It follows consumption of fish that have accumulated through the marine food chain a number of ciguatoxin analogues derived from gambiertoxins produced by the benthic dinoflagellate, *Gambierdiscus toxicus* (Fig. 8.1; Lewis and Holmes, 1993). CTX-1, the most potent sodium channel toxin known, is the major toxin found in carnivorous fish where it typically contributes ~90% of total lethality and poses a health risk at levels above 0.1 ppb (Murata *et al.*, 1990; Lewis *et al.*, 1991; Legrand *et al.*, 1992; Lewis and Sellin, 1992). Ciguatera can be debilitating and slowly resolving but is seldom fatal. In societies that depend heavily on reef fish for protein (e.g. atoll island countries of the Pacific) ciguatera can have major adverse socioeconomic impacts. In western societies outbreaks of ciguatera often attract media attention with a consequent negative impact on the marketing of seafood (Lewis, 1992a, 1994). Victims of ciguatera may seek compensation through the courts (Payne, 1994).

A cost-effective means of detecting ciguateric fish prior to consumption remains one of the few management options that can directly reduce the adverse impacts of ciguatera. Antibody-based assays appear to hold most promise since they can detect, under favourable circumstances, targeted compounds to 10^{-12} M and can be developed as cost-effective screens (Gazzaz *et al.*, 1992). However, the mouse bioassay is currently the assay of choice in many laboratories researching ciguatera, despite attendant concerns for animal welfare. This paper details the mouse bioassay method for estimating ciguatoxin in fish and discusses progress towards the development of alternative *in vivo* and *in vitro* assays for detecting ciguateric fish. Background is provided on the immunological, biochemical and chemical features of the ciguatoxins which are relevant to their differential detection.

IN VITRO BIOASSAYS

History of development of antibody based assays for ciguateric fish

The potential of an antibody-based screening assay for directly detecting ciguatoxin in fish flesh was first indicated by Hokama *et al.* (1977). Hokama has since led efforts to develop a rapid screen for ciguateric fish (Hokama, 1991, 1993a, 1993b). The original radioimmunoassay screened 88% of moray eel and 38% of other fish as toxic (>3.5 x 10^5 cpm/g; Kimura *et al.*, 1982) despite these fish rating as non-toxic by the mongoose assay. However, all fish rating toxic by the mongoose rated as toxic by the antibody assay, indicating the potential for this approach to detect ciguateric fish. This assay found 15% of 5,529 *Seriola dumerili* captured in Hawaiian waters tested positive, with the remaining fish, including those >9 kg, which normally are not marketed owing to their perceived higher risk of ciguatera, being consumed without incident (Kimura *et al.*, 1982). The quantity of additional *S. dumerili* entering the market increased by 68% as the direct result of the study. However, Kimura *et al.* (1982) found

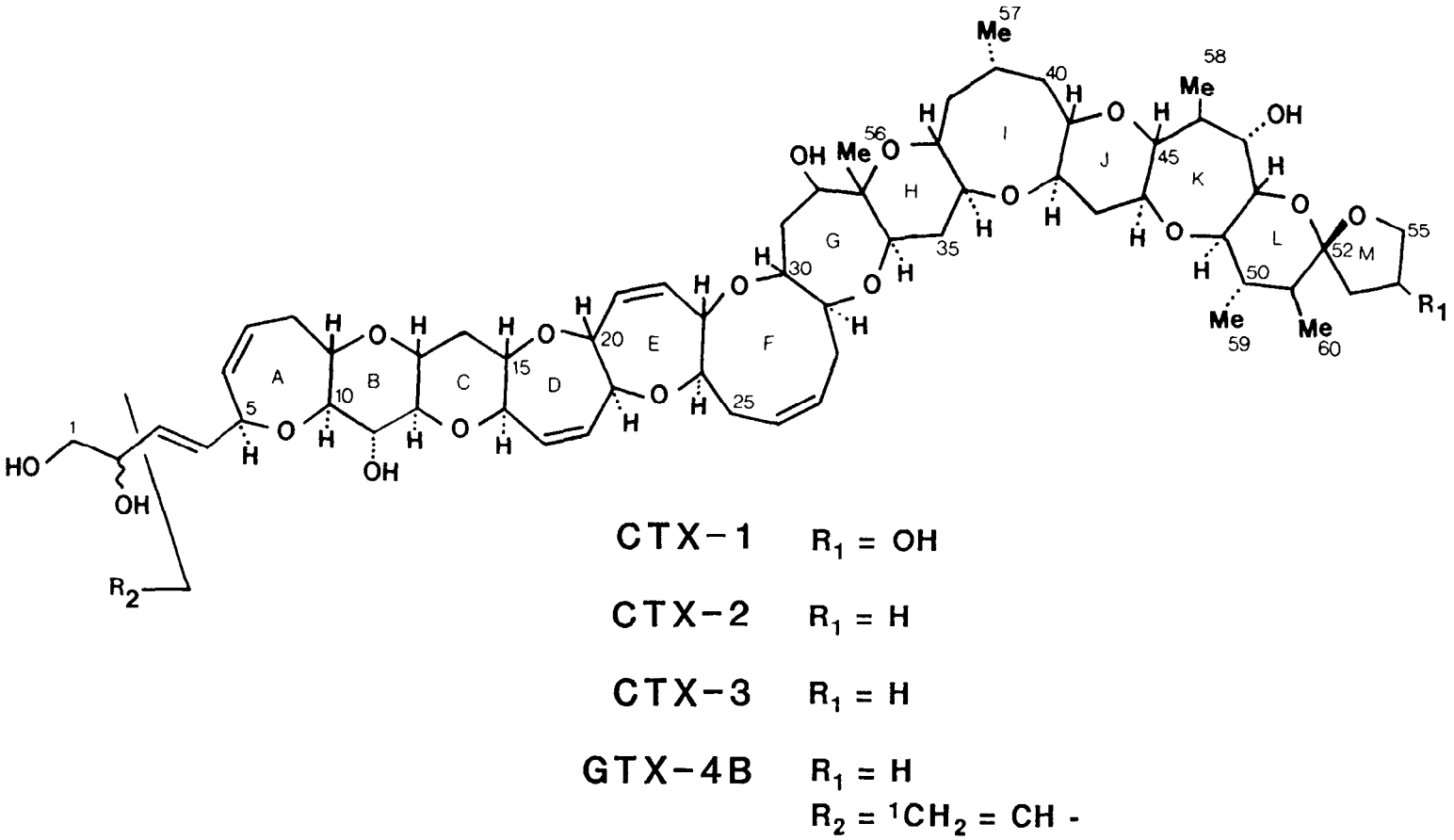

Figure 8.1. Structures of the major ciguatoxins (CTX) present in the flesh of ciguateric carnivorous fish in the Pacific and gambiertoxin-4B from *Gambierdiscus toxicus* and certain herbivorous species (Murata *et al.*, 1990; Lewis *et al.*, 1991; Legrand *et al.*, 1992). CTX-2 is 52-epi CTX-3 (Lewis *et al.*, 1991, 1993a). Stereochemistry of CTX-1 and CTX-3 at carbon 52 is shown here.

that 7% of fish (3 of 42) clinically implicated in ciguatera tested negative by the radioimmunoassay.

In 1984 the radioimmunoassay for ciguateric fish was replaced by a simpler enzyme immunoassay. In a binding inhibition assay this method was sensitive to as little as 5 pg of free ciguatoxin (Hokama *et al.*, 1983, 1984). The cross-reactivity of this assay with other polyether toxins was also documented. An enzyme labelled polyclonal antibody was subsequently used in a further simplified "stick-test" for ciguateric fish that rapidly distinguished toxic from non-toxic flesh samples (Hokama, 1985). However, six tests *per* fish appeared necessary for accurate determination of ciguateric fish that tested close to the borderline level. With the stick-test the rejection rate for *S. dumerili* was only 11% and *S. dumerili* testing non-toxic were consumed without incident. This assay responded directly to >1.0 ng of pure ciguatoxin *per* ml of methanol (Hokama, 1985).

These early studies all employed a polyclonal antibody raised to ciguatoxin in sheep. A disadvantage of such an approach is that for long-term antibody production a continual supply of antigen is required for booster injections. Monoclonal antibodies, on the other hand, can provide a continuous supply of a selected antibody. Hokama *et al.* (1985, 1989b) reported production of monoclonal antibodies to a related polyether toxin, okadaic acid, as well as to ciguatoxin (likely CTX-1). Using a monoclonal antibody (IgG) to ciguatoxin in a stick enzyme immunoassay, Hokama *et al.* (1989a) found that 98% of fish implicated in ciguatera (50 of 51) tested positive, while 55% of a random mix of fish, 55% of *Ctenochaetus striatus* and 44% of *S. dumerili* tested positive. A further simplified solid-phase immunobead assay for detection of ciguateric fish has recently been reported (Hokama, 1990). This latter approach appeared more sensitive than previous stick tests and is more amenable to field applications.

The monoclonal antibody to ciguatoxin used in these studies has been assessed for cross-reactivity to other polyether toxins (Hokama *et al.*, 1989b, 1992). The assay employing the antibody raised to ciguatoxin detected similar concentrations of pure ciguatoxin, okadaic and a synthesised fragment of okadaic acid (EC_{50} = ~0.5 ng of toxin *per* ml methanol) (Hokama *et al.*, 1992). Somewhat surprisingly, a monoclonal antibody obtained specific to okadaic acid was less sensitive at detecting okadaic acid (EC_{50} ~15 ng/ml) than the ciguatoxin antibody using the same assay format [somewhat different cross-reactivity was reported in an earlier study (Hokama *et al.*, 1989b)]. In a second experiment, pure ciguatoxin (EC_{50} ~1 ng/ml) or okadaic acid (EC_{50} ~3 ng/ml) added to a ciguatoxin antibody inhibited the subsequent binding of this antibody to sticks coated with an extract from a fish implicated in ciguatera (Hokama *et al.*, 1992). This result suggests these polyether toxins compete at a specific, saturable site on the IgG.

Continuing work on the development of a commercial screening assay for ciguateric fish is now being undertaken by Hawaii Chemtec International who purchased development rights for the Hokama stick test from the University of Hawaii. Recent progress towards the development of screening assays for ciguateric fish based on Hokama's antibody is discussed further in several recent studies (Park, 1992, 1994; Dickey *et al.*, 1994). Presently there is no commercially available test shown to reliably detect the levels of ciguatoxin which contaminate ciguateric fish — however, the approach has considerable merit and warrants further research.

Production of ciguatoxin antibodies

An antibody response is elicited in an animal following injection of an immunogenic antigen. The ciguatoxins (*m/z* = ~1,100) are relatively small haptens that are likely to be only weakly immunogenic (the absence of any protection developing in people repeatedly exposed to ciguatoxins through their diet supports this suggestion). A hapten can be made immunogenic by covalently conjugating it to a carrier protein that is highly immunogenic (Erlanger, 1980). Non-covalent associations with protein do not significantly enhance the immunogenicity of haptens, irrespective of whether Fruends' complete adjuvant is used (Layton *et al.*, 1987; Gazzaz *et al.*, 1992). A possible draw-back of using a hapten conjugated to a carrier protein is that the

antibodies obtained may not have high specificity for the unconjugated (native) form of the hapten.

The ciguatoxins found in fish possess a relatively reactive primary hydroxyl (Murata *et al.*, 1990; Lewis *et al.*, 1991; Lewis *et al.*, 1993a) which can be reacted with succinic anhydride to yield a hemisuccinate (see Baden *et al.*, 1984). The hemisuccinate so formed has a free carboxylic acid group through which ciguatoxin can now be linked to a suitable carrier protein (e.g. bovine serum albumin, keyhole limpet haemocyanin, ovalbumin) using a water soluble carbodiimide cross-linking reagent. Ciguatoxin covalently attached to a carrier protein in this way is expected to have considerably enhanced immunogenicity compared with the native ciguatoxin and such a complex may additionally have reduced potency, an important consideration for *in vivo* immunisation. The availability of only one easily accessible site on ciguatoxin for covalent conjugation limits (Mandal and Latif, 1988) the possibilities for producing a range of antibodies possessing differing selectivities for the various ciguatoxin analogues. To-date antibodies to ciguatoxin have not been obtained using an immunogen covalently attached to a carrier protein. However, the use of such an immunogen is presently under consideration in a number of laboratories. Attempts in our laboratory to produce a hemisuccinate of small quantities of CTX-1 (~50 μg) have met with limited success using succinic anhydride in dry pyridine at 80°C (Baden *et al.*, 1984), a method successfully employed to produce a hemisuccinate of <100 μg brevetoxin-3 (Lewis, Potomski and Gillespie, unpublished results). An alterative approach to immunising with ciguatoxin is to use synthesised partial structures as the hapten(s). Such an approach may produce antibodies that recognise different portions of the ciguatoxin moiety upon which a sandwich assay could be developed. Synthesised partial structures that compete with ciguatoxin for binding to antibody would allow the establishment of competitive binding assay which are typically more reliable than the non-competitive assays used to-date (Lewis, 1994).

To obtain maximal *in vivo* immune response with small quantities of immunogen, the immunogen should be emulsified in Fruends' complete adjuvant, or similar, prior to injection (Vaitukaitis, 1981; Smith *et al.*, 1992). Blood from suitably immunised animals is assessed for selective antibody titre against the compound of interest and collected when titres are high. For monoclonal antibody production, the spleen cells of immunised mice are fused with a murine myoloma cell line, with hybridoma(s) secreting ciguatoxin-specific immunoglobulins subsequently isolated using an appropriate screen. This approach follows well described procedures (see Galfre and Milstein, 1981; Goding, 1986; Peters and Baumgarten, 1992). Alternatively, monoclonal antibodies (mostly IgMs) may be obtained through *in vitro* immunisation procedures (Brazeau *et al.*, 1982; Van Ness *et al.*, 1984; Buchman *et al.*, 1985; Borrebaeck, 1986; Brams *et al.*, 1987; James and Bell, 1987; Borrebaeck and Glad, 1989). This approach allows the production of high specificity antibodies (including human antibodies) with small quantities of immunogen, and compared with *in vivo* immunisation, is less susceptible to immunogen toxicity. Modifications to standard *in vivo* immunisation protocols can also reduce the quantity of immunogen required (Vaitukaitis, 1981; Forest and Ross, 1993) and such approaches may be useful for the production of antibodies to ciguatoxin. The recently developed DNA based bacteriophage expression system offers an exciting alternative to conventional approaches to ciguatoxin antibody production.

To allow detection of potentially useful antibodies, a screen must be developed that is selective only for those antibodies that combine with high affinity to the compound being targeted (Lewis, 1994). Such a screen may utilise a hapten-protein conjugate attached through non-specific interactions to the plastic surface of microtitre plates. To avoid potential problems of cross-reactivity to carrier protein or to epitopes extending beyond the hapten itself, the protein *and* cross-linking reagent used for screening should be different from those used in the preparation of the immunogen. Again, any method employed to couple ciguatoxin to a carrier protein should not alter the structure of the targeted compound, otherwise antibodies may be selected which do not recognise the native compound. This is also important for compounds labelled for use as the competitor in competitive antibody binding assays. A careful choice of blockers (Tween 20, foetal calf serum, albumins etc) must also be made to ensure that the response is specific for the compound of interest (e.g. ciguatoxin). A number of pitfalls in

antibody production have been identified in the literature. One such pitfall is the ability of certain clones to express antibody which binds promiscuously to plastic (Conger *et al.*, 1988). We have noted that adding ciguatoxin solubilised in methanol causes an increase in the microtitre plate's affinity for IgG that is not related to the presence of ciguatoxin. This effect could not be blocked by traditional blockers and gave rise to a number of false positive results and the selection of inappropriate antibodies (Lewis, Potomski and Gillespie, unpublished results).

Screening assays employing antibodies that have both high affinity and selectivity for CTX-1 are essential if routine detection of the low levels of ciguatoxins (10^{-10} to 5 x 10^{-9} g CTX-1/g) contaminating the flesh of ciguateric fish is to be achieved. Ideally the chosen antibodies should have an affinity for any contaminating toxin that is directly proportional to its oral potency (to humans) and the antibody should not cross-react with compounds normally present in non-toxic fish. The high cross-reactivity of the ciguatoxin antibodies with less potent compounds, including the low polarity ciguatoxins and structurally dissimilar polyether toxins such as okadaic acid (Hokama *et al.*, 1989b, 1992), may in part explain the high number of false positive results obtained with antibody assays. Recent studies using steroid-antibody interactions as a model, have revealed that antibodies recognising apolar and functionally inert molecules (such as ciguatoxin) can have a high affinity binding site, but this site apparently cannot be engineered with high specificity that avoids cross-reactivity with related ligands (Arevalo *et al.*, 1993). In contrast to antibodies that recognise ciguatoxin, and somewhat surprisingly given the previous statement, antibodies raised to brevetoxin and okadaic acid have been found to possess low cross-reactivity to other polyethers, including ciguatoxins-1, -2, and -3 (Levine *et al.*, 1988; Lewis *et al.*, 1991). It remains to be confirmed if it is possible to obtain brevetoxin-antibodies that cross-react with the ciguatoxins.

In addition to the major ciguatoxins isolated, low potency forms arising from further biotransformation of these ciguatoxins may also accumulate in fishes to significant levels (Lewis and Holmes, 1993). While the low potency of these ciguatoxins has made their detection difficult by the mouse bioassay, such compounds may cross-react strongly with antibodies to CTX-1, to increase the probability of obtaining false positive results. Unfortunately, antibody-based assays respond depending on the relative affinity (specificity) of the antibody for each form of the toxin in a way that may only by chance be related to the potency of the different forms. Non-selective binding of ciguatoxin to IgG and non-selective binding of IgG to fish tissue (Parc *et al.*, 1979; Chanteau *et al.*, 1981; Emerson *et al.*, 1983) may present additional obstacles to the development of a successful screening assay for ciguateric fish. The external testing of prototype assays utilising polyclonal (Berger and Berger, 1979) and monoclonal antibodies (Lewis, unpublished result) have at times given results that are difficult to explain.

Sodium channel binding assays for ciguatoxins

The ciguatoxins are characterised by their high affinity binding (ED_{50} = 0.23–0.85 ng/ml) to voltage sensitive sodium channels (Lewis *et al.*, 1991). The binding affinity of each ciguatoxin for the sodium channel (ED_{50}) is proportional to its ip LD_{50} in mice (Lewis *et al.*, 1991), indicating that the lethal effects of the ciguatoxins likely stem from their action on sodium channels. This high affinity binding could be used to form the basis of a sensitive assay for ciguateric fish. The advantage of using sodium channels in a biosensor-type assay is that the assay response would be proportional to the level of sodium channel activator toxin in a mixture, *i.e.* it provides a direct measure of likely *in vivo* potency. However, the development of biosensors has been slow (Ogert *et al.*, 1992; Griffiths and Hall, 1993) despite considerable interest in the potential of this approach (Malmqvist, 1993).

Ciguatoxins bind to sodium channels causing them to open at normal cell resting membrane potentials. This results in an influx of sodium ions, cell depolarisation and the appearance of spontaneous action potentials in excitable cells. This sodium influx can be markedly enhanced by the addition of site 2 sodium channel activator toxins (veratridine etc) through an allosteric mechanism. The recently reported cell based assay for the ciguatoxins (Manger *et al.*, 1993, 1994a, 1994b) takes advantage of this phenomena to produce an assay

Table 8.1. Effects of structurally defined dinoflagellate polyether toxins and palytoxin administered intraperitoneally (i.p.) to 20 g mice (for doses of toxin <~20 MU)

Toxin	i.p. LD_{50} (µg/kg)	MU (ng)	Signs of intoxication in mice	Dose *vs* time to death	Min./max.[g] death time
CTX-1[a]	0.25	5	hypothermia below 33°C, piloerection, diarrhoea, lachrymation, hypersalivation, dyspnoea, wobbly upright gait, gasping, terminal convulsions with tail arching, death from respiratory failure	log (MU) = 3.3 log(1+T^{-1})	37 min/ ~24 hr
CTX-2[a]	2.3	9	as for CTX-1, plus progressive hind limb paralysis	log (MU) = 2.4 log(1+T^{-1})	53 min/ ~100 hr
CTX-3[a]	0.9	18	as for CTX-1, plus progressive hind limb paralysis	log (MU) = 3.9 log(1+T^{-1})	60 min/ ~26 hr
GTX-3C[b]	1.3	26	–	–	–
GTX-4B[c]	4.0	80	as for CTX-1, plus hind limb paralysis	–	–
MTX-1[d]	0.05	1	hypothermia, piloerection, dyspnoea, progressive paralysis from hind extending to fore limbs, mild gasping, mild convulsions preceding death >30 sec	log (MU) = 6.7 log(1+T^{-1})	72 min/ ~72 hr
MTX-2[d]	0.08	1.6	as for MTX-1	log (MU) = 4.0 log(1+T^{-1})	41 min/ ~72 hr
MTX-3[d]	~0.1	~2	as for MTX-1	log (MU) = 6.7 log(1+T^{-1})	72 min/ ~72 hr
OA[e]	210	4200	hypothermia, hind limb paralysis, dyspnoea and respiratory failure	log (MU) = 2.6 log(1+T^{-1})	47 min/ ~7 hr
PTX[f]	0.4	8	hypothermia, hind limb paralysis, diarrhoea, dyspnoea, convulsions, death from respiratory	log (MU) = 2.3 log(1+T^{-1})	4 min/ >2 hr

[a] CTX-1, -2 and -3 (= ciguatoxin-1, -2 and -3) are oxidised and accumulated in fish from the gambiertoxins produced by *Gambierdiscus toxicus* (Murata et al., 1990; Lewis *et al.*, 1991, 1993). Acid catalysed spiroisomerisation converts CTX-2 to the more potent CTX-3 (Lewis and Holmes, 1993)

[b] GTX-3C (= ciguatoxin-3C) is from *G. toxicus* (Satake *et al.*, 1993) and can also accumulate in fish (Legrand *et al.*, 1992)

[c] GTX-4B (= gambiertoxin-4B) is from *G. toxicus* (Murata *et al.*, 1990; Legrand *et al.*, 1992; Holmes *et al.*, 1991) and can also accumulate in fish (Legrand *et al.*, 1992)

[d] MTX-1, -2 and -3 (= maitotoxin-1, -2 and -3) are from *G. toxicus* (Holmes *et al.*, 1990; Murata *et al.*, 1993; Holmes and Lewis, 1994) but are unlikely to accumulate to significant levels in the flesh of fish (Lewis and Holmes, 1993). MTXs can induce the appearance of a slight watery anal secretion but do not cause diarrhoea

[e] OA (= okadaic acid) is a diarrhetic shellfish toxin from *Prorocentrum lima* and *P. concavum* (Tachibana *et al.*, 1981; Murakami *et al.*, 1982; Dickey *et al.*, 1990; dose–death time relationship calculated by author from data of Baut and Masselin, 1990); these and related toxins (e.g. dinophysis toxins) have not been shown to accumulate to significant levels in fish (Lewis and Holmes, 1993)

[f] PTX (= palytoxin) is from *Palythoa* spp. (Moore and Scheuer, 1971; dose–death time relationship calculated by author from data of Moore and Scheuer) and can accumulate in fish (Fukui *et al.*, 1987; Noguchi *et al.*, 1987; Kodama *et al.*, 1989)

[g] minimum time to death (min.) estimated as described by Molinengo (1979); maximum time to death (max.) estimated from effects of doses near the LD_{50} dose

MU = mouse unit, where 1 MU = LD_{50} ÷ 50 (ie. 1 MU is the LD_{50} dose for a 20 g mouse)

T = time to death in hr (measured to the nearest min); – data not available

that is highly sensitive to ciguatoxins and other site 5 sodium channel activator toxins. This assay is 10^4-fold more sensitive than the mouse assay for ciguatoxins (see Fig. 8.2) and is presently undergoing refinement and further validation at the Food and Drug Administration laboratories at Bothell (WA). A version of this cell-based assay also shows considerable potential for the detection of sodium channel blocking toxins such as the saxitoxins and tetrodotoxins (Kogure *et al.*, 1988; Gallacher and Birkbeck, 1992; Jellet *et al.*, 1992). Cell-based assays have the potential to be automated and miniaturised (Goguen and Kedersha, 1993) but require somewhat specialised laboratory equipment. A number of tissues also respond to low concentrations of ciguatoxins, including ilea (Lewis and Endean, 1984), atria (Lewis and Endean, 1986) and phrenic-nerve diaphragm (Lewis *et al.*, 1993) but their use has been limited to research applications. These tissues could also be developed into assays for ciguatoxins since each gives a response that is proportional to the level of sodium channel activator toxin(s) present. However, such assays are costly and cannot be automated.

The ciguatoxins bind quasi-irreversibly to site 5 on sodium channels, a site overlapping the brevetoxin binding site (Lombet *et al.*, 1987; Lewis *et al.*, 1991). Legrand *et al.* (1994) have recently reported an assay for ciguateric fish based on the ability of ciguatoxins to selectively inhibit the binding of ^{3}[H]-brevetoxin to sodium channels in rat brain synaptosomes (Lombet *et al.*, 1987; Lewis *et al.*, 1991). Both *in vitro* sodium channel assays described above are more sensitive than the mouse bioassay and have considerable potential to replace this assay for the detection of ciguatoxins in crude fish extracts. However, in their current format these assays are unlikely to be cost-effective for the routine screening of individual fish.

Ciguatoxin binding to other proteins

In addition to having a high affinity for sodium channels, the ciguatoxins have also been reported to have affinity for various proteins including IgG from a variety of sources and proteins from the liver and flesh of fish (Parc *et al.*, 1979; Emerson *et al.*, 1983; Vernoux *et al.*, 1985a; Hahn and Capra, 1992). The affinity of ciguatoxin for these proteins has not been quantified and these studies have failed to exclude the possibility that the binding being measured was not simply the binding of ciguatoxin to sodium channels present in the protein-containing extracts. Such an interaction is expected to be considerable in tissues rich in excitable cells (e.g. fish flesh). Binding of ciguatoxin to IgG was proposed as the basis for a proposed assay for ciguateric fish (Emerson *et al.*, 1983) but their results could not be reproduced with extracts of ciguateric fish from Australia (unpublished result) and further development of this approach has not been reported. A possible ciguatoxin binding protein has been detected in fish flesh and its appearance has been proposed to result from the exposure of fish to ciguatoxin (Hahn and Capra, 1992). The appearance of such a protein in fish may explain why fish are less susceptible to the ciguatoxins than to the closely related brevetoxins (Lewis, 1992b). Detection of such a protein in fish could provide the basis for a novel assay for ciguateric fish. However, ciguatoxin may bind non-specifically to hydrophobic regions of a range of proteins.

Any high affinity binding of ciguatoxins to proteins (including sodium channels) present in fish tissue could be used to immobilise ciguatoxin to a solid phase prior to its detection with a labelled antibody specific for ciguatoxin. This approach can be considered a type of sandwich assay and could allow the rapid detection of ciguatoxin in fish flesh without the need for a solvent extraction step. Such an approach warrants further investigation but requires that an antibody can "see" the portion of the ciguatoxin molecule that is not obscured as a consequence of its immobilisation on fish protein.

IN VIVO BIOASSAYS FOR CIGUATOXIN

The mouse bioassay, based on the method described by Banner *et al.* (1960, 1961), is presently the most widely used assay for the detection of ciguatoxins contaminating fish. This assay has recently been validated for the detection of ciguatoxins in up to 20 mg of ether extract from the flesh of fish (Lewis and Sellin, 1993). Step by step procedures for the extraction and partial purification of ciguatoxins from fish tissues and the bioassay of these extracts using mice are described in detail below.

Extraction of ciguatoxins from fish tissue

A flow diagram describing the steps involved in the extraction of ciguatoxins ready for mouse bioassay is shown in Fig. 8.3. Samples should be in good condition upon collection, clearly labelled and immediately frozen at ~–20°C for storage. Prior to extraction, samples are thawed (for each sample we typically store ~50 g of flesh for testing in other assays), cooked in a plastic cooking bag, chopped and/or minced to a fine slurry and either refrozen prior to extraction or extracted immediately after the sample has cooled. The cooking step (which could be accomplished using a microwave and a beaker) is included to denature proteins that would otherwise interfere with the physical process of homogenisation in acetone and reduce extraction efficiency. Ciguatoxin is extracted with acetone along with a wide range of other lipid soluble material. Typically samples are extracted by homogenisation for 15 min using an explosion-proof homogeniser (such as an air powered homogeniser fitted with a medium viscosity generator with protruding cutters). Samples are extracted twice with acetone at room temperature and the acetone solubles separated from the insoluble material by vacuum filtration using a Buchner flask and filter-paper lined funnel. The extract is then dried to a viscous slurry on a rotavapor. More rapid drying on the rotavapor may be achieved by keeping the first and second extracts separate until almost dry. Care needs to be taken to ensure that samples do not foam excessively. Small amounts of antifoaming agent (e.g. n-octanol) may be added to difficult samples. Closing a tap placed in the vacuum line entering the rotavapor can be useful to maintain the vacuum at a suitable level and reduces the risk of excessive foaming.

The subsequent liquid-liquid partitioning steps are described in detail in Fig. 8.3. Care needs to be taken to ensure that pressure in the separatory funnel is regularly released during shaking and that the correct layers are carefully collected. With this procedure ~63% of ciguatoxin is recovered from fish flesh using routine methods (Lewis and Sellin, 1993). Poor recovery of spiked maitotoxin from fish flesh has been observed (R.Lewis, unpublished result). Unfortunately, the mouse assay of 20 mg of an ether extract typically cannot detect the presence of ciguatoxins in low toxicity ciguateric fish (Lewis and Sellin, 1993). Several modifications to this approach have been described. Replacing the ethanol–water/ether partition with a 2 M NaCl/ether partition may improve sample clean-up 2-fold (Lewis and Sellin, 1993). There remains a need to develop a truly rapid, inexpensive extraction procedure for the ciguatoxins.

Extraction of toxins from benthic dinoflagellates

Many toxins in addition to those found in the tissues of ciguateric fish are likely to be found in benthic dinoflagellates (either wild or cultured) or in the gut contents of herbivorous fish which have a diet that contains benthic dinoflagellates. Collections of dinoflagellates from the wild typically involves the collection of macro-algae from 1–15 m, shaking to remove dinoflagellates and sieving to obtain a 45–150 μm size fraction which contains most of the *G. toxicus*. An air-lift vacuum apparatus allows collection of dinoflagellates from turf algae on coral reefs and is especially useful for sampling *G. toxicus* at sites where *Ctenochaetus striatus* is observed to feed (Lewis *et al.*, 1994a). Extracts from dinoflagellates or the gut contents of herbivorous fish can contain a mix of several toxins, especially maitotoxin which induces effects in mice often

Table 8.2. Mouse assay to detect ciguatoxin in extracts from suspect ciguateric fish.

Preparation of ether soluble extract for injection:

- dissolve ether extract in chloroform–methanol (97:3) up to a known volume[a]
- remove an aliquot containing the required weight of extract to be assayed
- remove solvent from the sample on a rotavapor and/or under a stream of N_2
- to the dried material, add 1–5% Tween 60/0.9% saline solution to give a final volume of 0.1–0.5 ml for each i.p. injection (ie. for each mouse)
- warm, whirlimix and/or sonicate to ensure extract dissolves or is evenly suspended prior to any transfers (use 5% Tween 60 saline to suspend difficult samples)

Mouse assay:

- obtain healthy 18–22 g mice of either sex[b], house at ~25°C (12:12 light–dark), and provide food and water *ad libitum*
- mark mice for easy identification (see Fig. 4) and place mice in paper-lined cages (do not re-use mice)
- withdraw required volume of the Tween suspension (0.1–0.5 ml) into a 1 ml insulin syringe or similar syringe with a fine short needle
- in the first instance, inject ≤20 mg of extract into mice *via* the intraperitoneal route
- for each mouse record; time of injection, weight, sex, extract (g) administered, time of onset and nature of signs, time of death (observe mice for at least 24 hr)
- use at least 2 mice *per* fraction *per* dose, retest at a lower estimated doses (~2 MU) when deaths occur within 40 min (ie. dose exceeds 10 MU of ciguatoxin)
- for extracts inducing hypothermia to <33°C, dyspnoea and gasping, as well as copious diarrhoea, lachrymation and/or hypersalivation, calculate MU injected from the time to death *vs* dose relationship for mixed ciguatoxins[c]
- non-lethal fractions at 20 mg inducing the above signs and poor weight gain at 4 days are estimated to contain 0.5 MU of ciguatoxin
- fractions inducing unexpected signs or atypical dose *vs* time to death relationships could be retested to assess oral potency [ciguatoxins are equipotent by the i.p. or oral route (Lewis *et al.*, 1993b)]

[a] polar solvents (e.g. chloroform–methanol 1:1) may improve solubilisation of ether extracts, while more polar solvents (e.g. methanol–water 9:1) may improve solubilisation of water and butanol soluble extracts

[b] Quackenbush mice can be bred by continuously keeping mating pairs together, reducing each litter to 10 around day 2, and weaning mice at 21 days. Breeding females can be kept for 6 months and males for 12 months. A sheltered, low noise environment is important for a successful breeding colony

[c] for the mixed ciguatoxins found in carnivorous fish, log MU = 2.3 log(1 + T^{-1}), where one MU = ~5 ng CTX-1 and time to death (T) is measured in hrs to the nearest minute (Lewis *et al.*, 1992). For routine assay of ciguatoxins, a dose vs time to death relationship should be established for each colony of mice and preferably for each species of fish to be assayed

mistaken for effects of ciguatoxins, despite clear differences (Table 8.1). It is recommended that additional purification is undertaken (at least silica gel chromatography) in an attempt to separate the various toxins and to improve the reliability of conclusions based on mouse bioassay signs. Toxins eluting differently from ciguatoxin during purification should be chemically characterised where possible (see later). Silica gel chromatography has been used to separate and further characterise the toxins present in the gut contents of herbivorous species (Yasumoto *et al.*, 1971; Lewis *et al.*, 1988, 1994a), wild *G. toxicus* (Murata *et al.*, 1990; Holmes *et al.*, 1991, 1994) and cultured *G. toxicus* (Holmes *et al.*, 1991; Babinchak *et al.*, 1994).

Modified extraction procedures may improve separation of maitotoxins and ciguatoxins (Yokayama *et al.*, 1988; Holmes *et al.*, 1990, 1991; Holmes and Lewis, 1992, 1994; Legrand *et al.*, 1992). Typically the maitotoxins are only poorly separated from the ciguatoxins by a diethyl ether-water partition. Modifications to this partition, either using a dichloromethane–60% aqueous methanol partition or a diethyl ether–25% aqueous ethanol partition can improve the separation between the ciguatoxins/gambiertoxins and the more polar maitotoxins (Satake *et al.*, 1993; Holmes and Lewis, 1994). The maitotoxins are poorly soluble in –20°C acetone and the majority of the maitotoxins can be separated by filtration from the ciguatoxins which are soluble in cold acetone. However, silica gel or florisil column chromatography is still required to achieve complete separation of the ciguatoxins/gambiertoxins and the maitotoxins after these clean-up steps.

It is important to bear in mind that the maitotoxins can easily overload silica columns, in part because they are prone to co-elute with dinoflagellate pigments present in crude extracts. Maitotoxins typically elute from silica with chloroform-methanol (1:1) or more polar solvents and a loading of at least 1 g of dinoflagellate extract *per* 300 g of silica is recommended to ensure that a portion of the maitotoxins does not elute with the ciguatoxins and gambiertoxins in the lower polarity fractions.

Careful observation of mice injected with fish or dinoflagellate extracts can help characterise known toxins (Table 8.1) or to indicate the presence of new toxins. The gambiertoxins can also be distinguished from the maitotoxins (which are sulphonated toxins) by desulphonating any maitotoxins in pyridine-dioxane (1:1) at 120°C for 4 hr (Holmes and Lewis, 1994). The potency of the maitotoxins intraperitoneally (i.p.) to mice is reduced by more than 100-fold by this procedure, whereas the gambiertoxins are unaffected. Assessment of oral potency and heat stability are useful additional criteria to ascertain the potential of a toxin to cause human poisoning. The maitotoxins are ~100-fold less potent by the oral route compared with the i.p. route, whereas the ciguatoxins are equipotent.

The mouse bioassay

A step by step description of the mouse bioassay for ciguatoxins is given in Table 8.2. Briefly, a portion (up to 20 mg) of each diethyl ether fraction is suspended in 0.5 ml 1–5% Tween 60/0.9% saline and assayed in duplicate by i.p. injection into 20 ± 2 g mice of either sex. Control mice receive Tween/saline alone. The i.p. injection is given as indicated in Fig. 8.4 a. For each marked mouse (Fig. 8.4 b), weight, sex, time of injection, quantity injected, signs of intoxication, rectal body temperature (optional) and the time to death are recorded. Mice should be observed and monitored closely for the first two hr, then intermittently over at least a 24 hr period, with mice close to death being monitored closely. A depression of growth at 4 days can also be used to indicate the presence of sub-lethal doses of ciguatoxin (Lewis *et al.*, 1993). The relationship between dose and time to death is used to quantify each fraction. For the mix of ciguatoxins typically found in carnivorous fish (Lewis and Sellin, 1992) this relationship is approximated by:

$$\log MU = 2.3 \log(1 + T^{-1})$$

where, MU = number of mouse units of ciguatoxin injected and T = time to death in hr (Lewis *et al.*, 1992b). One MU is the LD_{50} dose for a 20 g mouse which is equivalent to 5 ng CTX-1

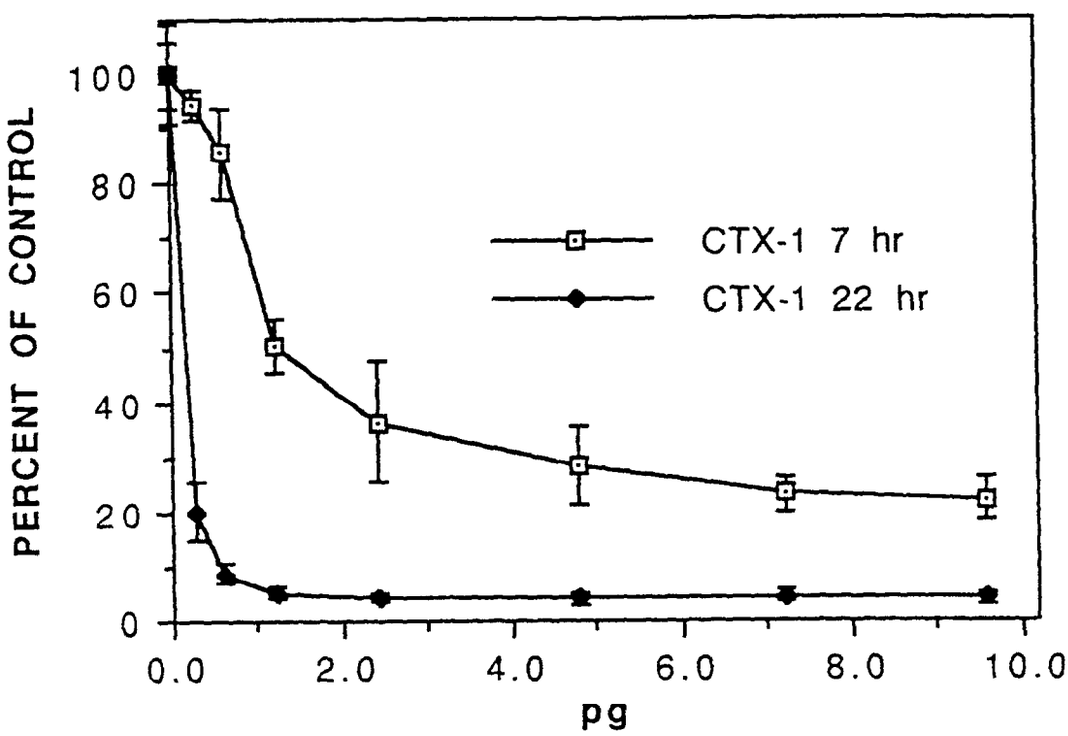

Figure 8.2. Cell-based assay response to pure ciguatoxin-1 (CTX-1) after 7 and 24 hr exposure. Data from Manger *et al.* (1994b). Control (100%) represents cell survival at 7 hr or 22 hr in the absence of CTX-1. Sensitivities (ED_{50}s) of 1 pg (7 hr) or <0.25 pg (24 hr) of CTX-1 *per* well were recorded in this experiment.

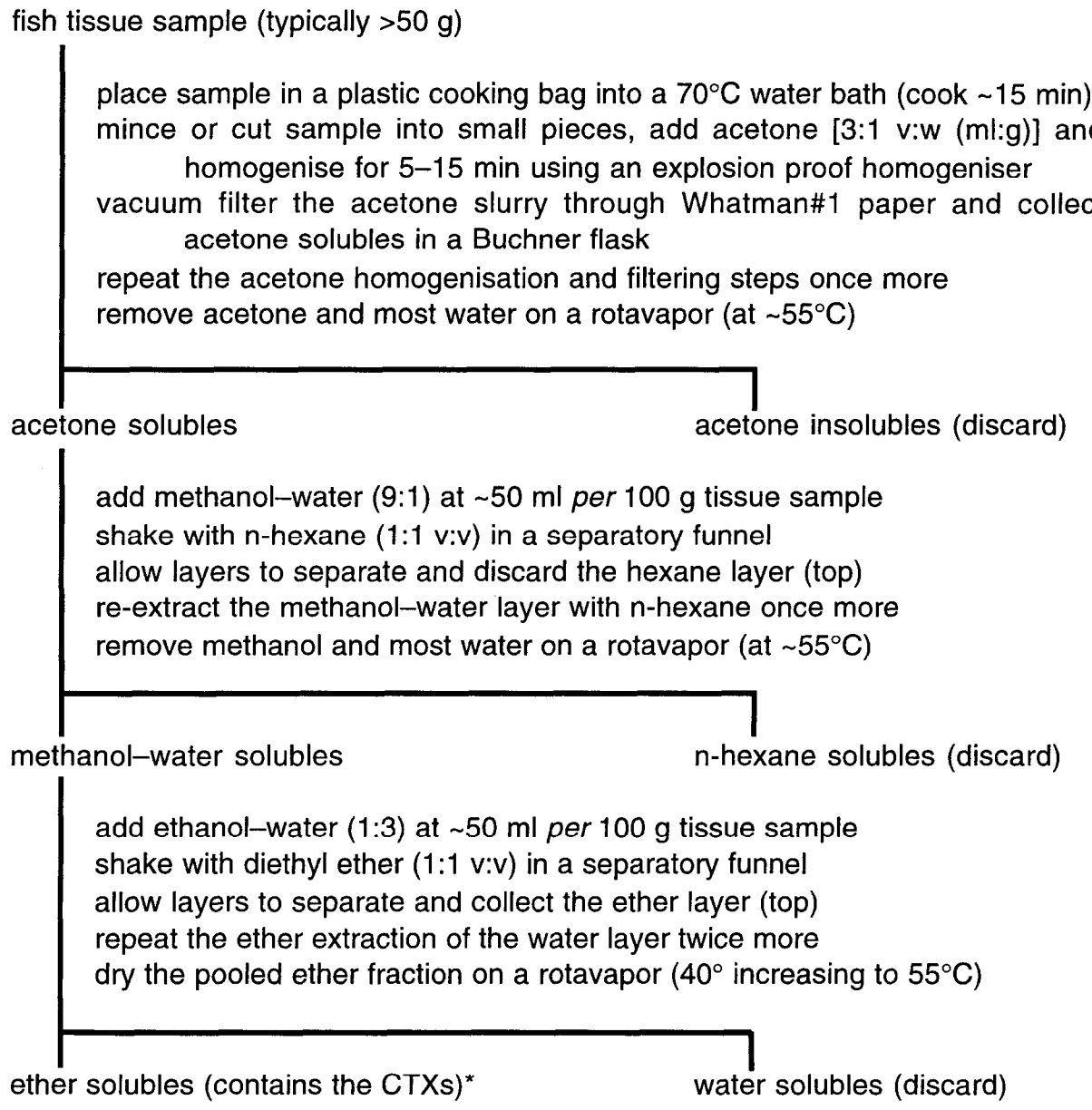

Figure 8.3. Generalised scheme for the extraction and partial purification of the ciguatoxins (and gambiertoxins) from fish tissue. The ether soluble material (*) contains the ciguatoxins (and any gambiertoxins) and is ready for testing with the mouse bioassay and further purification. Fractions required for testing should be stored at –20°C, either dry under N_2 or dried and then made to a known volume with chloroform–methanol (97:3). Those fraction marked to be discarded can be dried and assayed if it is suspected that toxins are present which have different polarity (solubility) to the ciguatoxins and gambiertoxins. The water bath temperatures indicated work with a tap water cooling system and a weak vacuum system (e.g. a water aspirator). Somewhat lower bath temperatures and higher vacuums can be used with colder traps (e.g. dry ice / ethanol or dry ice / acetone). A tap placed in the vacuum line before the rotavapor can be used to easily maintain vacuum at the correct level and gives a closed system which ensures solvents do not enter vacuum pumps or waste water outflows. Chloroform–methanol (97:3) can be used to transfer samples from large flasks (used to dry large or difficult samples, especially acetone/water, methanol water or water containing fractions) to smaller flasks for final drying and sample weighing. Both solvents to be used in a partition should be used transfer samples efficiently to the separatory funnel from rotavapor flasks etc.

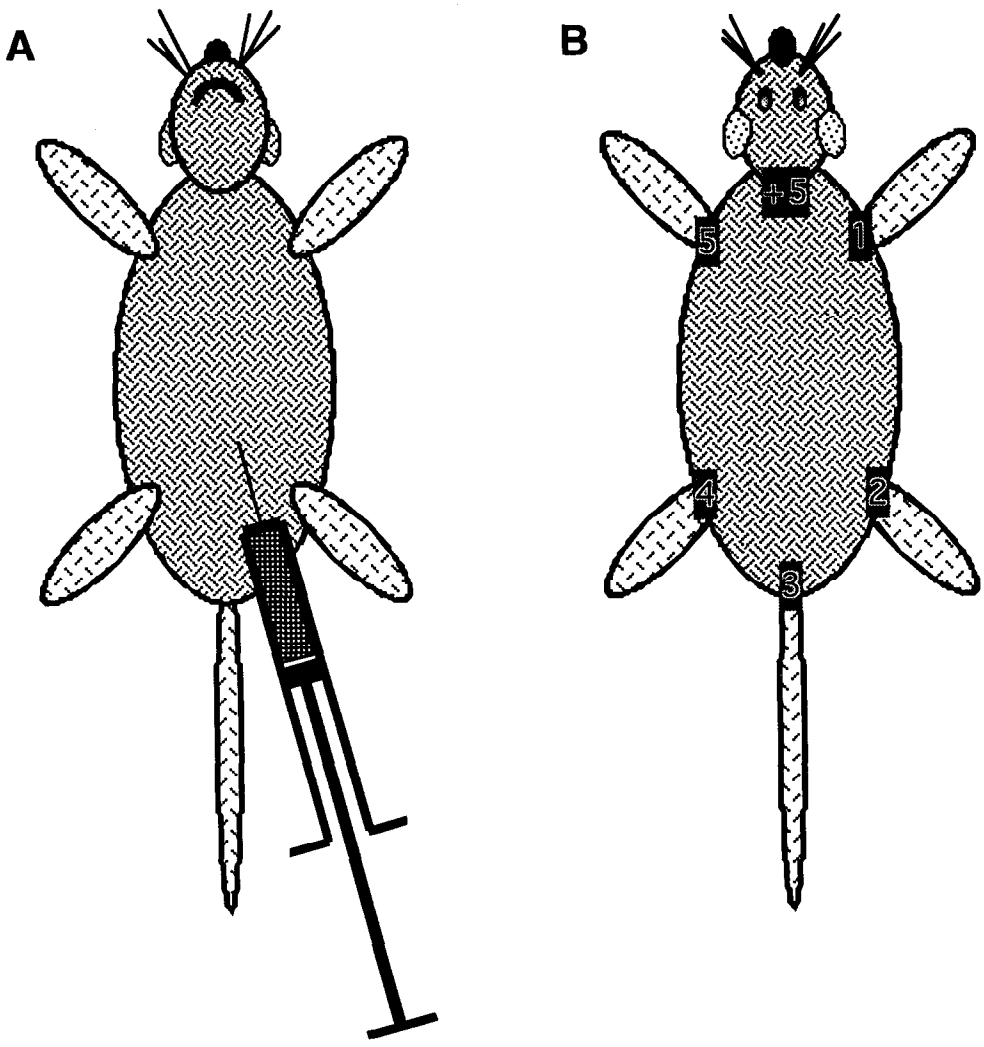

Figure 8.4. The mouse bioassay. (a) Site for i.p. injection of extracts into the perotonium of mice (ventral view). Mice can be handled for injection by pushing down and then gripping the back of the neck (at site +5) between the index finger and the thumb, at the same time holding the base of the tail onto the palm of the same hand with one or more of the remaining fingers. This can be achieved by first holding the tail with the other hand and pulling away from the head while the mouse is gripping with its front legs onto its wire cage top or similar. Someone familiar with the procedure should guide initial injections. (b) A suggested location of marks to identify injected mice (dorsal view). Using a permanent oil-based marker, one mark is used to indicate mice numbered 1–5, while a second mark on the head (near the +5 site) is used to indicate mice 6–10. Different coloured markers can be used to indicate additional groups of 10 mice.

(Lewis *et al.*, 1991). Table 8.1 gives the dose *vs* time to death relationship, signs of intoxication and the LD_{50} for various structurally defined polyether toxins and palytoxin. The mouse assay of ether extracts of fish tissue is considered to be a reliable method of characterising and quantifying the presence of ciguatoxin. However, reliable characterisation requires that the signs of intoxication in mice are consistent with ciguatoxin, especially the presence of hypothermia below 33°C, dyspnoea with gasping, as well as at least severe diarrhoea or lachrymation or hypersalivation, preferably with an estimate, derived from testing several doses, that the dose *vs* time to death relationship approximates the relationship for ciguatoxin (Lewis and Sellin, 1993). Additional signs of mouse intoxication have been reported by Hoffman *et al.* (1983) for partially purified extract from a Caribbean fish; however, some of these additional signs are not easily observed and are often absent. Unfortunately, the Caribbean ciguatoxin(s) remain structurally undefined. It is important to note that i.p. administration of 20 mg of crude ether extract from non-toxic fish can induce signs of mild intoxication in mice that could be misinterpreted (Lewis and Sellin, 1993).

For accurate quantification of dose, a specific dose *vs* time to death relationship should be established using mice from the locally available breeding colony, preferably for each source of ciguatoxin to be assayed. The strain and housing conditions for mice can influence sensitivity to ciguatoxin, in addition to any differences caused by the presence of different toxins or different ratios of the same toxins (unpublished observations). A dose *vs* time to death relationship is obtained by injecting ~12 mice (19–21 g) with doses between 1 and 20 MU of toxin. The use of the appropriate dose *vs* time to death relationship allows the accurate quantification of a particular toxin or mix of toxins with a minimum number of mice (typically 2 mice only are required) and can quantify unknown potency extracts over a wide range of doses (1–20 MU) with acceptable accuracy if doses giving death times <40 min are retested at lower doses (Table 8.2). This approach to estimating the LD_{50} of a fraction avoids a number of problems associated with classical LD_{50} estimations, where additional mice are required for initial range-finding and many more mice receive close to the minimum lethal dose and consequently are affected longer by the toxin.

In addition to the ether soluble fraction, the hexane and 25% aqueous ethanol soluble fractions can be assayed if toxin(s) less or more polar than the ciguatoxins (and gambiertoxins) are suspected. Quantification and characterisation of these toxins requires purification to homogeneity and chemical characterisation (see below). Given that putatively toxin-free extracts at 1 g/kg (dose weight/mouse body weight) can induce signs of intoxication following i.p. injection into mice (Lewis and Sellin, 1993), the presence of specific toxin(s) in crude extracts given at i.p. doses above 1 g/kg requires independent confirmation. The presence of toxins likely to be involved in ciguatera should be questioned if such doses are not orally potent [the ciguatoxins are similarly potent whether administered i.p or oral to mice (Lewis *et al.*, 1991, 1993b)] or if the suspected toxins cannot be purified by chromatography with reasonable recovery. It is noteworthy that free unsaturated fatty acids from marine species have i.p. LD_{50}s as low as 300 mg/kg (Takagi *et al.*, 1984).

Alternative in vivo *bioassays*

A number of other animal assays have been reported for the assay of ciguatoxins. These bioassays are in use in only a few laboratories and each has its strengths and weaknesses. The relative merits of the chicken, cat, mongoose, brine shrimp, mosquito and diptera larvae assays are briefly discussed below.

The chicken assay developed by Vernoux *et al.* (1985b) provides a rapid means of assaying the toxicity of fish liver by administering minced portions of liver directly into the crop of young chickens at 10% of the body weight. Administration of fish flesh is physically more difficult but can be accomplished on most fresh samples. The mongoose (Banner *et al.*, 1960) and cat (Hessel *et al.*, 1960; Bagnis *et al.*, 1985; Lewis, 1987) can also directly assay the whole flesh of fish without the need for extraction but require considerably more sample for assay (5–15% fed) than the chicken. The chicken, mongoose and cat assays are semi-quantitative,

sensitive and ciguatoxin induces characteristic signs of intoxication. However, these test species are typically not available through laboratory animal suppliers or are costly, and results may be compromised because of disease. The continuing use of large animal assays for detection of the ciguatoxins is increasingly difficult to justify on ethical grounds.

The first non-vertebrate assay developed for detecting ciguatoxins was the brine shrimp assay (Granade *et al.*, 1976). This assay has been more recently used in Parc's laboratory to confirm the presence of ciguatoxin and related toxins in fish extracts (Hungerford, 1993). However, attempts to confirm the usefulness of this assay by testing ether extracts from highly toxic ciguateric fish from Queensland were unsuccessful (R. Lewis, unpublished result, 1980). False positive results were caused by the toxic effects on brine shrimp of the Tween 60 recommended to emulsify the extract and no toxic effect attributable to ciguatoxin could be detected. The mosquito bioassay has been extensively used in French Polynesia to assay levels of ciguatoxin in fish implicated in human poisoning (Bagnis *et al.*, 1985, 1987). This bioassay correlates with the cat, mouse and human responses. However, few laboratories perform this assay, perhaps because of difficulties obtaining and housing mosquitos and a lack of familiarity in handling and recognising signs characteristic of intoxication by ciguatoxins. Recently, Labrousse *et al.* (1992) proposed a diptera larvae assay for detecting ciguatoxins. The assay involves the direct feeding of suspect fish flesh to meat eating fly larvae, with reduced weight gain (compared with controls) or death indicating that ciguatoxin is present. The assay appears sensitive, inexpensive, rapid and relatively easy to establish with limited laboratory resources. If validated, the diptera larvae assay could replace the mouse bioassay in the absence of alternative *in vitro* tests.

CHEMICAL METHODS FOR DETECTING CIGUATOXINS

Chemical features of the ciguatoxins

The ciguatoxins (Fig. 8.1) are lipid-soluble toxins consisting of 13 rings fused by ether linkages into a mostly rigid, ladder-like structure (Murata *et al.*, 1990; Lewis *et al.*, 1991, 1993a). They are relatively inert molecules which remain toxic after cooking and exposure to mild acidic and basic conditions. An assay selective for CTX-1 has general utility for the detection of ciguateric fish, given that (i) CTX-1 is the major toxin (on the basis of both quantity and total toxicity) present in fish, except certain herbivorous species which accumulate mostly gambiertoxins and less polar ciguatoxins, (ii) the involvement in human poisonings of toxins other than those of the ciguatoxin class remains an unsubstantiated and unlikely possibility, and (iii) carnivorous fish cause most cases of ciguatera.

Extraction of ciguatoxins and initial clean-up

Depending on their toxicity, ciguateric fish contain from 0.1 to 5 ppb CTX-1 (Lewis and Sellin, 1992; Lewis, 1992b) or up to 10-fold higher levels of the less potent ciguatoxins (CTX-2 and -3) and gambiertoxins. This extraction and clean-up procedures are likely to be necessary to concentrate the ciguatoxins prior to detection. The ciguatoxins are sufficiently hydrophobic to be insoluble in water but can be extracted from fish flesh with organic solvents of intermediate polarity such as methanol, chloroform or acetone. However, even with a several step clean-up procedure, involving the removal of both low polarity lipids and water-soluble material (Fig. 8.3), the ciguatoxins are still present in the remaining lipid mixture at relatively low levels of from 30 to 5,000 ppb.

Several so called 'rapid' extraction procedures for ciguateric fish have been reported. The method of Pompon and Bagnis (1984) appears to work in the mosquito assay but these extracts caused unexplained rapid deaths in mice. The methods of Legrand *et al.* (1993) and

Babinchak *et al.* (1994) give improved clean-up but neither could be considered sufficiently cost-effective nor rapid to be used for the routine screening of fish entering the marketplace. The R.E.M.™ (rapid extraction method) developed by Hawaii Chemtect is reported to give improved selectivity for the toxins involved in ciguatera but confirmatory data or details on the method are scant. There is considerable scope to develop improved rapid extraction methods that minimise interfering matrix effects. Such extraction procedures would need to be tailored for a specific assay.

Chemical detection of the ciguatoxins

Less than 5 ng of pure CTX-1 can be detected by monitoring eluants from high performance liquid chromatography (HPLC) with a sensitive UV detector (Lewis, unpublished results). However, since the ciguatoxins do not possess a distinctive UV chromophore, it is not possible to develop a method for selectively detecting ciguatoxin from other lipids present in a crude lipid extract from fish by monitoring the HPLC eluant with a UV detector. As discussed previously, the major ciguatoxins found in carnivorous fish possess a relatively reactive primary hydroxyl through which labels could be attached to enhance detectability. Since numerous compounds in a lipid extract from fish might also react with such a label, detection of the labelled ciguatoxins must be done in combination with a subsequent separation technique. HPLC coupled to fluorescence detection provides a high sensitivity method that has the potential to detect natural levels of ciguatoxins in crude extracts from fish flesh. Such an analytical detection methodology could be used to validate positive (or negative) responses obtained by antibody-based assays. Dickey *et al.* (1992) and Yasumoto *et al.* (1993) have reported encouraging results by labelling ciguatoxin with novel coumarin-based fluorescent reagents or the fluorescent 1-anthroylnitrile, respectively, prior to HPLC separation and fluorescence detection. These probes are available from Molecular Probes Inc., Eugene, OR, USA. However, further development of the extraction and clean-up steps is required if the ciguatoxins are to be detected in readily prepared extracts. HPLC linked to a fluorescence detector can detect in relatively crude extracts ≥40 ng diarrhetic shellfish toxins/g shellfish (Lee *et al.*, 1987) and ≥13 ng aflatoxin/g peanut butter or corn (Park *et al.*, 1990). However, two orders of magnitude greater sensitivity is required if ciguateric fish are to be detected.

HPLC coupled to selective-ion monitoring ionspray mass spectrometry (MS) is an alternative to fluorescent detection of ciguatoxin in HPLC eluants. This approach has shown considerable potential for the detection of labelled diarrhetic shellfish toxins (Pleasance *et al.*, 1992). Preliminary studies with CTX-1 indicate that such an approach could form the basis of a confirmatory analytical assay for ciguatoxins in fish (Lewis *et al.*, 1994b). Labelling ciguatoxin with an easily protonated probe (e.g. diethylaminocoumarin-carbamate) may increase the sensitivity of selective ion monitoring by ionspray MS, with such an approach providing an alternative confirmatory method to fluorescence monitoring. Preliminary results with this approach have been encouraging. Labelled CTX-1 could be detected by Ionspray MS when pure but further work is required to obtain the required sensitivity to allow ciguatoxins to be detected in crude extracts of fish (unpublished results).

NMR (nuclear magnetic resonance) and mass spectrometry (MS) of ciguatoxins

If sufficient purified toxin is available, NMR (one-dimensional NMR typically requires >25 μg of pure compound) and Ionspray MS (typically requiring >0.1 μg of purified compound) can be utilised for structure confirmation or for the characterisation of unknown toxins once purified. A general method for purifying the gambiertoxins or ciguatoxins to homogeneity is detailed in Fig. 8.5. Using material prepared in this way (Lewis *et al.*, 1991) we have obtained reference one-dimensional (Fig. 8.6) and two-dimensional HOHAHA spectra (Fig. 8.7) of CTX-1. These spectra, together with the proton and carbon chemical shifts (Table 8.3) facilitate the comparison of unknowns with the spectra of CTX-1. Detailed analysis of HOHAHA, DQF-COSY and

Table 8.3. ^{13}C and ^{1}H NMR chemical shifts for CTX-1 at 30°C (400 MHz, Pyridine-d_5).

Position	^{13}C(ppm)	^{1}H(ppm)	Position	^{13}C(ppm)	^{1}H(ppm)	Position	^{13}C(ppm)	^{1}H(ppm)
1	67.1	3.96	**21**	132.3	5.67	**41**	81.5	3.21
2	72.9	4.68	**22**	135.9	6.04	**42**	84.0	3.35
3	132.0	6.36	**23**	85.7	4.02	**43**	41.4	1.78. 2.59
4	131.0	6.36	**24**	85.0	3.64	**44**	74.8	4.47
5	78.0[2]	4.86	**25**	32.5	~2.2, 2.96	**45**	87.6	3.20
6	136.0	5.91	**26**	128.2	5.97	**46**	44.0	2.59
7	126.8	5.78	**27**	128.2	5.96	**47**	77.0	4.21
8	34.6	2.54, 2.73	**28**	32.7	~2.3, 2.93	**48**	72.6	4.06
9	76.6	3.50	**29**	83.9	3.76	**49**	77.9	3.96
10	87.8	3.75	**30**	85.4	3.63	**50**	39.0	2.01
11	74.1	4.10	**31**	40.0	~2.52, ~2.52	**51**	42.2	1.68
12	82.0	3.43	**32**	74.5	4.16	**52**	109.7[3]	-
13	73.6	3.34	**33**	78.6[3]	-	**53**	45.9	2.34, 2.40
14	37.5	1.85, 2.56	**34**	80.8	3.31	**54**	70.7[2]	4.86
15	79.7	3.55	**35**	36.4	1.92, 2.26	**55**	75.1	4.18, 4.18
16	80.5	4.03	**36**	81.0	3.34	**56**	9.7	1.37
17	133.6	5.74	**37**	73.1	3.50	**57**	28.3	0.94
18	131.1	5.89	**38**	46.8	1.54, 1.84	**58**	20.2	1.32
19	83.3	4.07	**39**	27.8	1.91	**59**	16.2	1.31
20	83.6	4.21	**40**	46.1	1.71, 2.03	**60**	13.9	1.23

1 ^{13}C and ^{1}H NMR chemical shifts derived from the HMQC experiment at 400 MHz, unless otherwise indicated.

2 ^{1}H chemical shifts at 4.86 ppm for two protons were obscured by the water resonance (^{13}C values from Murata *et al.*, 1992)

3 ^{13}C chemical shift values for quaternary carbons from the HMBC experiment at 30°C (500 MHz, pyridine-d_5)

ROESY spectra has provided independent support for the structure proposed by Murata *et al.* (1990). Spectra shown here were obtained on 0.45 mg of CTX-1 in pyridine-d_5 (Figs 8.5–8.6) with 500 or 600 MHz spectrometers. Typical acquisition parameters for the two-dimensional experiments include a sweep width of ~6000 Hz, >72 scan *per* experiment, ~512 experiments in t_1, and 4096 complex data points acquired in t_2. Data are zero-filled (final matrix consisted of 1k x 4k points) and apodised using a shifted sine bell squared filters (DQF-COSY) or a combination of a negative exponential followed by a Hamming (HOHAHA) on t_1 data. A ~6 Hz gaussian deconvolution on t_2 data is used to optimise the signal to noise in this dimension.

NMR and/or MS techniques similar to those described above have been used to characterise toxins present in fish viscera (Murata *et al.*, 1990; Lewis *et al.*, 1991) and flesh (Lewis and Sellin, 1992) and gambiertoxins present in wild and cultured *G. toxicus* extracts (Murata *et al.*, 1990; Satake *et al.*, 1993). If the acid labile ciguatoxins and gambiertoxins (e.g. CTX-2 and gambiertoxin-4A) are suspected to be major toxins, the purification scheme should be modified by replacing the silica gel column as well as possibly avoiding chloroform or other acidic solvents (Lewis and Holmes, 1993). A non-acidic replacement for silica gel is a Florisil, which can be eluted successively with hexane-acetone (4:1), acetone-methanol (9:1) and methanol (Satake *et al.*, 1993). Gambiertoxins (and ciguatoxins) elute in the acetone–methanol (9:1) fraction. It is recommended that these more specialised experiments be done in collaboration to ensure the best possible results are obtained.

CONCLUSIONS

A major advance in the management of ciguatera will come with the development of a validated, cost-effective assay that detects ciguatoxins contaminating fish. In this review, assays currently in use and progress and obstacles to a rapid *in vitro* assay are described. Ciguatera results predominantly from the effects of the ciguatoxin class of sodium channel activator toxins, especially CTX-1, the most potent sodium channel toxin, which predominates in ciguateric carnivorous fish. Levels of CTX-1 in fish flesh of 0.1 ppb (10^{-10} mole/kg) and above result in human poisoning. Ciguatera may also result from somewhat higher levels of the less potent gambiertoxins and less oxidised ciguatoxins found in herbivorous species. Other toxins produced by benthic dinoflagellates, including okadaic acid, maitotoxin and cyanobacterial toxins, have no proven role in ciguatera.

The recently validated mouse assay is the most widely used assay to assess levels of ciguatoxin in extracts from fish. Other *in vivo* assays (e.g. the chicken, cat, mongoose, mosquito, brine shrimp and diptera larvae assays) are less widely used and most require further validation. *In vitro* cell-based assays that measure the effects of ciguatoxin-induced sodium channel opening or the inhibition of [^{3}H]-brevetoxin sodium channels are more sensitive than *in vivo* methods for the detection of ciguatoxins and importantly give a response that relates to toxin potency. These recently reported *in vitro* assays (and perhaps the diptera larvae assay) could replace the mouse bioassay, with its attendant concerns for animal welfare, but require further validation. Antibody based assays still hold much potential as the basis of cost-effective screens for ciguateric fish but may suffer from a lack of specificity, with the presence of less potent ciguatoxins or cross reacting low-potency compounds potentially giving false positive results. In addition, it is yet to be proven that such assays have sufficient sensitivity to detect ciguatoxin down to 0.1 ppb directly in fish flesh or in rapidly prepared crude extracts.

The ciguatoxins do not possess a useful chromophore for selective spectroscopic detection but contain a relatively reactive primary hydroxyl through which (after appropriate clean-up) a label could be attached prior to detection. Detectors (e.g. fluorescence or ionspray mass spectrometry) coupled to optimised high performance liquid chromatography may provide the required sensitivity for analytical detection of derivatised ciguatoxins in crude extracts of fish and could be used to validate responses obtained by rapid screening assays. Reference nuclear

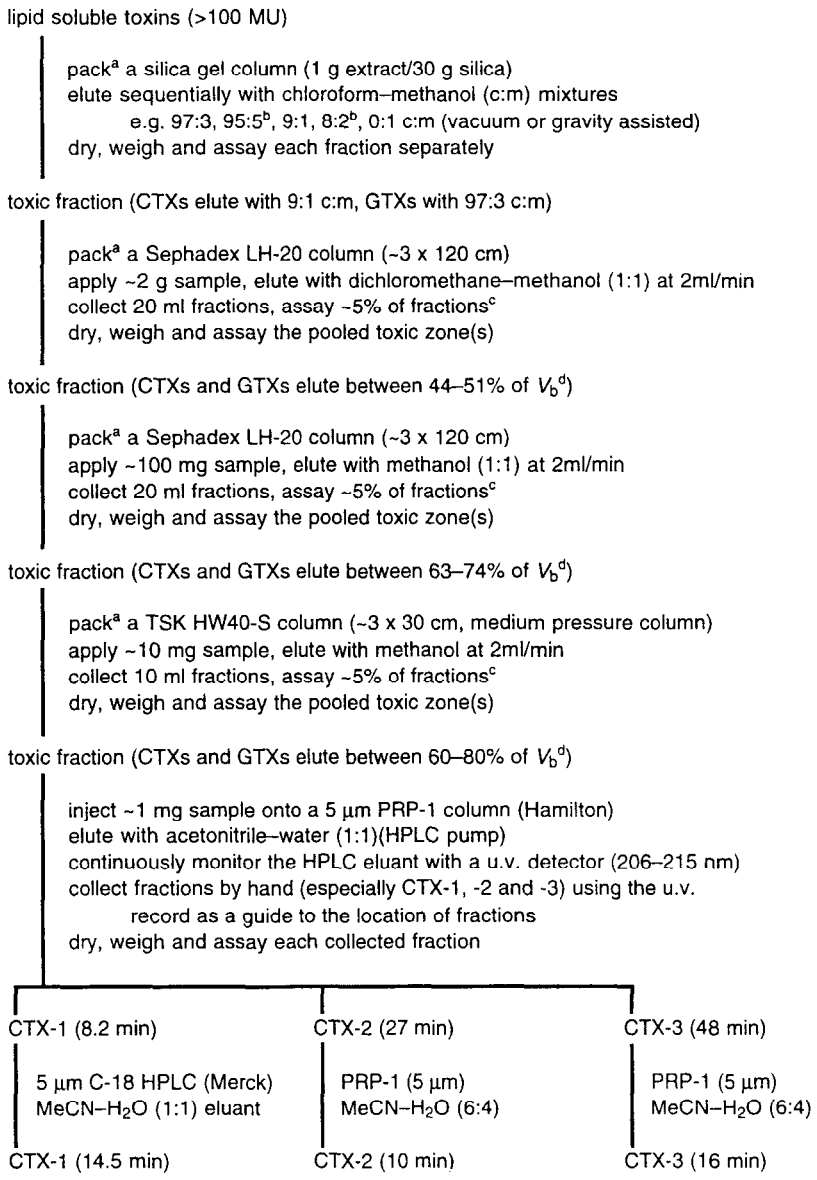

Figure 8.5. Generalised scheme for purifying ciguatoxins and gambiertoxins. Prior to the first chromatographic step, the ether soluble material can be further cleaned by filtering off precipitates, mostly polar impurities and the majority of any maitotoxins, which form in acetone at –20°C. Fractions are dried on a rotavapor, except the HPLC fractions which should be carefully dried under a stream high purity N_2 (in a warm water bath) to avoid chances for contamination on the rotavapor. (a) Columns packed with dry, thin-layer-chromatography grade silicic acid (Lewis et al., 1992), or as a slurry of silica gel, Sephadex LH-20 (Pharmacia) or TSK HW40-S (Merck). The silica gel column is eluted with 2 ml (97:3, 95:5, 8:2, 0:1 c:m) or 4 ml (9:1 c:m) of solvent *per* g of silica. A pump is the prefered solvent delivery method; however, gravity can be used to elute the Sephadex LH-20 columns. (b) Ciguatoxins eluting in the 95:5 and 8:2 c:m fractions (and the side fractions of each of the other columns) can be pooled and reapplied to a silica column to improve recovery. (c) Toxic zone(s) are located by assaying every second tube, working in from either side of the expected toxic zone and where possible avoiding the assay of the middle of toxic zones. (d) V_b is the column bed volume (ml). The time of elution of ciguatoxins from the PRP-1 column (5 µm, 150 x 4.1 mm, Hamilton) at 0.5 ml/min, and a Li ChroCART C-18 column (5 µm, 250 x 4 mm, Merck) at 1.0 ml/min are indicated. Elution times can be estimated for a range of solvents from their K' vs solvent strength relationship (Holmes and Lewis, 1992) but times vary slightly between runs depending on the precise solvent composition and column equilibration. HPLC eluants are monitored at 206–215 nm and the location of toxic zone determined by mouse assay. Fractions required for testing should be stored at –20°C, either dry under N_2 or dried and then made to a known volume in chloroform–methanol (97:3). Sample transfer of purified material can be achieved with methanol and chloroform–methanol mixtures (dichloromethane can replace chloroform).

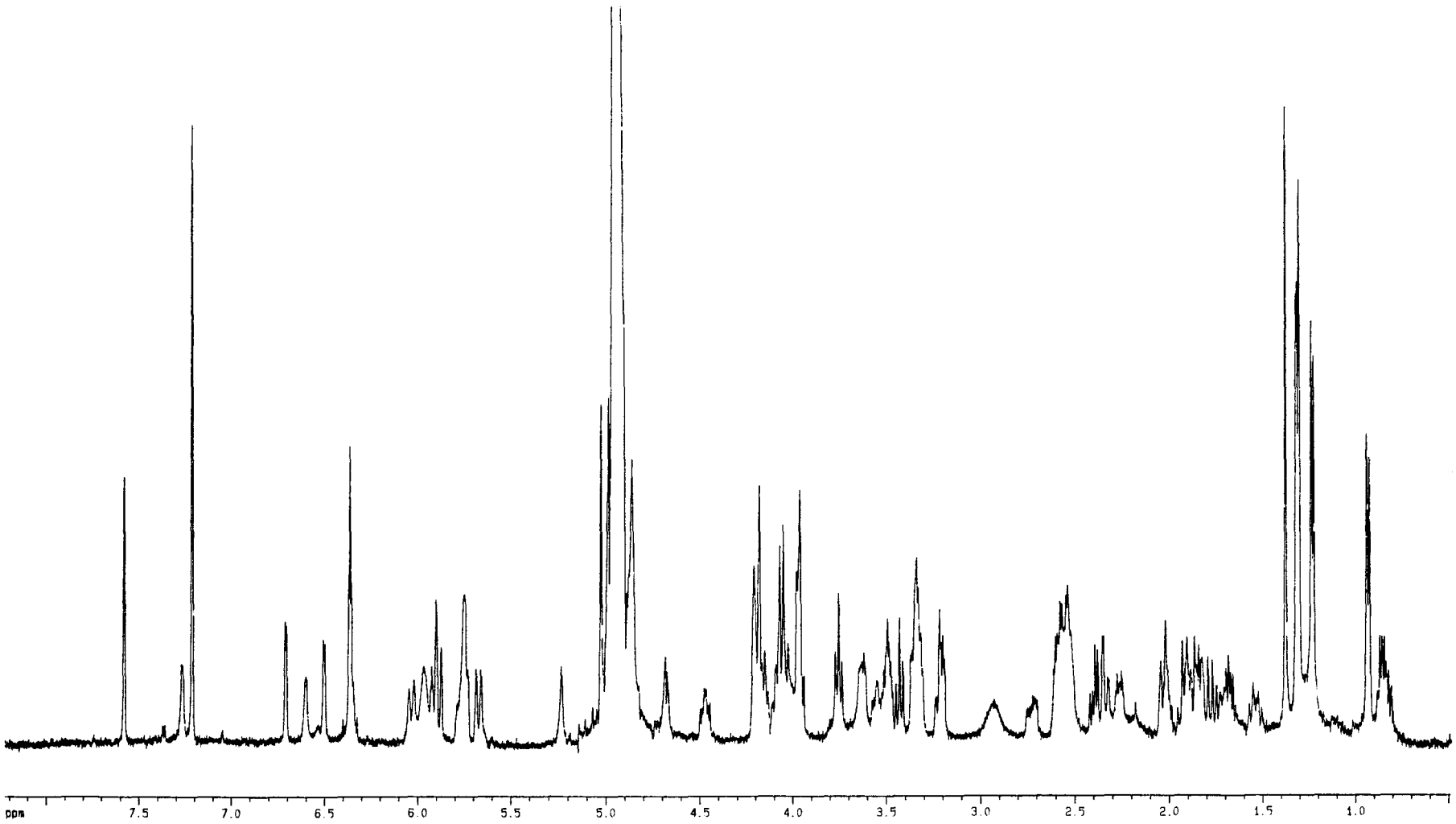

Figure 8.6. One-dimensional 500 MHz ^{1}H NMR spectra of ciguatoxin-1 in pyridine-d_5 at 30°C. ^{1}H chemical shifts are measured down-field of tetramethylsilane.

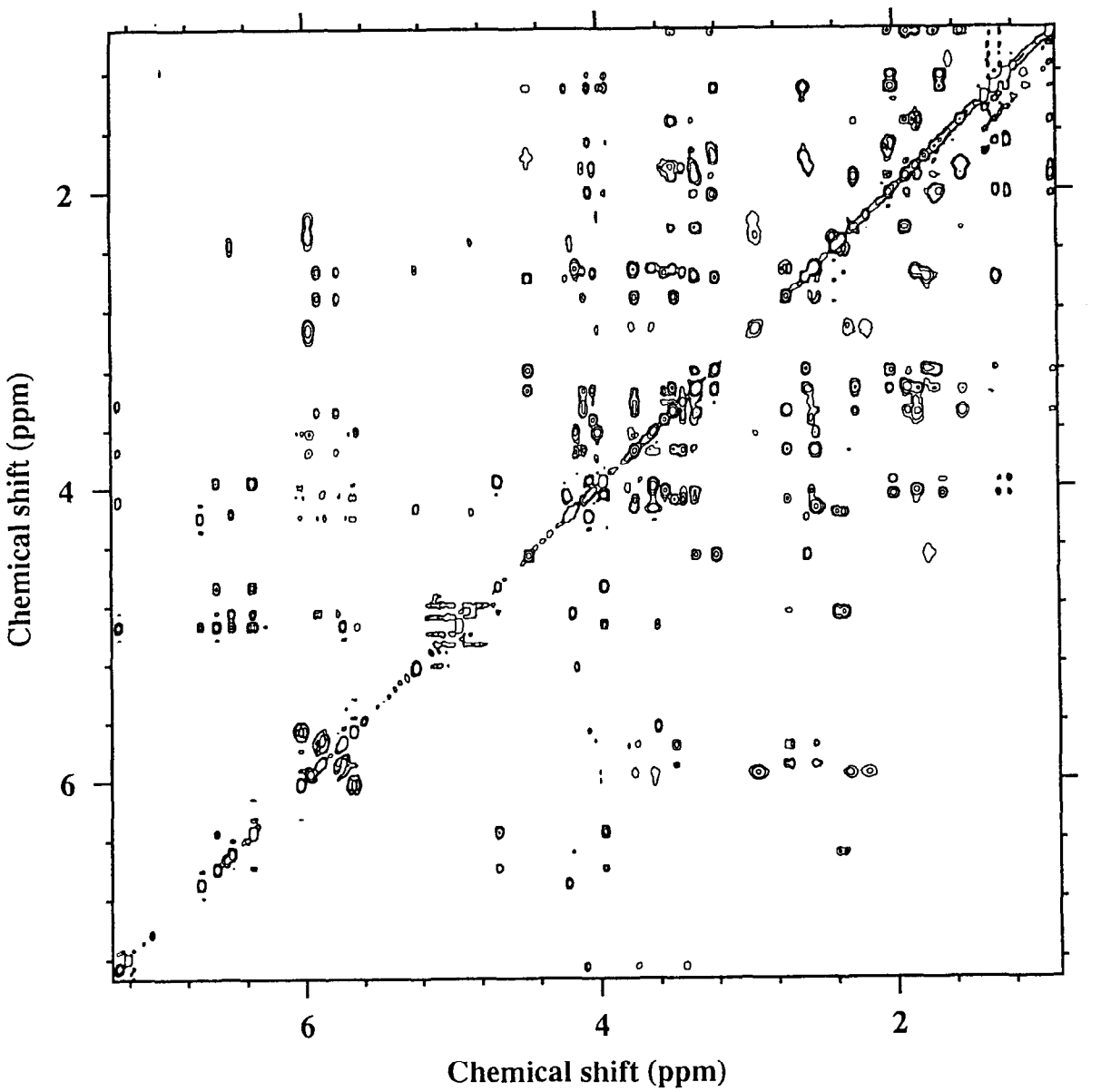

Figure 8.7.Two-dimensional 600 MHz HOHAHA (TOCSY) spectra of ciguatoxin-1 measured in pyridine-d_5 at 25°C. ^1H chemical shifts are measured down-field of tetramethylsilane.

magnetic resonance spectra of CTX-1, purified according to a generalised purification scheme for ciguatoxins and gambiertoxins, are provided to facilitate chemical characterisation of the ciguatoxins.

Clearly major advances are still required before ciguatoxin can be detected as part of a routine monitoring scheme to reduce the risk of ciguatera, a disease caused by one of the most orally potent toxins known. Important recent advances in knowledge of the chemistry of the toxins involved in ciguateric fish have laid the foundations for such advances.

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9. Cyanobacterial Toxins

W.W. Carmichael

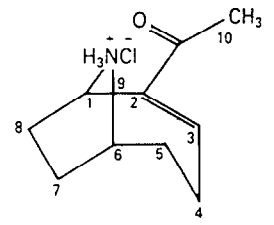
While algae responsible for producing toxins are found in the divisions Chrysophyta (class Prymnesiophyceae), Pyrrhophyta (class Dinophyceae or dinoflagellates) and Cyanophyta (cyanobacteria or blue-green algae), it is the latter that cause most of the problems in freshwater environments (Carmichael 1992). Several species and strains of cyanobacteria cause an often acute and potentially fatal condition from drinking water that contains high concentrations of toxic cells. Fatalities and severe illness of livestock (including birds), pets, wildlife and fish from heavy growths of waterblooms of blue-green algae are known to occur in almost all countries of the world. Most poisonings occur among terrestrial animals drinking algal infested freshwater supplies, but marine animals, especially maricultured fish, are also affected (Carmichael and Falconer 1993, Lambert *et al.* 1994, Anderson *et al.* 1993).

ETIOLOGY AND TOXICOSIS

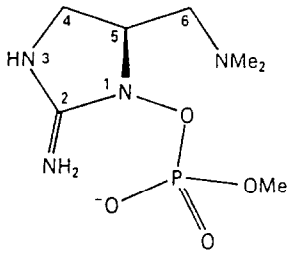
Although toxic strains within species of *Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis, Nodularia, Nostoc* and *Oscillatoria* are responsible for most toxic episodes, there are over 30 species of cyanobacteria that can be associated with toxic waterblooms (Skulberg *et al.* 1993). Neurotoxic alkaloids (called anatoxins) can be produced by *Anabaena, Aphanizomenon* and *Oscillatoria* while paralytic shellfish poisons (PSP) can be produced by *Anabaena, Aphanizomenon* and *Lyngbya* (Fig. 9.1). Hepatotoxic peptides (called microcystins and nodularins) can be produced by *Anabaena, Microcystis, Nodularia, Nostoc* and *Oscillatoria* (Fig. 9.2). *Cylindrospermopsis* and *Umezakia* can produce a potent hepatotoxic alkaloid called cylindrospermopsin. Some genera, especially *Anabaena*, can produce both neuro and hepatotoxins.

Poisoning usually does not occur unless there is a heavy waterbloom that forms a dense surface scum. Factors contributing to heavy waterblooms are nutrient rich eutrophic to hypereutrophic water experiencing warm, sunny weather. Agriculture practices and urban activities which lead to nutrient enrichment often contribute to waterbloom formation. Light winds or wind conditions that lead to leeward shore concentration of cyanobacteria in areas where livestock drink augments the problem. Experiments with both toxin groups have revealed a sharp dose-response curve with up to 90% of the lethal dose being ingested without measurable effect. Animal size and species sensitivity influence the degree of intoxication. Monogastric animals are less sensitive than ruminants and avian species. Depending upon bloom densities and toxin content animals ingest several milliliters to several liters to experience acute or lethal toxicity.

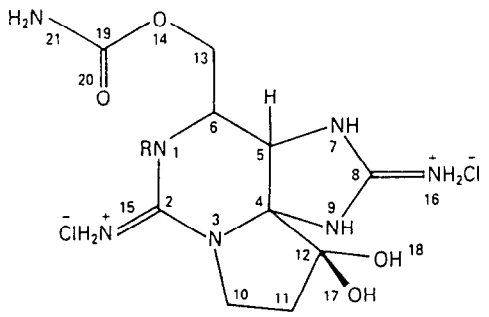
While the species sensitivity and signs of poisoning can vary somewhat depending upon the type of exposure, the gross and histopathology for the hepatopeptides is quite similar between affected species. Death from the cyclic peptide-induced hepatotoxicosis is generally accepted as being the result of intrahepatic hemorrhage and hypovolemic shock (Table 9.1). This conclusion is based upon large increases in liver weight (up to 100% in small laboratory test animals) as a percent of body weight as well as in hepatic hemoglobin and iron content that account for blood loss sufficient to induce irreversible shock. In animals that live more than few hours hyperkalemia and/or hypoglycemia may lead to death by liver failure within a few days (Beasley *et al.* 1989).



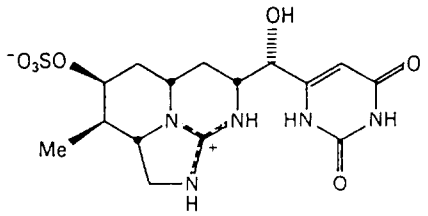
Anatoxin-a hydrochloride



Anatoxin-a(s)

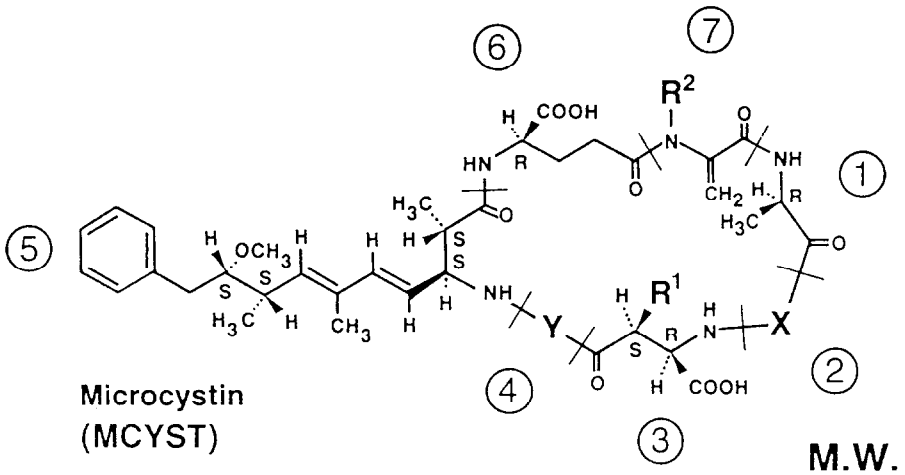


R = H; saxitoxin dihydrochloride
R = OH; neosaxitoxin dihydrochloride



Cylindrospermopsin

Figure 9.1. Structure of anatoxin-a (produced by some species and strains of *Anabaena and Oscillatoria)*, anatoxin-a(s) (produced by some strains of *Anabaena flos-aquae)*, saxitoxin, neosaxitoxin produced by some strains of *Anabaena* and *Aphanizomenon* and cylindrospermopsin produced by *Cylindrospermospsis raciborskii* (Carmichael and Falconer 1993) and *Umezakia natans* (Harada *et al.* 1994).



	MCYST-LA: X = Leu; $R^1 = CH_3$; Y = Ala; $R^2 = CH_3$	909
	MCYST-YA: X = Tyr; $R^1 = CH_3$; Y = Ala; $R^2 = CH_3$	959
	MCYST-LR: X = Leu; $R^1 = CH_3$; Y = Arg; $R^2 = CH_3$	994
desmethyl 3-	MCYST-LR: X = Leu; $R^1 = H$; Y = Arg; $R^2 = CH_3$	980
	MCYST-YM: X = Tyr; $R^1 = CH_3$; Y = Met; $R^2 = CH_3$	1035
	MCYST-RR: X = Arg; $R^1 = CH_3$; Y = Arg; $R^2 = CH_3$	1037
desmethyl 3-	MCYST-RR: X = Arg; $R^1 = H$; Y = Arg; $R^2 = CH_3$	1023
desmethyl 3,7-	MCYST-RR: X = Arg; $R^1 = H$; Y = Arg; $R^2 = H$	1009
	MCYST-YR: X = Tyr; $R^1 = CH_3$; Y = Arg; $R^2 = CH_3$	1044

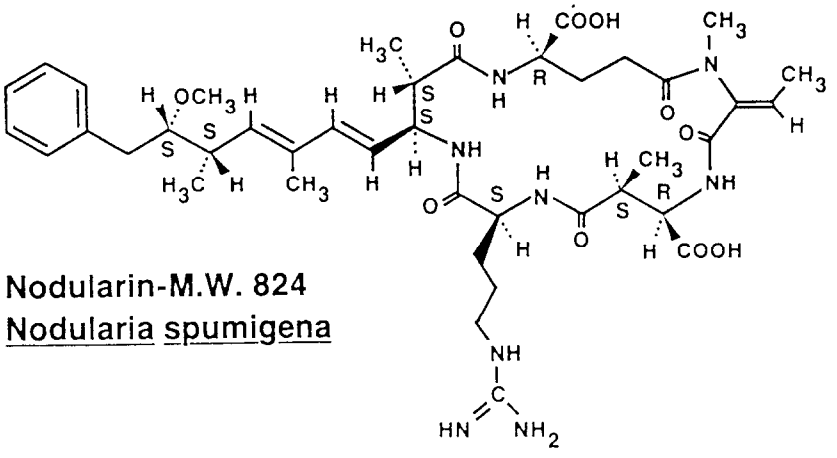


Figure 9.2. Structure of some of the more than 40 microcystins (produced by some species and strains of *Anabaena, Microcystis , Nostoc* and *Oscillatoria)* and nodularin produced by some strains of *Nodularia spumigena* (Carmichael and Falconer 1993).

Table 9.1. Animal hepatotoxicosis by cyanobacterial toxins (after Carmichael and Falconer 1993).

Animal	Clinical signs and lesions
Cattle, sheep	Hepatotoxicosis - clinical signs: recumbency/weakness, diarrhea, tachypnea/dyspnea, trembling, photosensitization, aberrant behavior, ataxia, pale mucous membranes, algae on skin/hair, weight loss, tachycardia, anorexia. Lesions include: liver enlarged, congested, mottled or friable, enteritis/hemorrhage, edema, anemia, algae in digestive tract, diffuse centrilobular hepatocyte degeneration.
Dogs	Hepatotoxicosis - clinical signs: abdominal discomfort, recumbency, diarrhea, vomiting, secretions from the eyes and mouth, anorexia, ataxia, coma. Lesions include: swelling/mottling of the liver, hemorrhagic enteritis, pulmonary edema, algae in the intestine.
Birds (turkeys, ducks, geese)	Hepatotoxicosis - clinical signs: restlessness, eye blinking, defecation, clonic spasms. Lesions include: hepatic enlargement/ hemorrhage, pulmonary edema, enteritis; algae in digestive tract.
Fish (rainbow trout)	Hepatotoxicosis - clinical signs: non-toxic when fish were immersed in a culture of M. aeruginosa; died following i.p. administration with hepatic necrosis.
Monkey (vervet)	Hepatotoxicosis - clinical signs: no prodromal signs from oral dosing before death. Lesions include: liver necrosis and hemorrhage.
Rhinoceros	Hepatotoxicosis - lesions include: hepatic enlargement, hemorrhage and necrosis.

Neurotoxicosis, with death resulting in minutes to a few hours, from respiratory arrest may result from ingestion of the neurotoxic alkaloid producing cyanobacteria. Species and strains of *Anabaena, Aphanizomenon* and *Oscillatoria* can produce a potent postsynaptic cholinergic (nicotinic) agonist called anatoxin-a that causes a depolarizing neuromuscular blockade. Strains of *Anabaena flos-aquae* can produce an irreversible organophosphate anticholinesterase called anatoxin-a(s). Strains of *Anabaena circinalis, Aphanizomenon flos-aquae* and *Lyngbya wollei* can produce the potent presynaptic sodium channel blockers called paralytic shellfish poisons (saxitoxins).

CLINICAL FINDINGS AND LESIONS

With microcystin poisoning one of the earliest effects (15-30 minutes) is an elevation in the serum concentrations of bile acids, alkaline phosphatase, gamma glutamyltransferase and aspartate aminotransferase. White blood cell counts will increase along with an increase in time for certain clotting factors to occur. Death may occur within a few hours (usually within 4-24 hours), up to a few days. It may be preceded by coma, muscle tremors, paddling and labored breathing. Watery or bloody diarrhea may also be seen. Gross lesions include hepatic enlargement due mostly to intrahepatic hemorrhage. Intact clumps of greenish algae can be found in the stomach and digestive track as well as a greenish algal stain on the mouth, nose, legs and feet. In time course laboratory studies hepatic necrosis begins centrilobularly and proceeds to the periportal regions. Hepatocytes are disassociated and rounded. Following death debris from disassociated hepatocytes can be found in pulmonary vessels and even in the kidney.

Clinical signs of neurotoxicosis progress from muscle fasciculations, decreased movement, abdominal breathing, cyanosis, convulsion and death. When anatoxin-a is involved signs in avian species are similar but include opisthotonus ("s" shaped neck). With smaller animals death is often preceded by leaping movements while in large ones collapse and sudden death occur. Animals, especially cattle and horses, that survive acute poisonings of either liver or neurotoxins may experience photosensitizations in areas exposed to light (nose, ears and back), that lead to hair loss followed by sloughing of the skin (Carmichael and Schwartz 1984).

DIAGNOSIS

Diagnosis is based primarily upon history (recent contact with an algal bloom), signs of poisoning and postmortem findings. Microscopic analysis of the water sample will reveal the presence of the toxigenic cyanobacteria. Although there are nontoxic and toxic strains of all the known toxic species it is not possible to identify a toxic strain by visual examination (Skulberg *et al.* 1993). Some agencies are equipped to analyze for the toxins either by chemical or biological assay. Newer methods of immunoassay are also available. Samples of the waterbloom should be taken as soon as possible to confirm the presence of the toxigenic species and analyze for toxins.

CONTROL

Removal of animals from the affected water supply is essential. If no other water supply is available animals should be allowed to drink only from shore areas kept free, by prevailing winds, of dense surface scums of algae. Some efforts have been made to erect surface barriers (logs or floating plastic booms) to keep shore areas free of surface scum but these are not very successful. Where permitted by federal and/or local agencies Cyanobacteria can be controlled by adding copper sulfate ($CuSO_4$) or other algicidal treatments to the water. The usual treatment is from 0.2 to 0.4 PPM equivalent to: 0.65 to 1.3 oz. per 10,000 gallons of water or 1.4 to 2.8 lb. per acre-foot of water. Livestock (especially sheep) should not be watered for at least 5 days (sometimes up to 2 - 3 weeks depending upon degradation rate: Jones *et al.* 1994) after the last visible evidence of the algae bloom. Since $CuSO_4$ and other herbicides can have effects on non-target organisms it is best used to prevent bloom formation. Special care should be taken to avoid water that has dead algae cells, either from algicide treatment or natural aging of the bloom, since most toxin free in the water occurs only after breakdown of the intact algae cells.

TREATMENT

Following removal from the contaminated water supply, affected animals should be placed in a protected area out of direct sunlight. Ample quantities of water and good quality feed should be made available. Since the toxins have a steep dose response, surviving animals have a very good chance for recovery. While therapies for cyanobacterial poisonings have not been investigated in detail, activated charcoal slurry is likely to be of benefit. An ion exchange resin such as cholestyramine (Questran, Mead Johnson) has also proven useful in laboratory studies to absorb the toxins from the digestive tract (Beasley *et al.* 1989). In laboratory studies certain bile acid transport blockers such as cyclosporin-A, rifampin and silymarin have been injected prior to dosing of microcystin and proven useful to prevent hepatotoxicity (summarized in Carmichael 1992). No therapeutic antagonist has been found effective against anatoxin-a or the saxitoxins but atropine and activated charcoal are effective in reducing the muscarinic effects of the anticholinesterase anatoxin-a(s).

MONITORING AND RISK ASSESSMENT

Cyanobacterial waterblooms can present an intermittent or continuous problem in water supplies. In addition to toxicity these blooms can cause taste and odor episodes in drinking and recreational waters. The blooms, especially when they accumulate on the water surface, are often easily visible making it possible to visually (or even by remote satellite sensing) monitor them. Several U.S. and Australian states, and some European and Asian countries have organized and advertised an "algae watch" to monitor waterbloom formation. In Australia the New South Wales Blue-green Algae Task Force (1992) have adopted a three-level alert system based on blue-green algal cell counts in water. Level 1 is 500-2000 cells ml^{-1}, when water authorities are alerted and sampling increased. Level 2 is 2000-15,000 cells ml^{-1}, when toxicity testing is carried out, water filtration plant operators advised to take precautionary action, agriculture agencies advised. Level 3 is above 15,000 cells ml^{-1} of toxic blue-green algae in a persistent bloom. In this situation if activated carbon is not available the water may be declared unsafe for human consumption.

A recent publication by the National Health and Medical Research Council of Australia acknowledged that due to apparent variability in individual sensitivities to toxins, it may not be possible to specify a "safe level" of cyanobacteria in water used for recreation or bathing purposes (Ressom *et al.* 1994). However, the authors concluded that it is reasonable to assume that recreational contact with "visible levels" of cyanobacteria represents a potential public health problem.

A number of tools are available for managing recreation in situations where toxic blooms are likely to occur. These include:

- The placement of permanent warning signs in situations where blooms are known to regularly recur.
- The erection of temporary warning signs during known bloom events.
- The placement of permanent signs with event-specific information.
- Newspaper and media announcements.
- Closure of specific facilities.
- Policing of recreational restrictions/warnings.
- Restriction of public access (closure of the waterbody).
- Relocation of recreational facilities to areas with lower potential for bloom development/accumulation.

Currently there are no international criteria for assessing the risk of toxic cyanobacteria in recreational or drinking water supplies. To date three countries, Australia, Canada and Great Britain, are moving to establish no-adverse or maximum acceptable levels for microcystins in drinking water supplies. No guidelines are being developed for the cyanobacterial neurotoxins because they are not considered as widespread and they do not appear to pose the same degree of risk from chronic toxicity, including tumor promotion, as the microcystins and nodularins do.

In Australia, a no-adverse-effect guideline level for microcystins has been published by Jones *et al.* (1993) and corroborated by Falconer *et al.* (1994). Data for their studies comes from the published literature on microcystin occurrence, toxicity and structure studies plus animal model data, using pigs as a model for human injury. The guideline level that resulted from Falconer's work, including the incorporation of a safety factor for tumor promotion is 1.0 µg microcystins or nodularins/l. Based upon the use of *Microcystis* cells for the pig feeding trials, this concentration corresponds to about 5000 cells/ml.

A similar number for a maximum acceptable concentration (MAC) was presented in a Criteria Document on microcystin-LR; Canadian Drinking Water, June 1994. This draft document, which is still under review, calculated a recommended MAC of 0.5 mg/l for microcystin-LR. Microcystin-LR is the first microcystin chemically identified and is found as the dominant microcystin in most countries (Japan and Australia are notable exceptions) reporting toxic episodes involving microcystins. The document also recommends that in the absence of potency equivalency values for other microcystins, 1 µg/l of total microcystins be the MAC in drinking water. It also recommends developing guidelines for other cyanobacterial toxins at a later date as needed.

DETECTION AND ANALYSIS OF TOXINS

Several methods are now available for detection and analysis of cyanobacterial toxins but none are considered by official agencies as standard. Harada (1995) presents a four-level approach to the detection and analysis of microcystins that can also serve as a model for the other cyanotoxins. His approach involves screening, cleanup, identification and quantitative analysis (Fig. 9.3). To this list should also be added sample collection. Collection of waterbloom samples is with plastic or glass containers. For toxicity studies using a mouse bioassay about 0.5 g dry weight of cells is required to do a statistically significant LD_{50}. This usually means collecting 1-2 l of light to moderate waterbloom to as little as 50-100 ml of a

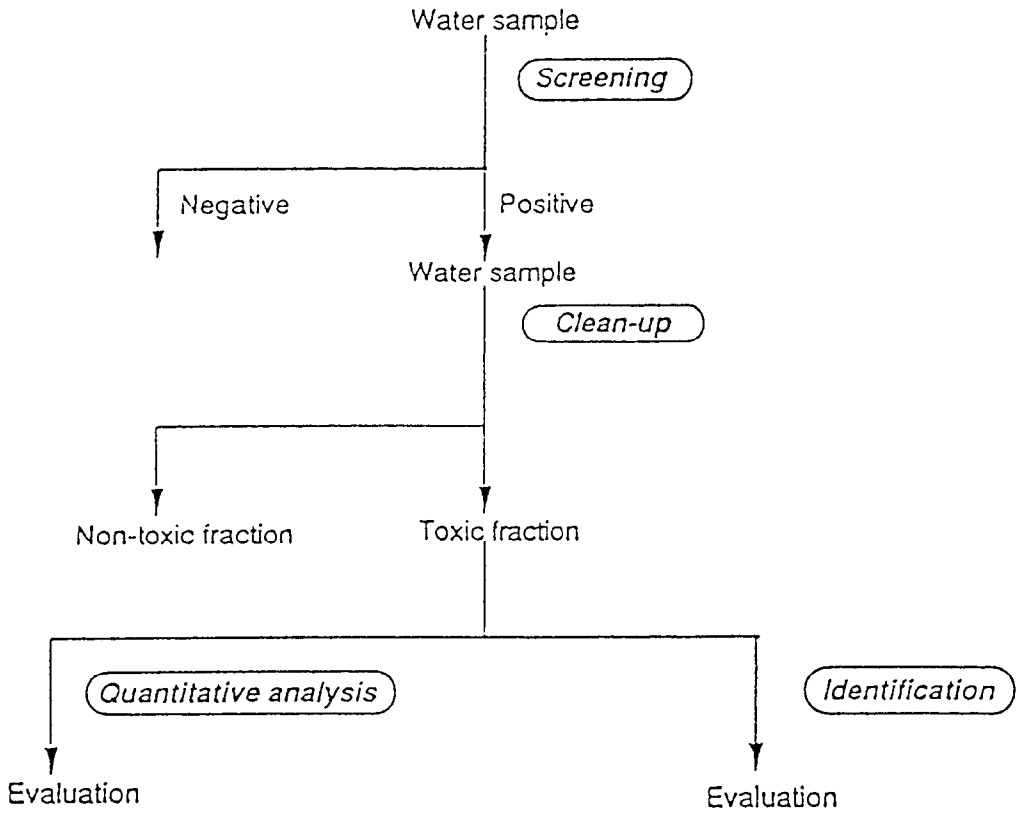


Figure 9.3. Flow diagram procedure for investigating cyanobacterial toxins in water (Harada 1995).

heavy surface accumulation. For the newer more sensitive assays only a few ml of bloom sample are needed. The author has found that the use of reversed phase (C-18) silica cartridges (i.e. Bond Elut®-Varian or Sep-Pak®-Waters) are very useful to concentrate waterbloom samples for later elution of microcystins followed by immunoassay.

Screening methods involve the traditional mouse bioassay or the more sensitive enzyme linked immunosorbent assay (ELISA) (Chu *et al.* 1990) and protein phosphatase inhibition assay (An and Carmichael 1994). Immunoassays rely on polyclonal or monoclonal antibodies raised against microcystin-LR. Cross reactivity against all the known microcystins has not been checked but polyclonals are effective against many of the microcystins (An and Carmichael 1994) with a few microcystins having been tested on the monoclonals (Nagata *et al.* 1995).

Immunoassay kits are becoming available for use in laboratories equipped with color metric plate readers. These kits, while complete with reagents, conjugates, etc.. have not been validated for all lab conditions, matrices, etc., under which they might be used. Therefore, some interlaboratory validation is recommended.

A. An ELISA Kit for Microcystin/Nodularin

Contact: Dr. Titan Fan
Immunosystems
Millipore Corp.
4 Washington Ave.
Scarborough, Maine 04074
Tel. 207-883-9900
FAX 207-883-8088

B. An ELISA Kit for Microcystin

Contact: Kyouichi Sekine
Kenkyu Kaihatsu Honbu
Mitsubiashi Kagaku BCL
Shimura 3-30-1
Itabashi-ku
Tokyo 174, Japan
Tel. +81-3-5994-2413
FAX +81-3-5994-2931

The mouse bioassay can be used to screen for both the neurotoxins and hepatotoxins of cyanobacteria because the signs of poisoning for these two toxin groups are very different. The method is used to determine the minimum amount of toxin required to kill a mouse--the LD_{50} or LDmin or it can be used to measure PSP's of cyanobacteria by the standard AOAC (1990) method. The major disadvantages of the test are that it does not detect toxins at low concentrations (Fig. 9.4), especially in finished drinking waters, and has limited application at water utilities which often have no facilities for maintaining experimental animals. The mouse bioassay does remain, however, a useful screening method for the neurotoxic anatoxin-a and -a(s) which have no other rapid screen available.

In order to carry out accurate identification and analysis from water and cyanobacteria sample cleanup is important. Tsuji *et al.* (1994) have analyzed several C-18 silica based cartridge systems and concluded that for water samples 10% water-0.1% TFA in methanol provides effective elution of microcystins while eliminating unwanted background compounds. Lawton *et al.* (1994) has also done extensive analysis of sample preparation procedures prior to HPLC-photodiode array detection of microcystins and concluded that 0.1% TFA in methanol was useful to elute microcystins from C-18 silica cartridges.

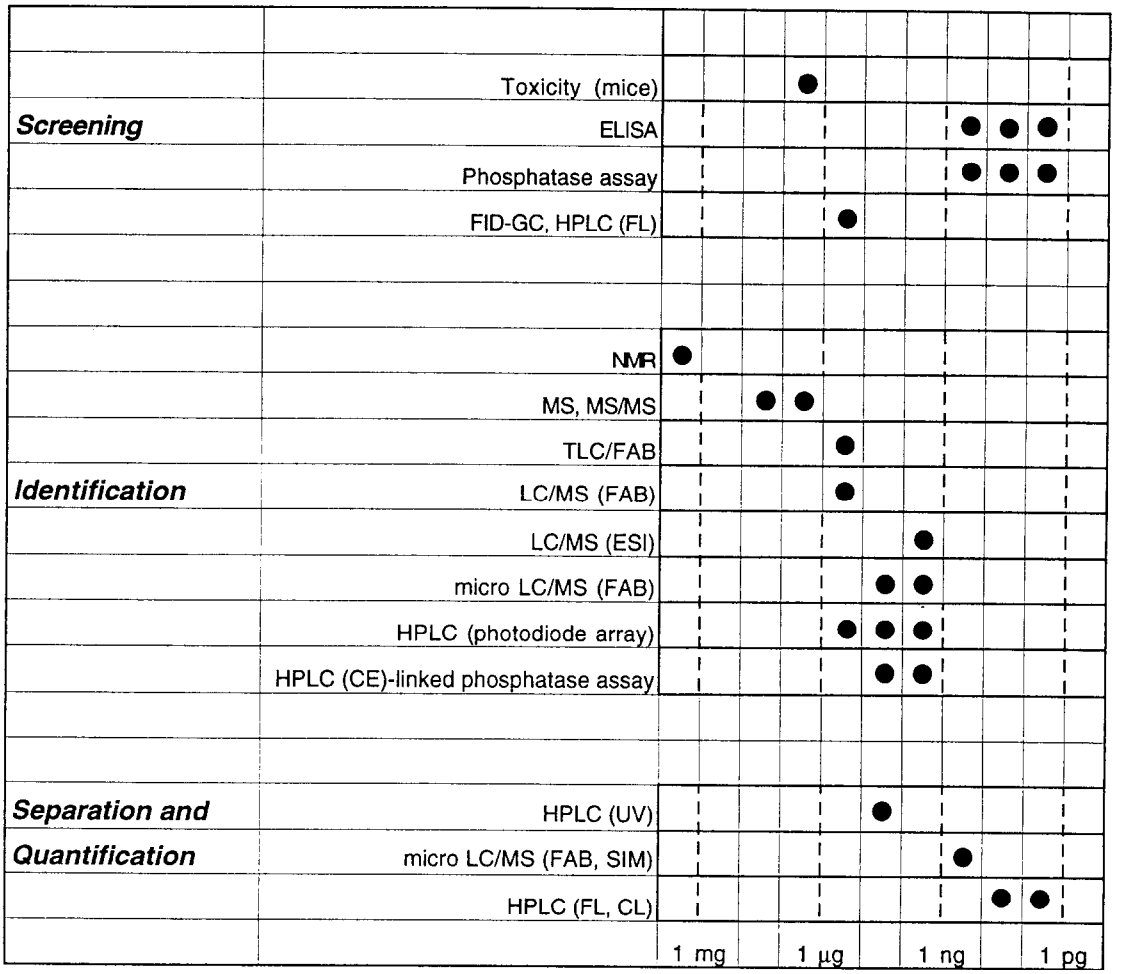
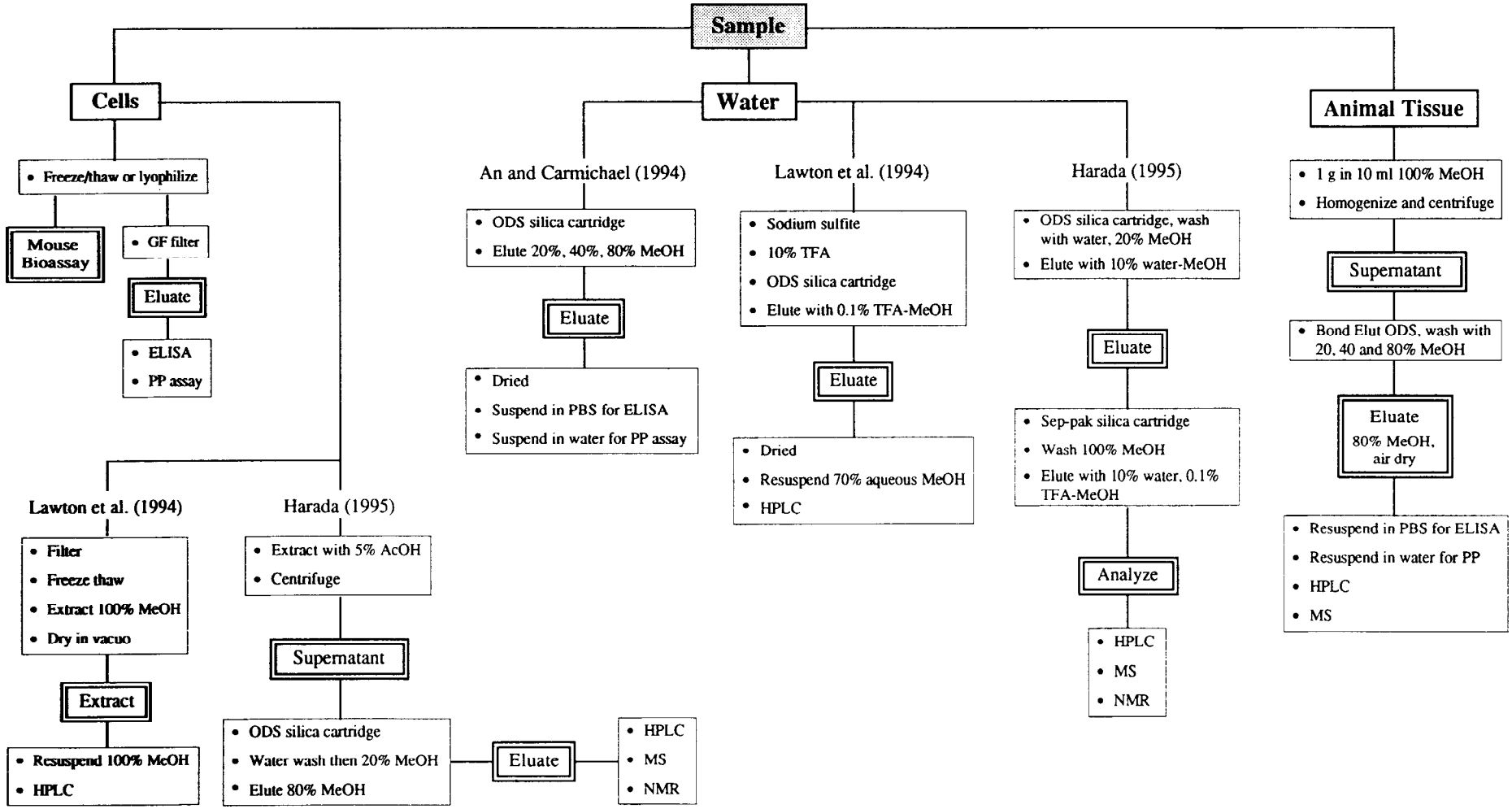


Figure 9.4. Methods and detection limits for screening and analysis of microcystins and nodularins (Harada 1995). Multiple dots for a given assay procedure indicate the range over which the method is sensitive. This will depend upon the toxin content of the sample and the type of instrument being used.

Table 9.2. Procedure for Microcystin and Nodularin detection and analysis based upon the screening method of An and Carmichael (1994) and the analysis method of Harada (1995) and Lawton *et al.* (1994).



Following sample cleanup, identification and analysis of cyanobacterial toxins uses several types of standard analytical procedures. For anatoxin-a HPLC, MS and NMR procedures are available (Mahmood and Carmichael 1987, Harada *et al.* 1993). For saxitoxin and their derivatives the fluorescence HPLC method of Oshima *et al.* (1993) has been used for analysis of Australian *Anabaena circinalis* (Humpage *et al.* 1994; Negri and Jones 1995; Negri *et al.*, 1995) and *Lyngbya wollei* (Carmichael *et al.*, unpublished).

Since microcystins and nodularins are the most common cyanobacterial toxins present in water supplies they have been the subject of several analysis methods. These methods include gel chromatography, thin layer chromatography, high performance thin layer and liquid chromatography, mass spectroscopy and nuclear magnetic resonance. Detectors for HPLC include ultraviolet (UV), photodiode array (PDA), fluorescence (FL) and chemiluminescence (CL). For a more detailed description of the methods for detection and analysis of cyanobacterial toxins refer to Harada (1995) and Fig. 9.4. A method for HPLC-PDA analysis of microcystins in raw and finished water was recently presented by Lawton *et al.* (1994) while the health effects of microcystins are nicely summarized by Lambert *et al.* (1994). Table 9.2 summarizes methods available for microcystin and nodularin screening, cleanup and analysis.

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10. *In Vitro* Biochemical Methods and Mammalian Bioassays for Phycotoxins

A.D. Cembella, L. Milenkovic, G. Doucette and M. Fernandez

MAJOR PHYCOTOXIN SYNDROMES AND MODE OF ACTION

The phycotoxins of principal concern to seafood safety involving human health and ecotoxicology in marine trophic webs include a wide diversity of compounds produced primarily by eukaryotic microalgae and cyanobacteria. These biologically active compounds range from low molecular weight components, such as the amino acid analogue, domoic acid (mol. wt. = 312), to complex polycyclic macromolecules, e.g. the cyclic polyether, maitotoxin (mol. wt. = 3422). Many phycotoxic syndromes result from simultaneous exposure to several closely-related but structurally distinct toxin derivatives. Toxin potencies are also highly variable, not only between different classes of phycotoxins, but also among congeners of the same toxin family. Differences in toxin structure and potency, and complications introduced by complex organic matrices, clearly pose a challenge to the development of *in vitro* biochemical and cellular diagnostic tests. Such methods should be capable of simultaneously detecting the presence of multiple toxin derivatives and estimating total toxicity referable to that derived from whole animal bioassays. A brief description of the toxin syndromes and respective biological activity are offered below, particularly as they relate to the function of *in vitro* methods and mammalian bioassays. Further details on the chemical structures, pharmacology, analytical methods, and toxicity for specific phycotoxins are found in relevant chapters elsewhere in this volume (Chapters 5, 6, 7, 8 and 9).

Paralytic Shellfish Poisoning (PSP)

Paralytic shellfish poisoning (PSP) is a neurotoxic syndrome resulting primarily from the blockage of neuronal and muscular Na^+ channels. Binding to the Na^+ channel prevents propagation of the action potential which is essential to the conduction of nerve impulse and muscle contraction. In vertebrates, the peripheral nervous system is particularly affected; typical symptoms of poisoning include tingling and numbness of the extremities, progressing to muscular incoordination, respiratory distress and muscular paralysis leading to death by asphyxiation in extreme cases. The PSP toxins include saxitoxin (STX) and approximately two dozen naturally occurring tetrahydropurine derivatives: a) highly potent carbamate toxins, including (STX), neosaxitoxin (NEO), gonyautoxins (GTX1-GTX4), b) weakly toxic N-sulfocarbamoyl toxins (B1, B2, C1-C4) and c) decarbamoyl (dc-) analogues of intermediate toxicity. Analysis of these compounds in natural matrices is complicated by the presence of multiple components which vary in specific toxicity (by up to two orders of magnitude), the susceptibility to chemical conversion during sample processing or storage, and the potential for substantial bioconversion which can occur in shellfish tissues. These differences in specific toxicity among the various toxin derivatives must be taken into account when determining total toxicity using assay methods which purport to yield an estimate of net toxicity.

Amnesic Shellfish Poisoning (ASP)

The memory loss associated with extreme cases of human intoxication from shellfish contaminated by domoic acid (DA) led to the description of the phycotoxic syndrome known as amnesic shellfish poisoning (ASP). Domoic acid is an excitatory amino acid derivative acting as a glutamate agonist on the kainate receptors of the central nervous system (CNS). This secondary amino acid is considered to be a more potent neuroexcitor than kainic acid, which when systemically injected into specific parts of the brain is known to have degenerative effects. Domoic acid is considered to be the primary toxin involved in ASP, although isomeric forms (e.g., iso-domoic acid) of lesser potency also occur naturally.

Diarrhetic Shellfish Poisoning (DSP)

Several of the components associated with the DSP toxin complex cause severe gastrointestinal disturbances in mammals when delivered orally. In humans, typical symptoms following the consumption of DSP toxin-contaminated shellfish include acute diarrhea, nausea, vomiting and, in some cases, abdominal pain. Although no human mortalities from DSP have been reported, the affliction can be highly debilitating for several days. Among the polyether compounds which belong to the DSP toxin complex, diarrhegenic effects have only been proven for okadaic acid (OA) and the dinophysistoxins DTX1 and DTX3, whereas the macrolide lactone pectenotoxins (PTX1-4) cause liver necrosis and sulfated yessotoxin (YTX) damages cardiac muscle after intraperitoneal injection into experimental mice. Certain diarrhegenic toxins (OA and DTX1) are potent inhibitors of at least two sub-classes of protein phosphatases (PP1 and PP2A) and this mode of action may be linked to the observed diarrhea, degenerative changes in absorptive epithelium of the small intestine, and to tumor promotion.

Neurological Shellfish Poisoning (NSP)

The toxins implicated in neurological shellfish poisoning (NSP), known collectively as "brevetoxins", are considered to be primarily ichthyotoxins. In humans, the symptoms of NSP intoxication include respiratory distress, as well as eye and nasal membrane irritation, caused principally by exposure to sea-spray aerosols and by direct contact with toxic blooms while swimming. The brevetoxins are also accumulated in shellfish, which when consumed by humans, cause a toxic syndrome somewhat similar to PSP intoxication. Many of these lipid-soluble cyclic polyether compounds have been characterized, including brevetoxins A, B and C (BTX A, B and C) [synonyms: PbTx-1, PbTx-2 and PbTx-8, respectively], GB3, 5 and 6 [synonyms: PbTx-3, PbTx-4 and PbTx-5, respectively] and PbTx-6 and PbTx-7, but undoubtedly there exist other undescribed derivatives. Due to the chemical lability of the brevetoxins, analysis of these compounds continues to be problematic. All of these derivatives exert their toxic effect by specific binding to site-5 of voltage-sensitive Na^+ channels, leading to channel activation at normal resting potential.

Ciguatera Fish Poisoning (CFP)

Ciguatera fish poisoning (CFP) is a complex syndrome in humans who have consumed certain fish inhabiting or feeding upon coral reef areas, principally in the tropical Pacific and Caribbean region. Symptoms can include gastroenteritis, skin itching, cardiovascular disorders, peripheral neuropathy and CNS disfunction; some of the latter effects can persist for several months following exposure or recur as transient episodes. The toxin components involved in CFP are large polyether molecules characterized operationally by their relative hydrophilic properties, as the lipophilic ciguatoxins (CTX) and the relatively polar maitotoxin (MTX). Maitotoxin is known to have a profound excitory effect on voltage-sensitive Ca^{++} channels. The neurotoxic

effects of CTX derivatives are due to enhancement of membrane permeability to Na^+ ions by opening voltage-dependent Na^+ channels. Ciguatoxins and brevetoxins share a common binding site on voltage-dependent Na^+ channels, and this may account for similarities in neuropathological symptoms in mammals.

Part A. *In Vitro* Biochemical and Cellular Assays

A.D. Cembella, L.V. Milenkovic and G.J. Doucette

INTRODUCTION

The development of *in vitro* techniques, including immunoassays, enzyme assays and cytotoxicity tests, offers one of the most promising approaches to the detection of phycotoxins in complex organic matrices, such as extracts of shellfish tissues and toxic microalgae. In principle, such methods have several inherent advantages over sophisticated chemical analytical alternatives (HPLC-FD, LC-MS, CE-MS, etc.) for routine phycotoxin monitoring, in that they can be configured to yield extremely high sensitivity (theoretical detection limit typically $<10^{-12}$ M) and specificity towards the target toxin analyte, for a comparatively low investment in hardware, operating cost, and technical training.

In vitro diagnostic test kits are expensive to develop, however, when manufactured in quantity, the cost per analysis becomes very competitive with other methods. In many cases, relatively crude extracts may be assayed, without resorting to the extensive clean-up procedures often required for instrumental analysis by physico-chemical methods. Biochemical assays should prove to be valuable to mariculturists and regulatory personnel to identify the presence of phycotoxins in shellfish, finfish and natural assemblages of toxic phytoplankton. When produced as rapid detection kits, certain assays could be conducted by field technicians on shipboard, at dockside or in local laboratories. As a preliminary screening for phycotoxins, mariculturists and fishermen might choose to perform assays upon landing their catch, since extraction and preparation of samples for these techniques is often rudimentary.

In comparison, most advanced instrumental analyses can only be performed in centralized laboratories due to the complexity of operation and the requirement for sample clean-up. Using microtiter plates and automated plate readers, *in vitro* microassays can permit the rapid parallel screening of large numbers of samples (>100 per day), whereas most analytical instrumental detection methods are limited to sequential injection, even when equipped for automated operation. In many regulatory programs, the phycotoxins of concern are well known and resolution of each individual component in the suite of related toxins may not always be necessary. Thus, a well calibrated *in vitro* test can serve a valuable role as the first line of defence in a phycotoxin monitoring program, with only key samples being subjected to further analysis by instrumental methods for toxin confirmation or structural elucidation.

By monitoring the phytoplankton for signs of phycotoxins, rapid assays can be employed as an early warning before significant toxin accumulation occurs in shellfish. Taxonomic characterization of potentially toxic species by microscopy is often inadequate, due to the lack of equipment or trained personnel in commercial mariculture operations and regulatory facilities. *In vitro* diagnostic methods may be of particular significance in cases where both toxic and non-toxic phytoplankton strains of the same species co-occur or when toxic species cannot easily be identified based upon morphological criteria.

Cell culture (cytotoxicity) bioassays, immunoassays, and enzymatic tests are a less controversial alternative to mammalian bioassays of whole animals, which are increasingly subject to legislative restriction for use in routine toxin monitoring programs because of animal rights concerns. The dependence upon mammalian bioassays to yield "biologically relevant" toxicity data might be significantly reduced without compromising public health and seafood safety if *in vitro* assay methods were employed.

In vitro assay methods may be broadly categorized into two general sub-types - functional versus structural assays. Functional assays are based upon the biochemical action of the toxin (e.g., binding to the ion channels of neuroreceptors), and hence quantitation will tend

to correlate well with the specific toxicity of the analyte. In the case of matrices which contain several toxic components with a similar mode of biological activity, but which vary in specific potency, such assays should yield an accurate estimate of net toxicity.

In contrast, structural assays are dependent upon the conformational interaction of the analyte (toxin) with the assay recognition factor (e.g., epitopic binding sites in immunoassays). Thus cross-reactivity in such structural immunoassays is limited to components with compatible epitopic sites and often does not reflect relative biological activity or specific toxicity. This lack of broad-spectrum cross-reactivity for toxic, naturally occurring analogs is a major drawback to the use of quantitative immunoassays for screening phycotoxins in naturally contaminated samples.

Although the application of such assays usually does not require the use of sophisticated technology, this is not the case for their development; costs of trained labour, materials, laboratory facilities, bulk production of diagnostic reagents, mass production of test kits, collaborative testing, patenting, certification, and marketing can often be excessive - perhaps exceeding the potential for cost recovery. There are also inherent limitations of many of *in vitro* methods, including the susceptibility for generating false positive responses for target phycotoxins due to the presence of toxicologically inactive congeners, or failure to detect all toxigenic components when a complex suite of toxin analogues is present. Inconsistencies in crossreactivity are particularly acute with immunodiagnostic tests. Such assay methods generally yield only an integrated quantitative value representing a group of toxins, whereas the components may vary widely in specific toxicity. Thus direct comparison with "toxicity" as determined from mammalian bioassays may be confounded. Finally, although availability of certified toxins for preparation and validation of *in vitro* diagnostic techniques has increased in recent years, efforts to develop broad-spectrum assays has been somewhat limited by the supply of particular phycotoxin analogues.

The importance of determining the degree of specific versus non-specific binding for *in vitro* assay techniques cannot be overemphasized. Non-specific binding (to either antibody or neuroreceptor) can be defined as that due to extraneous interaction with the ligand (i.e., toxin) resulting from the presence of bindable non-target components in the sample matrix.. Spurious binding of components (fatty acids, proteins, etc.) which are not related to the analytes of interest (toxins) can occur in immunodiagnostic tests as well as in neuroreceptor and cytotoxicity assays. Experimentally, non-specific binding must be taken into account by using the controls indicated in the appendices. Typically, the assay is conducted at a saturation concentration of unlabelled toxin and the non-specific binding factor is subtracted from the results prior to generation of competitive displacement curves.

In spite of the attributes of *in vitro* assays, there are surprising few of these techniques in current routine use in phycotoxin monitoring programs. The technology is advancing so rapidly, however, that this chapter will focus on techniques which have reached the advanced prototype stage or are in commercial production, rather than providing a review of the latest developments.

IMMUNOASSAYS

In the last few decades, there have been several concerted attempts to produce reliable immunodiagnostic test kits for various phycotoxins. Many of these efforts have been hampered by the lack of purified toxins for conjugation and difficulties in producing stable immunogens from relatively low molecular weight toxins such as STX or domoic acid. Low molecular weight components with little intrinsic antigenic activity (haptens) must be coupled to a carrier (typically a protein) prior to inoculation. Since toxin conjugates for immunization are typically prepared from only a single readily available derivative, whereas toxigenic phytoplankton and affected target species usually contain a suite of chemically related derivatives, crossreactivity is important in the development of immunological methods. The high acute toxicity of certain

phycotoxins within mammalian systems is a further complication to achieving an adequate antibody titre upon immunization. Nevertheless, the sensitivity of the such immunodiagnostic tests is typically orders of magnitude greater than the corresponding mouse bioassay or HPLC method - even picogram quantities of toxins are detectable.

Immunoassays for phycotoxin detection have been prepared from both monoclonal and polyclonal antibodies. In general, polyclonal antibodies are more rapidly and inexpensively produced than monoclonals and their higher affinity for multiple epitopic sites tends to yield a heterogenous broad spectrum assay, i.e. superior cross-reactivity with related antigens. Monoclonal antibodies are more suited for single epitope detection and since they are generated from "immortal" cell lines they can be consistently produced with low batch-to-batch variability albeit with lower stability than their polyclonal counterparts.

A wide array of different assay configurations may be used for immunodiagnostic tests - these include direct- and indirect-coupling, competitive interaction and "sandwich" assays (see Fig. 10.1 for a schematic of these approaches). Detection systems for immunoassays commonly make use of a radio-label (RIA), a coupled enzyme reaction (EIA), or a fluorescent marker (FIA), but other detection modes including chemiluminescence may also be employed.

Early efforts to produce polyclonal antisera to STX (Johnson *et al.*, 1964) using bovine serum albumin as the carrier protein yielded a relatively labile conjugate and poor activity. Several RIA methods (Carlson *et al.*, 1994; Davio *et al.*, 1985) and enzyme-linked immunosorbent (ELISA) assays (Chu and Fan, 1985; Davio *et al.*, 1985) described in the literature have employed various cross-linking agents and STX derivativization reactions to produce antibodies. In all cases, toxin conjugates for antibody production were prepared only from STX (or via a synthetic derivative, e.g. saxitoxinol). Where crossreactivity with other PSP toxins was evaluated, a critical deficiency in most immunoassays has been the weak cross-reactivity with the NEO sub-group (N-1-OH toxins) (Carlson *et al.*, 1984; Chu and Fan 1985; Usleber *et al.*, 1991).

An absorption-inhibition ELISA technique for the detection of PSP toxins, based upon a polyclonal antibody to STX, has been prepared by covalent linkage of the hapten STX to a synthetic carrier polypeptide polyalanine-lysine (Pal) (Cembella *et al.*, 1990). When configured as a rapid diagnostic immunoassay kit (**SAXITOXIN TEST**^{R}, Institut Armand-Frappier) the assay uses immobilized STX fixed to polystyrene batons to competitively bind free STX-antibody from the toxic sample-antibody incubation mixture (see Fig. 10.2). In the second step, the batons are incubated in horseradish- peroxidase-conjugate, followed by a final development of a colored reaction product in a cuvette containing the enzyme substrate solution. The optical density of the colored product is determined spectrophotometrically at 450 nm, with the color intensity varying inversely with the STX concentration (i.e., zero optical density signifies complete STX absorption, > 64 µg STXeq/100 g shellfish tissue). The toxin content of highly toxic samples is determined by serial dilution of the AOAC (1984) extracts in the reaction buffer.

The polyclonal antibody to STX incorporated into the **SAXITOXIN TEST**^{R} kit exhibits relatively broad antigen specificity and cross-reacts well with at least two gonyautoxins (GTX2 and GTX3), but there is no cross-reactivity to the low potency N-sulfocarbamoyl toxins (Cembella *et al.*, 1990). Binding affinity (relative to STX) in a 5 min incubation for various purified carbamate toxins is as follows: GTX3 (87%), GTX2 (74%), and NEO (60%). This cross-reactivity pattern suggests that binding occurs primarily through the guanidinium groups associated with the central nucleus, with both the N-1,2,3 and N-7,8,9 guanidinium rings serving as recognition sites for antibody coupling, whereas the SO_3^- group at the N-21 position of the N-sulfocarbamoyl toxins effectively blocks attachment to the antibody. For total toxin quantitation (in µgSTXeq) this failure to cross-react with N-sulfocarbamoyl toxins may not be a critical weakness because these derivatives are substantially less potent than the carbamate toxins and they could be readily converted by acid hydrolysis into their respective carbamate analogues prior to assaying. Other microbial toxins, including domoic acid, okadaic acid and *Staphylococcus* enterotoxin B do not cross-react in this immunoassay.

When tested against the HPLC-FD method and conventional AOAC mouse bioassays on toxic shellfish, the **SAXITOXIN TEST**^{R} kit yielded comparable results in the critical

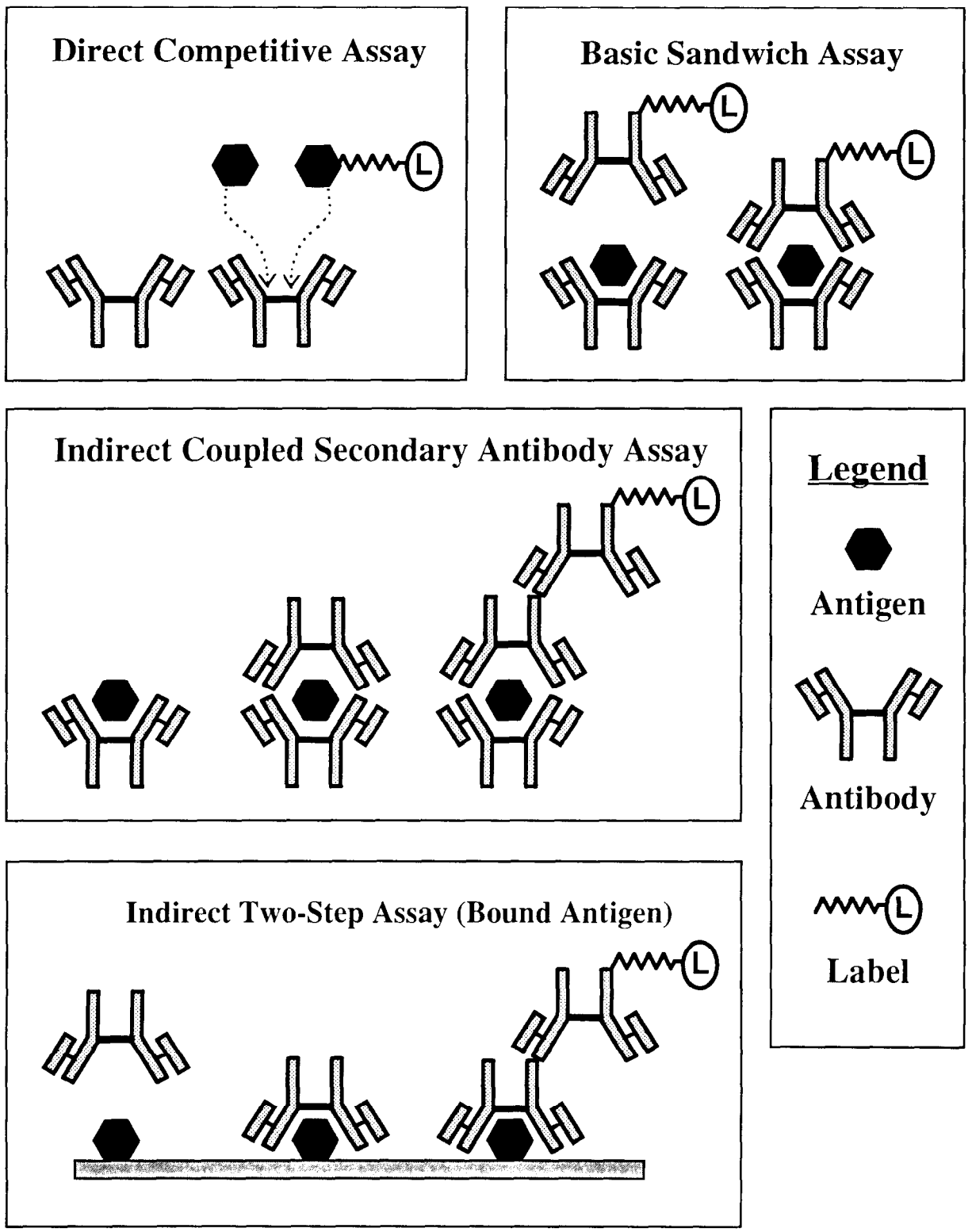


Figure 10.1. Schematic diagram showing alternative structural immunoassay configurations.

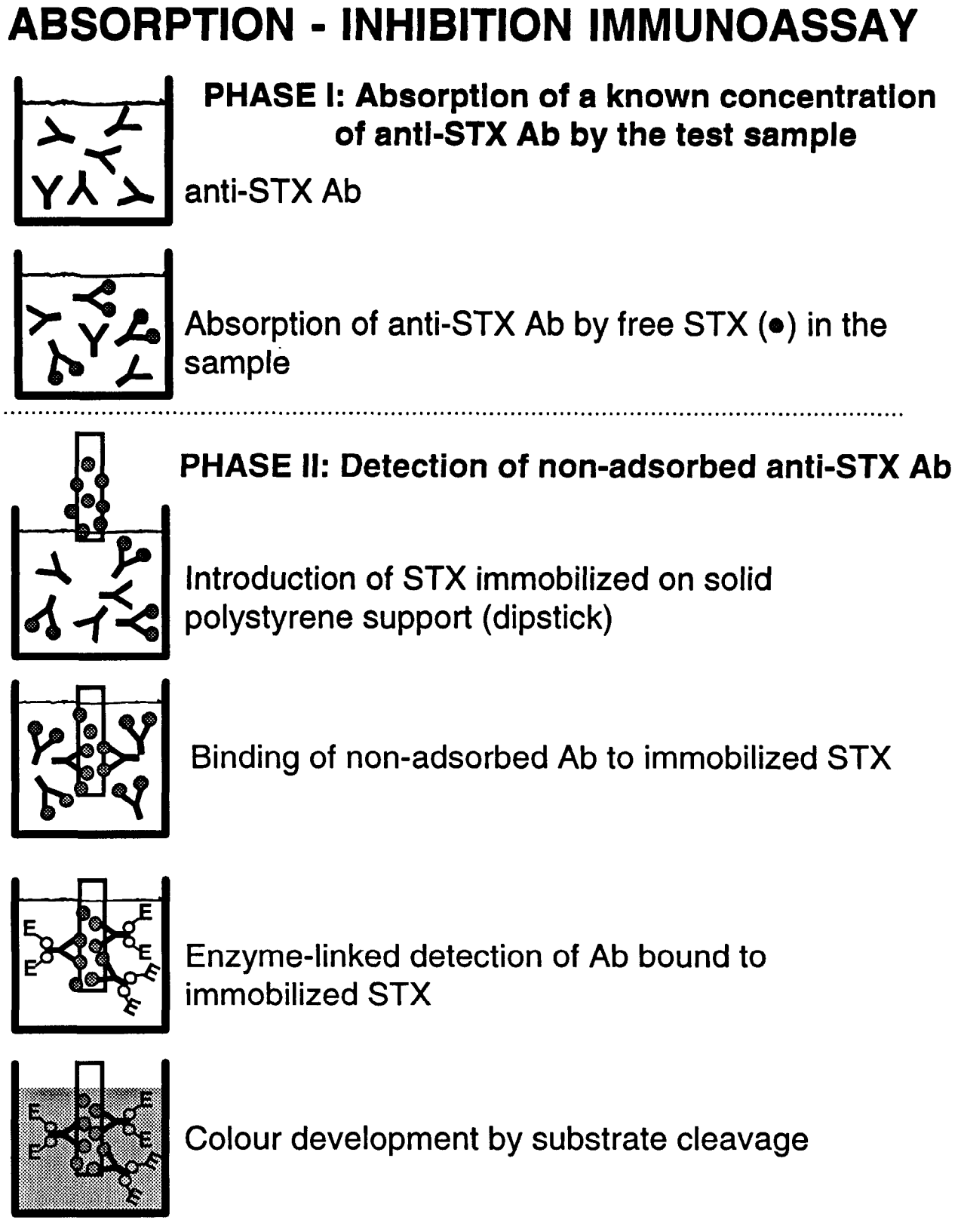


Figure 10.2. A flow diagram illustrating the step-wise protocol for a competitive absorption-inhibition immunoassay, such as the SAXITOXIN TEST^{R} kit.

toxicity range below 200 µg STXeq/100 g shellfish tissue with no false positive responses (A. Cembella and G. Lamoureux, unpubl. data). Unfortunately, detailed collaborative studies were not completed and the kit is no longer produced commercially for shellfish toxin assays. This polyclonal antibody was shown to be useful in assaying for PSP toxins in laboratory reference cultures of phytoplankton, including gonyaulacoid dinoflagellates isolated from both temperate and sub-tropical areas, and in natural phytoplankton assemblages in which *Alexandrium* spp. were dominant (Cembella and Lamoureux, 1993).

A direct EIA prepared from a polyclonal anti-STX antibody, configured as a microtitration plate ELISA and in a test strip assay, by conjugation of STX to horseradish peroxidase showed high sensitivity (3-4 ng/g tissue) for the detection of STX in shellfish (Usleber *et al.*, 1991). Further developments which compared the effect of heterologous PSP toxin-enzyme conjugates and crossreactivity with purified toxins (Usleber *et al.*, 1994) indicated that the direct EIA method was generally superior in sensitivity to the indirect technique. A modified assay for qualitative screening incorporates the use of a membrane filter in a competitive enzyme-linked immunofiltration assay (ELIFA), a simple and rapid assay which could be performed outside of a well-equipped laboratory (Usleber *et al.*, 1995). Although the cross-reactivity against NEO is poor, STX, GTX2/GTX3 and dcSTX are readily detectible in shellfish at or below the 80 µg/100 g tissue level.

A related ELISA method for the detection of STX in shellfish is commercially available as a test kit (**RIDASCREEN**^{R}, R-Biopharm) and this immunoassay has been subjected to collaborative testing against other methods by the European Commission's Measurement and Testing Programme (BCR) (Van Egmond *et al.*, 1994). Not surprisingly, the ELISA test yielded an overestimate of "STX" when used to assay for STX spiked into an mussel tissue matrix containing several other PSP toxins, probably due to undefined crossreactivity. For shellfish extracts, the method is useful for assaying STX but can only be employed semi-quantitatively.

An immunodiagnostic test for the presence of domoic acid in mammalian serum and urine has been developed (Newsome *et al.*, 1991), using polyclonal antibodies produced in rabbits. As a clinical research assay, the RIA is rather complex to perform and the ELISA method gives inconsistent results with various serum dilutions. Neither of the detection systems has yet been subjected to extensive collaborative testing for use as a routine technique, therefore the technique will not be described in further detail.

There are several immunodiagnostic methods available for the detection of DSP toxins, configured as either RIA (Levine *et al.*, 1988) or ELISA (Chin *et al.*, 1995; Park, 1995; Uda *et al.*, 1989) tests, all of which incorporate antibodies prepared against a single diarrheagenic agent okadaic acid (OA). The competitive binding [^{3}H]-labelling RIA procedure for OA detection (Levine *et al.*, 1988) is highly sensitive (detection limit: 0.2 pmol OA) and shows no competitive inhibition against a variety of aquatic biotoxins, including maitotoxin, aplysiatoxin, palytoxin, brevetoxin B and lyngbyatoxin A, but the method is too time-consuming and complex to be considered as a routine assay.

Typically ELISA tests configured for OA quantitation show some cross-reactivity with certain other DSP toxins, particularly DTX-1, although the affinity for other analogues may vary markedly. The **DSP-Check** ELISA test kit (UBE Industries, Tokyo, Japan) has been widely used throughout the world for screening DSP toxins (OA and DTX1) at a claimed detection limit of 20 ng/g. However, many investigators have reported inconsistencies including false positive responses when applied to either phytoplankton or shellfish samples. The monoclonal antibody in the **DSP-Check** test kit cross-reacts with DTX1 at a level comparable to OA, but pectenotoxins and yessotoxin are unreactive (Usagawa *et al.*, 1989). The **Rougier Bio-Tech** ELISA test kit utilizes an anti-OA monoclonal antibody and an anti-idiotypic antibody which competes with OA for binding sites on the anti-OA antibody (Shestowsky *et al.*, 1993). The antibody in this test kit exhibits a much higher sensitivity (10-20 fold) for OA than either DTX-1 or DTX-2, and methyl-, diol- and alcohol-derivatives of OA will also bind to the antibody, whereas DTX-3 and brevetoxin-1 (T34) do not cross-react at all. This latter test kit has undergone extensive comparison with alternative analytical methods for DSP toxins such as HPLC and LC-MS, and was found to be rather reliable for OA quantitation in both mussel

extracts and phytoplankton (Chin *et al.*, 1995). Commercial sources for DSP toxin test kits and constituent antibodies are presented in Appendix VI. Protocols for the use of the Rougier **Bio-Tech** ELISA test kit are provided in Appendix II(b) of this chapter.

Initial efforts to develop an immunodiagnostic method for CTX detection in fish tissues were based upon ^{125}iodine-labelled immunoglobulins (Ig) produced from sheep serum and incorporated into a direct RIA test (Hokama *et al.*, 1977). This RIA technique was evaluated as a potential screening technique for ciguatoxic fish and was found to detect CTX in 93% of clinically documented ciguatera cases (Kimura *et al.*, 1982). When configured as an EIA test, the results were similarly encouraging for screening of CTX in fish tissues, and competitive inhibition by a variety of related polyether toxins, including OA, MTX, and PbTx, was indicated (Hokama *et al.*, 1984). The EIA technique using heterologous sheep anti-CTX antibody was eventually incorporated into a simple "poke stick" test for the detection of polyether toxins in intact fish flesh (Hokama *et al.*, 1987), but the test has not been subjected to a rigorous inter-calibration study and the reliability is uncertain. Other work on these sheep immunoglobulins (Berger and Berger, 1979) and on putative CTX antibodies prepared from rabbit serum and mouse ascites (Chanteau *et al.*, 1981) involving enzyme coupling reactions as a detection system (ELISA), failed to confirm antibody activity which correlated with ciguatoxicity. A direct RIA using Ig purified from mouse ascitic fluid (Parc *et al.*, 1979) also revealed severe deficiencies in using polyclonal sera to detect CTX, because of non-specific physicochemical interference with antigen-antibody complexation.

In the most recent development, the **Ciguatect** immunodiagnostic method for ciguatoxins incorporates solid support immunobead technology and is based upon monoclonal antibodies which cross-react with these lipophilic polyether toxins (Park, 1995). Significantly, the chemical similarity in the polyether skeleton of CTX and OA permits for the screening of OA down to a detection limit of 50 pg/g fish tissue. The lack of CTX standards has hampered the determination of relative crossreactivity with various derivatives, therefore, the **Ciguatect** test can only be used as a general screening method to select samples for further analysis. The rate of false positive responses has not yet been determined. Further descriptions of immunoassay and chemical analytical methods for CTX are in Chapter 8.

For research purposes, RIA methods have been developed for the detection of the brevetoxins associated with NSP (Baden *et al.*, 1984; Levine and Shimizu, 1992), and work has also advanced in the preparation of a reliable monoclonal antibody ELISA test for these compounds (Trainer and Baden, 1991). The monoclonal antibodies employed in these immunodiagnostic tests were prepared against brevetoxins (PbTx) but because they also exhibit some cross-reactivity with CTX, they can be used (non-quantitatively) to detect either class of toxins. The RIA technique for PbTX (Baden *et al.*, 1995) is based on the competitive displacement of [^{3}H]-PbTx-3 from complexation with the antibody [see detailed protocol in Appendix II(d)]. In the ELISA method, the same anti-brevetoxin antibodies are coupled with rabbit anti-goat IgG-horseradish peroxidase linked after binding of the primary antibody to the toxin [procedure given in Appendix II(c)].

In initial trials, BSA was linked to PbTx-3 to form the antigen and this serum was found to bind competitively to PbTx-2 and PbTx-3 (Baden *et al.*, 1984). Since the assay is structural rather than functional, the antibody also binds to non-toxic PbTx derivatives with similar binding affinity. When keyhole limpet haemocyanin (KLH) was substituted for BSA, the higher toxin to protein ratios of 75-100 fmol per fmol KLH yielded more efficient antibody production (Trainer and Baden, 1991). Procedures for isolation and purification of the resulting antibody are presented in Appendix VI.

Recent studies on epitopic recognition using naturally occurring and synthetic brevetoxin derivatives with two different anti-PbTx sera indicated that single antibody assays may not be adequate for detecting toxin metabolites (Poli *et al.*, 1995). Tests are being developed to utilize more than one antibody specifically designed for recognition of different regions of the polyether ladder (Melinek *et al.*, 1994).

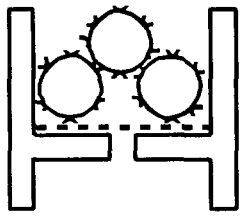
ENZYME-INHIBITION ASSAYS

Functional *in vitro* enzymatic assays for phycotoxin detection are comparatively rare, probably as a reflection of the general paucity of detailed knowledge of the reaction kinetics and key enzymes associated with toxin biosynthesis and metabolism. Nevertheless, Holmes (1991) has successfully exploited the specific inhibition of protein phosphatase Type 1 (PP1) and Type 2A (PP2A) by certain DSP toxin analogues (OA and DTX1) in the development of a phosphatase radioassay using ^{32}P phosphorylase. Although this original technique, which is coupled with toxin fractionation by liquid chromatography, is not in wide circulation as a regulatory tool, it has been used frequently in screening the phosphatase inhibition activity of putatively phycotoxic compounds and partially purified extracts of phytoplankton and shellfish. When applied to the analysis of naturally-contaminated mussel tissue, extracts of cultured *Prorocentrum lima*, and net tow material from natural phytoplankton assemblages, the assay revealed that the PP1/PP2A inhibition activity exceeded that which could be accounted for by total levels of OA and DTX1 - suggesting the presence of cryptic but potentially diarrhegenic toxins (Luu *et al.*, 1993). In its current format, this assay is based on the inhibition of PP1 by OA (Kinoshita *et al.*, 1995) with a sensitivity as low as 10 fg OA/100 g tissue; additional research is continuing on the use of a mutant PP1 which promises to improve the sensitivity of this test by an order of magnitude (Zhang *et al.*, 1994). This enzyme inhibition assay is also useful for the detection of microcystins, a class of phycotoxins produced by certain cyanobacteria (see Chapter 9), and other toxins capable of inhibiting PP1. The complete procedure for this assay is given in Appendix III.

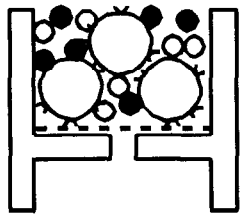
NEURORECEPTOR BINDING ASSAYS

Receptor assays were developed initially to investigate the properties of ion conducting channels, and to characterize the interaction of various ligands with their channel receptors. Despite the variations noted above, many of the marine biotoxins of interest here do share a common characteristic. The toxins responsible for PSP, ASP, NSP and CFP all exert their first order toxic effects by binding to a certain class of biological receptors and (except for ASP which targets the glutamate receptor) these receptors are exclusively either voltage-dependent Na^+ or Ca^{++} channels. It is this highly specific interaction with naturally occurring receptors which forms the basis of the receptor assay approach to the detection of marine biotoxins. In practical terms, any modifications to a toxin molecule which interfere with its binding to the receptor and thus its detection by a receptor-based assay, would also compromise its ability to elicit a toxic response. Detection of a toxin is therefore based on its functional activity rather than on recognition of a structural component, as in the case of an antibody-based assay. Another important component of the toxin-receptor association is the high affinity of this interaction. The receptors that bind marine biotoxins do so with an affinity that matches, or in some cases exceeds, that characterizing antibody-antigen binding. Moreover, the affinity of a toxin for its receptor (binding constants in nM range) is directly proportional to its toxic potency. Thus, for a mixture of toxic congeners, a receptor-based assay will yield a response representative of the integrated potencies of those toxins present.

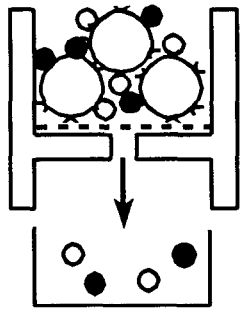
In the case of STX, use of radio-receptor assays has provided a detailed description of toxin binding kinetics and estimates of Na^+ channel densities in excitable membranes. The first attempt to utilize a receptor binding for the detection of STX employed [^3H]-labelled STX in a competitive displacement assay, but was tested only on samples prepared in isotonic buffer and human plasma (Davio and Fontelo, 1984). The method was later modified by Vieytes *et al.* (1993) to increase the sample capacity through the use of microtiter plates, and it was shown to be capable of detecting PSP toxins in shellfish extracts.The STX radio-receptor binding assay is essentially a competitive displacement assay in which radiolabelled and unlabelled STX and/or



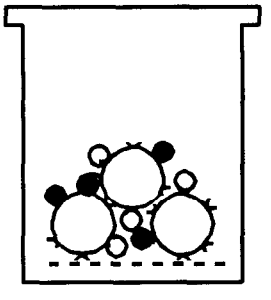
1. Rat brain synaptosome preparation containing receptor sites



2. Incubation of [^{3}H]STX + unlabeled STX standard or sample extract + rat brain synaptosomes



3. Unbound toxin removed by washing and vacuum filtration



4. Filter punched out of plate into vial; [^{3}H]STX bound to receptor sites determined on scintillation counter

Figure 10.3. Schematic diagram of STX radio-receptor assay performed using the Millipore MultiScreen^{TM} Assay System. The view represents a cross-section of a microtiter plate well, with dashed lines indicating the filter membrane. Solid circles show [^{3}H]STX; open circles indicate unlabelled toxin in standard or sample extract (see text and Appendix IV(a) for additional details).

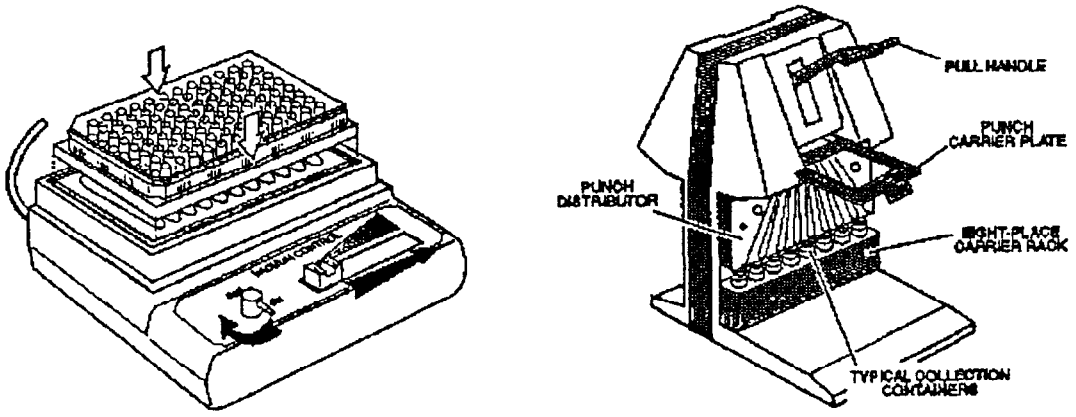


Figure 10.4. Schematic drawing of several components of the Millipore MultiScreen™ Assay System. The vacuum manifold holds specially designed, microtiter filter plates and allows for the simultaneous exchange of solutions in the wells by simple filtration. Filters are transferred to scintillation vials using the multiple punch assembly. The filter plates are set in a carrier, a template of disposable punch tips is placed on top of the plate, and the pull handle is depressed. This action drives one row of punch tips through a row of filters, excising the filters from the plate and allowing them to fall down the punch distributer runways into individual vials held in the collection container. At this point, liquid scintillant is added to the vials in preparation for counting on a scintillation counter [see text and Appendix IV(a)].

Table 10.1. Correlation of Potency with Radioimmunoassay and Synaptosome Assay

Toxin	Toxin concentration (nM)		
	ED_{50} radioimmunoassay†	LD_{50} fish bioassay‡	ED_{50} synaptosomes*
PbTx-1	93(90-130)	4.4 (3.8-5.0)	3.5 (3-5)
PbTx-7	92 (90-120)	4.9 (2.2-10.7)	4.1 (4-7)
PbTx-3	20 (16-25)	10.9 (7.2-22.0)	12.0 (10-15)
PbTx-2	22 (18-29)	21.8 (17.2-35.0)	17.0 (15-18)

†Average of three experiments; procedure from Baden et al. (1984)
‡Median lethal dose (n=3): *Gambusia* fish assay, 20 ml water and 60 min assay time
*Average of three experiments
Ranges are given in parentheses for RIA and synaptosomes; 95% confidence interval for the fish bioassay is given in parentheses

its derivatives compete for a given number of available receptor sites in a preparation of rat brain synaptosomes. The percent reduction in radiolabelled STX binding is directly proportional to the amount of unlabelled toxin present in either a certified reference standard or an unknown sample.

The STX radio-receptor binding assay has been further refined (Charleston Laboratory, U.S. National Marine Fisheries Service) to simplify the protocol and enhance the overall efficiency of the assay (Doucette *et al.*, 1994). This technique, described below in general terms (see Fig. 10.3), has been extensively validated for use with extracts from a variety of shellfish species, and it has performed extremely well in comparisons with the AOAC mouse bioassay. A detailed protocol, including the necessary equipment, supplies and reagents, are given in Appendices IV(a) and VI.

The assay is formatted for use with the MultiScreen™ Assay System (Millipore; Fig. 10.4), and is performed in a microtiter plate with wells occluded by hydrophilic, low protein binding Durapore® membranes, which facilitate the exchange of solutions by vacuum filtration on a manifold designed specifically for these membrane plates. To conduct an assay, a volume of [^{3}H]-STX is added to each well such that its final concentration approximates the dissociation constant, K_D (0.5-5 nM for STX). An equal volume of the reference standard or sample is then added, followed by *ca.* four volumes of rat brain synaptosome preparation. The assay components are incubated, then rinsed with buffer to remove any unbound labelled or unlabelled toxin, and the filters are transferred into scintillation vials using a dedicated punch apparatus (Millipore) that excises simultaneously one row of eight filters. The filters are allowed to stand overnight in liquid scintillant prior to determining the radioactivity retained on the membrane by standard liquid scintillation counting. The quantity of toxin in a sample is obtained from a binding competition curve, which is a semi-log plot of percent total binding of the [^{3}H]-STX against a range of unlabelled STX standard concentrations (Fig. 10.5).

As noted above, the receptor assay response reflects the integrated toxic potency of all PSP toxins (or other Site 1 Na^+ channel blockers; e.g., tetrodotoxin) present in a sample relative to purified STX, and is thus expressed in terms of STX equivalents (STXeq). The limit of detection for this assay is ca. 4 ng STXeq/mL in the original sample extract, which is several orders of magnitude more sensitive than the mouse bioassay. The STX receptor binding assay can generate results highly consistent with those produced by the mouse assay.

Synaptosome binding assays for PbTx and CTX are based on the interaction with site-5 on the voltage sensitive Na^+ channel. A radioassay based upon competitive binding with tritiated brevetoxin ([^{3}H]-PbTx-3)] is described in detail in Appendix IV(b). As with the STX neuroreceptor assay, the extent of binding to the Na^+ channel is directly related to toxicity, therefore the results are comparable to the mouse bioassay. The functional synaptosome binding assay was compared to the RIA and fish bioassay for brevetoxin (Baden *et al.*, 1988) and the results of this comparison are presented in Table 10.1.

Work is progressing towards the use of isolated or reconstituted ion channels as a 'sensor' for detecting voltage sensitive Na^+ channel toxins (Trainer *et al.*, 1995). These techniques promise to improve the sensitivity of the existing bioassay methods while retaining the ability to distinguish between toxin classes (e.g., Na^+ channel activators vs. blockers). An advantage to employing cloned receptors is that the use of vertebrate animals is avoided, and ultimately the problem of obtaining a consistent source of receptors for various assays may be solved.

A competitive receptor binding assay for domoic acid, using frog (*Rana pipiens*) brain synaptosomes, is based upon binding competition with radiolabelled [^{3}H]-kainic acid for the kainate/quisqualate glutamate receptor (Van Dolah *et al.*, 1994). This sensitive method (IC_{50}: 0.89_0.07) shows high promise as a rapid automated assay for domoic acid in a contaminated seafood and toxic phytoplankton samples. However, it is not described here in detail as a routine method because it is still in the latter stages of pharmacological trials.

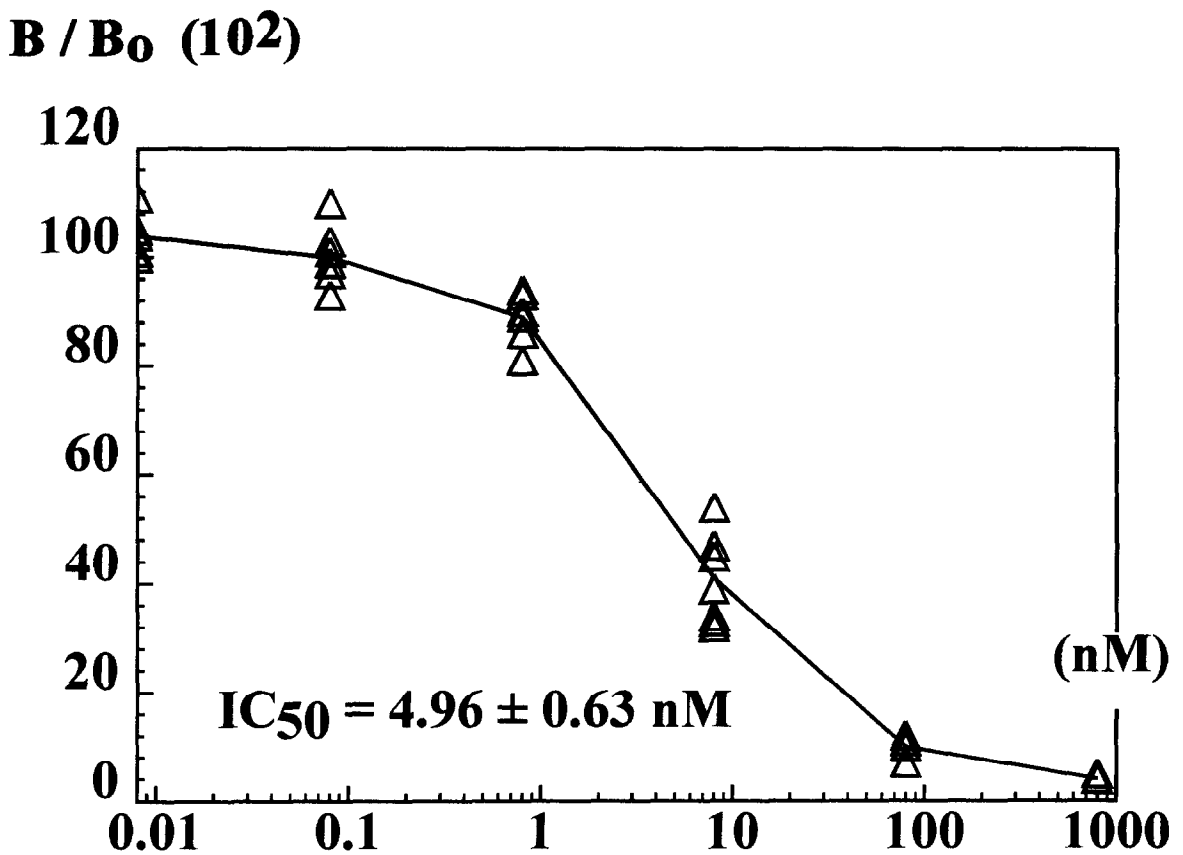


Figure 10.5. Compilation of seven competitive displacement curves, demonstrating low inter-assay variability. Percent total binding of [^{3}H]STX (B/B_0) is plotted against the concentration of unlabelled STX. The STX concentration which yields 50% total binding (i.e., IC_{50}) is *ca.* 5 nM.

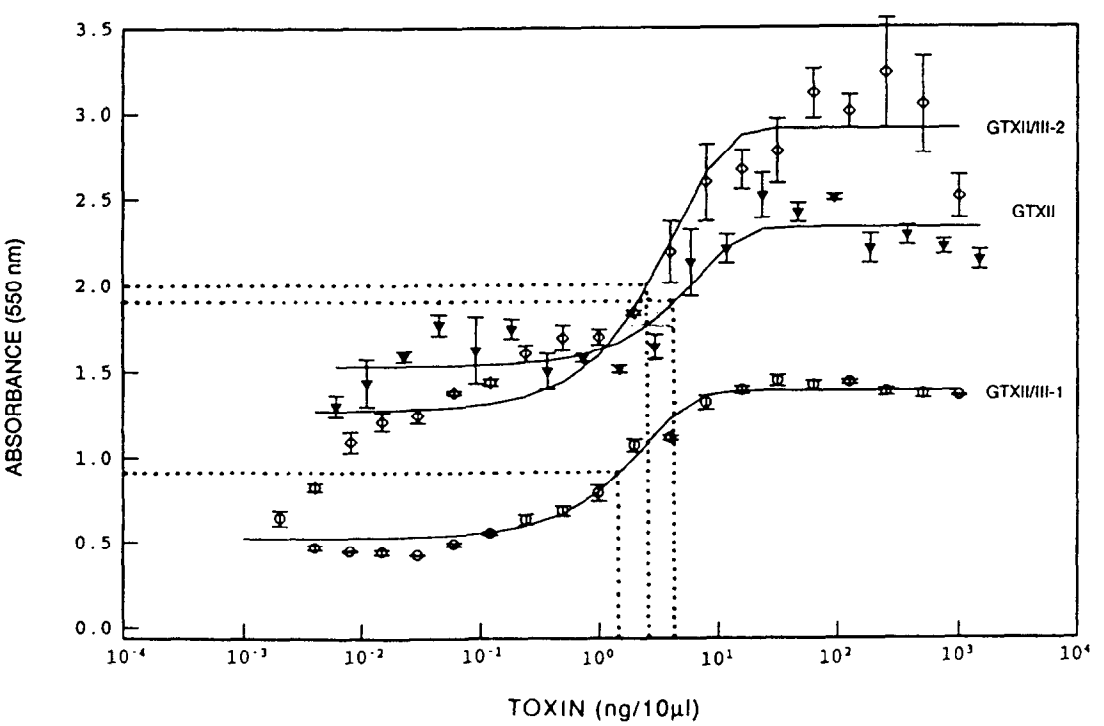


Figure 10.6. Dose-response profiles (absorbance of adherent stained cells± SD *v.* concentration) of pure gonyautoxin-2 (GTX2) and two GTX2/GTX3 mixtures at different molar ratios: (a) 1:2.5 and (b) 4:1. Each of the three profiles was obtained from a different bio-assay. Dotted lines show the EC_{50} concentrations calculated for each preparation.

CYTOTOXICITY BIOASSAYS

Cytotoxicity bioassays for several of the major phycotoxins are now well established in a few laboratories and they show great promise as a rapid screening technique. In general, the quantities of cultured cells required for such bioassays can be readily scaled-up to deal with a sudden increase in demand and the maintenance costs for continuing the cell line are modest. None of these assays has yet been subject to rigorous collaborative testing to warrant certification as an internationally recognized reference method, but inter-laboratory comparisons are underway.

A tissue culture technique using an established mouse neuroblastoma cell line (Neuro-2A; ATCC, CCL131) has been developed for the assay of Na^+ channel blocking toxins (Kogure *et al.*, 1988). The original assay was based upon the microscopic examination of cell morphology and survival after incubation with reagents and putative Na^+ channel blockers. This assay has been subsequently adapted and refined for rapid screening of these toxins by incorporating the use of a scanning spectrophotometer for colorimetric detection in microtiter plates (Gallacher and Birkbeck, 1992; Jellett *et al.* 1992, 1995). A toxicological evaluation using purified reference toxins (Jellett *et al.*, 1995) has indicated that the cellular assay yields EC_{50} values for STX, NEO, GTX2 and epimerized mixtures of GTX2/GTX3 (Fig. 10.6) which correlate quite well with their specific toxicity as determined by intraperitoneal mouse assays.

The method is now commercially available as the **MIST™** technology [see Appendix V(a) for description of the protocol] and requires only microliter quantities of sample for analysis. The two alkaloids ouabain and veratridine when added in combination to the neuroblastoma cell culture increase the influx of Na^+ ions into the cells causing swelling and cell death. However, if a Na^+ channel blocking agent is introduced, the antagonistic effect prevents Na^+ ion influx and the cells remain morphologically normal and in good growth condition. After staining with crystal violet, the intensity of the color reaction is proportional to the quantity of Na^+ channel blocker present. An alternative assay based upon the same principle, which was developed for the detection of Na^+ channel blocking activity in bacterial culture supernatant, but could be readily reconfigured for detection of these toxins in algal or shellfish extracts, employs Neutral red as the vital stain (Gallacher and Birkbeck, 1992). These structural assays target a specific subcellular unit (site-1 Na^+ channel), thus the method is highly specific and sensitive for channel blocking agents which bind to this site (e.g., PSP toxins or tetrodotoxin).

Recent developments have extended this tissue culture assay to the site-5 Na^+ channel activating toxins (PbTx and CTX) (Manger *et al.*, 1993, 1995). The cytotoxicity assay detection limit for PbTx is 250 pg and for purified CTX the sensitivity is in the low picogram or even sub-picogram range. Sodium channel activating toxins such as PbTx and CTX can be detected in 4 to 6 h, but the sensitivity can be enhanced with incubation for 22 h. Cytotoxicity is assessed using a colorimetric detection system, whereby active cells reduce a tetrazolium compound (MTT) to a blue formazan product (Manger *et al.*, 1995). Other cytotoxicity methods have used XTT (a water soluble formazane reagent) for colorimetric determination of cell survival (Yasumoto *et al.*, 1995).

Cell cultures assays have also proven useful in screening for DSP toxicity in extract of putatively toxic algae and shellfish. In the ciliate assay, *Tetrahymena pyriformis* cells are exposed by adding partially-purified DSP extract to cultured cells and incubating for 24 hours (Shiraki *et al.*, 1985). The concentration-dependant inhibitory effects on growth and acid phosphatase activity of the ciliate are used as an index of DSP toxicity.

In an alternative assay, freshly prepared rat hepatocytes are exposed to a lipid DSP toxin extract or to purified toxins, and the resultant damage to cell morphology (e.g., "blebbing" and surface irregularities) are observed microscopically (Aune *et al.*, 1991). The rate of release of lactate dehydrogenase can also be measured. Exposure to purified toxins yields distinctive results: OA and DTX1 cause severe blebbing and surface changes, whereas pectenotoxin-1 induces loss of microvilli and yessotoxin yields formation of only tiny blebs.

A quantitative cytotoxicity bioassay using a human cell line (KB) derived from an epidermoid carcinoma was shown to be effective in detecting OA in mussel samples at a lower sensitivity limit of 50 ng OA/g digestive gland tissue in a 24 h endpoint assay (Tubaro *et al.*, 1995). This dose-dependent cytotoxicity assay is based upon the metabolic conversion of a tetrazolium dye (MTT) to yield a blue-coloured formazan reaction product which can be read for absorbance with a microplate scanning spectrophotometer.

APPENDICES

The appendices contain basic protocols for the determination of phycotoxins by *in vivo* diagnostic methods described in the text. To avoid duplication, certain complementary information on the availability of reference materials and reagents may be found in Chapters 5, 6, 7 and 8.

APPENDIX I: EXTRACTION METHODS AND SAMPLE PREPARATION

This section provides guidance on the extraction and preparation of phycotoxins for assay by *in vitro* biochemical or cytological methods. Since most of these *in vitro* assays are validated and compared with alternative analytical techniques and mammalian bioassays by screening extracts prepared by standardized protocols, such as the AOAC (1984) method for PSP toxins and the procedure of Lee *et al.* (1987) for DSP toxins, the detailed procedures can be obtained in Chapters 5, 6, 7 and 8. Only special notes and extraction procedures not referenced elsewhere in this manual are given here.

a) Extraction and Preparation of PSP Toxins for *in vitro* assays

i) Shellfish Samples for PSP Toxin Synaptosome Binding Assay

As for the cytotoxicity assays and immunodiagnostic techniques, shellfish extracts prepared according to standard AOAC (1984) procedures can be used in the synaptosome binding assay with no "clean-up". However, it is *IMPORTANT* that the pH of the samples is not lower than 3.0 - 3.5, since this can result in a *false positive* response. Furthermore, as much particulate material as possible should be removed either by centrifugation and/or filtration prior to performing the assay, otherwise the filter plate will clog rapidly.

ii) Phytoplankton Samples for PSP Toxin Immunoassay

This method was developed for use with the **SAXITOXIN TEST**[R] immunoassay, but provides further details and modifications not available in the original publication (Cembella and Lamoureux, 1993).

1. When preparing phytoplankton samples for extraction mix thoroughly by swirling to homogeneously suspend cells. With natural phytoplankton assemblages (e.g., from net tows) determine the species composition and obtain cell counts of each putatively toxic species (e.g., *Alexandrium*, *Gymnodinium catenatum* and *Pyrodinium*) in the cell concentrate. Where toxic species are not present in chains of cells, the net tow concentrate may be sieved (150 μm mesh) to remove zooplankton. For either natural plankton samples or cultured cells, perform microscopic cell counts in duplicate using a Palmer-Maloney or Sedgewick-Rafter counting chamber (100X magnification). Calculate and record the volume

of plankton suspension required to give *ca.* 1 X 10^5 cells of the potentially toxic species and harvest by centrifugation. As a pre-centrifugation step, a 20 µm mesh Nitex net may be used for cell concentration, instead of the initial centrifugation described in step 2.
2. Centrifuge suspension in a large volume (50 or 250 mL) conical bottom plastic centrifuge tube at low speed (5,000 X *g*) for 15 min in a refrigerated centrifuge to collect pellet. Carefully remove the supernatant by aspiration taking care not to disturb the pellet, using a long Pasteur pipette.
3. Resuspend the pellet in 5 mL cold seawater, transfer to a 15 mL conical bottom centrifuge tube and re-centrifuge for 5 min at 5,000 X *g* or until a solid pellet is formed. Remove the supernatant carefully with a Pasteur pipette and freeze the pellet (<-30°C) for future extraction or extract immediately.
4. For extraction, add 3.0 mL of 0.1 M acetic acid to the fresh or frozen cell pellet and sonicate with an ultrasonicator at maximum intensity (10 X 10 sec bursts) using a microtip probe. Immerse the bottom of the centrifuge tube in an ice-ETOH bath for cooling.
5. Confirm by microscopic examination that the cells are completely disrupted. If not, repeat the sonication as in step 4.
6. Centrifuge the sonicated extract for 10 min @ 10,000 X g and collect the supernatant.
7. Pass supernatant through a 13 mm (0.45 µm) filter (HAWP Millipore or polycarbonate equivalent) mounted on a 5 mL Luer-lock syringe.
8. Retain the filtrate in a cryovial and store at 4°C prior to assay. Depending upon the toxicity, samples may require dilution of 1:10 - 1:1000 in buffer supplied with the immunoassay kit.
9. To improve quantitation with kits based upon anti-STX antibodies, sub-samples (200 µL) may be heated in sealed glass reaction vials in a heating block at 100°C for 10 min after addition of 1:10 volume of 1 M HCl. This treatment hydrolyzes the N-sulfocarbamoyl toxins to carbamate derivatives, thereby enhancing crossreactivity.

b) Extraction and Preparation of Lipid-Soluble Phycotoxins for *in vitro* assays

Extraction of shellfish tissue samples for DSP methods typically follows a procedure developed by Lee *et al.* (1987) (see also Chapter 6). This method uses extraction with 80% aqueous methanol followed by centrifugation at 3,000 x *g* for 15 min. The supernatant is stored at -20°C until needed. For ELISA analysis, samples must be diluted to reduce the methanol concentration, but further clean-up, as is necessary prior to instrumental (e.g., HPLC) analysis, is not required. For samples prepared according to the method of Lee *et al.* (1987), dilute extracts 1:1 with dH_2O for a final concentration of 40% aqueous methanol. Use 40% methanol in Tris-buffered saline (TBS) for all subsequent sample dilutions.

For the protein phosphatase inhibition assay for DSP toxins and microcystin-LR, shellfish tissue samples, homogenize in phosphate buffered saline (PBS) or in 80% aqueous methanol. Centrifuge for 15 min at 5000 x *g*. Dry supernatant in vacuo, washing 3 times with acetone. Use 1 g equivalent of tissue per microtiter plate well.

The American Public Health Association (APHA) extraction procedure for shellfish tissue was recently compared with an alternative protocol developed for extraction of a novel NSP toxin (Hannah *et al.*, 1995). The APHA method (Irwin, 1970; Table 4.4) uses a diethyl ether extraction, whereas the Hannah (1995) method involves an acetone extraction followed by a dichloromethane partition. Recovery (based on mouse bioassay toxicity) was consistently higher using the acetone extraction than with the APHA method.

For ELISA assay of dinoflagellate samples for DSP toxins, extract concentrated biomass with 100% methanol at a 10 + 1 (v/w) solvent-to-tissue ratio (see also Chapter 6 for alternative). Sonicate sample (or shake extensively on a Vortex mixer) and centrifuge as above. For analysis, dilute extracts 1:2.5 with TBS. Use 40% methanol prepared in TBS for all subsequent sample dilutions.

APPENDIX II: IMMUNOASSAYS

a) ELISA for PSP Toxins

The details of the use of the rapid kit formats for the **SAXITOXIN TEST**^{R} (Institut Armand-Frappier) and **RIDASCREEN**^{R} (R-Biopharm GmBH) are provided in the instructional literature supplied with the kits.

b) ELISA for DSP Toxins

This assay is based on a competitive displacement reaction with mouse anti-OA anti-idiotypic 1/59 monoclonal antibody (mAb) competing for the same binding sites as OA on anti-OA 6/50 mAb. Bound anti-OA 6/50 mAb is determined via colorimetric detection using an enzyme-conjugated secondary antibody.

Reagents:

1. Extraction solvents
   80% aqueous methanol
   100% aqueous methanol
2. Dilution solvent
   40% methanol in Tris-buffered saline (TBS)
3. Monoclonal Antibodies (mAb)
   Anti-okadaic acid 6/50 mAb (100 ng/mL) in 40% methanol
   Anti-okadaic acid anti-idiotypic 1/59 mAb (5 µg/mL) in 0.05 M Na citrate buffer, pH 9.6 (From Calbiochem, LaJolla, CA or produced according to Shestowsky *et al.*, 1993)
4. ELISA Wash Solution
   0.05 % Tween-20 in TBS (v/v)
5. Blocking Agent
   1 % milk in TBS
6. Substrate Peroxidase-conjugated anti-mouse IgG Fc fragment-specific antiserum (Sigma Chemical Co., St. Louis, MO) in 1% milk in TBS
7. Color Reagent
   0.03% H_2O_2 in 0.1% o-phenylenediamine (OPD) (Sigma) and 0.1 M Na citrate buffer (pH 7.0)
8. Stop Reagent
   3 N H_2SO_4 (Fisher)
9. OA standard solutions
   0-1 µg/mL in 40% methanol

Procedure:

1. Plate preparation: Coat microtiter plates (e.g., Immulon I, Dynatech Laboratories, Chantilly, VA) overnight at 4°C with anti-idiotypic 1/59 IgG (5 µg/mL) in 0.05 M Na_2CO_3-$NaHCO_3$ buffer, pH 9.6.
2. Wash off unbound antibody with Wash Solution.
3. Saturate plate with Blocking Agent to block any remaining sites. Incubate 1 hour at 37°C. Wash plate.
4. Add 50 µL 6/50 IgG and 50 µL standard or sample. Incubate 1 hour at 37°C. Wash Plate.
5. Add 100 µL Substrate to each well. Incubate 1 hour at 37°C. Wash Plate.
6. Add 100 µL Color Reagent. Cover plate. Incubate 30 min at room temperature.
7. Add 50 µL Stop Reagent.
8. Measure absorbance at 492 nm on a microplate reader.
9. Data Analysis. Correlate concentration and optical density using linear regression analysis.

c) ELISA for NSP and CFP Toxins

To date, the ELISA test kit has not been demonstrated for fish or shellfish tissue samples. Therefore, it is recommended that this test procedure only be used for dinoflagellate extracts.

Reagents:

1. Phosphate buffered saline (PBS)-Blotto, pH 7.2,
   10 mM Na_2HPO_4
   0.15 M NaCl
   40 g/L nonfat dry milk [alternative: 0.25% bovine serum albumin (BSA)]
   0.01% NaN_3 (sodium azide)
2. ELISA wash buffer, pH 7.2
   0.5 M NaCl; 20 mM Na_2HPO_4
3. Carbonate Buffer, pH 9.6
   0.1 M $NaHCO_3$
4. Horseradish Peroxidase (HRP) Substrate
   20 mL 0.1 M Na citrate, pH 4.2
   1 mL 40 mM ABTS (2,2'-azino-bis-2-ethylbenzthiazoline-6-sulfonic acid)
   20 µL 30% H_2O_2
5. Alkaline Phosphatase (AP) Substrate
   100 µL BCIP (5-bromo-4-chloro-3-indolyl phosphate) (50 mg/mL stock in dimethyl formamide, DMF); store at 4°C
   200 µL NBT (nitro blue tetrazolium) (200 mg in 2 mL DMF, 2 mL distilled H_2O; store at 4°C)
   30 mL Tris buffer: 0.1 M Tris base, pH 9.5; 0.1 M NaCl; 50 mM $MgCl_2$
6. SDS (sodium dodecyl sulfate), 10%
7. Goat anti-PbTx antibody purification for this ELISA is as in the RIA described below.
8. Rabbit anti-goat serum linked to HRP (Accurate Chemical Corp., Westbury, NY).

Procedure:

This assay protocol follows a four step procedure illustrated in Trainer and Baden (1991):

1. Adsorption of sample to microtiter plate.
   a. Add serial dilutions of sample (100 µL) to 96-well microtiter plates and allow to equilibrate overnight at 4°C or for 2 h at room temperature in PBS buffer. Cover plates with Saran wrap.
   b. Rinse plate 3 times with 200 µL ELISA wash buffer in each well.
2. Non-specific blocking of additional sites.
   Block wells 1 hour with 100 µL PBS-Blotto. Cover plate with Saran wrap. Other acceptable blockers are 1% pre-immune serum and 1% gelatin (Knox, unflavored). Rinse as in step 1b.
3. Specific binding of primary and secondary antibodies to toxin.
   a. Incubate >1 h with primary goat antibody diluted in PBS-Blotto (100 µL in each well). Cover plate with Saran wrap. Rinse as in step 1b.
   b. Incubate with diluted secondary antibody (rabbit anti-goat linked to HRP at a 1:1000 dilution) in PBS-Blotto for at least 1 h (100 µL per well). Cover plate with Saran wrap. Rinse as in step 1b. Suggested dilution factors are 1:500-1:4000 for HRP-linked antibodies and 1:2000-1:5000 for AP-linked antibodies. Optimal dilutions can be determined using a checkerboard assay.
4. Color Development and Measurement.
   a. Add 100 µL of appropriate enzyme substrate into each well. Develop for about 15 min in the dark. HRP antibody is visualized with ABTS solution.
   b. Stop reaction after about 30 min by adding 25 µL of 10% SDS per well.
   c. Measure absorbance at 405 nm.
5. For data analysis, correlate concentration and optical density using linear regression.

Notes: All reaction rates can be increased by increasing incubation temperatures. Blocker (PBS-Blotto) should only block nonspecific binding. Too high a concentration of blocker may also block specific interaction. Washes should use a greater volume of buffer in each well than the previous incubation step. Plate should be developed in the dark since all dyes used are light sensitive.

d) RIA for NSP and CFP Toxins

Antibody Preparation/Purification

Antigens are constructed using keyhole limpet haemocyanin (KLH) as a carrier protein employing procedures outlined in Baden *et al.* (1984), Trainer and Baden (1991), Levine and Shimizu (1992). Antibodies may be produced in either goats or rabbits. The following procedures for antibody preparation and purification are applicable for serum processing after bleeding the animal.

Ammonium Sulfate Precipitation

1. Dilute plasma from bleeds with an equal volume of saturated neutral ammonium sulfate, refrigerate overnight, and centrifuge at 3000 x *g* for 30 min.
2. Save the supernatant solution and discard the pellet. Add an equal volume of ammonium sulfate again to the supernatant. This results in a precipitate which is supernatant by centrifugation at 5000 x *g* for 1 h.
3. Re-dissolve the pellet in 0.3 volumes of PBS, pH 7.4, and dialyze against 3 changes of PBS overnight, then freeze at -20°C until needed.

Protein G Purification

Wash Buffer

0.01 M Na_2HPO_4
0.15 M NaCl
0.01 M EDTA, pH 7.0

Elution Buffer

0.5 M acetic acid, pH 3.0

1. Use a preparative HPLC pump connected to a protein G column (Genex GammaBind Plus) for this step. Equilibrate the column with Wash Buffer and load the dialyzed ammonium sulfate-precipitated antibody.
2. Wash the adsorbed IgG with 10 column volumes of Wash Buffer.
3. Elute the antibody with approximately 1 column volume of Elution Buffer and immediately neutralize with 1 M Tris base.
4. Dialyse eluted IgG at 4°C against 3 changes of PBS overnight, and lyophilize in 100 mL aliquots, then store at -20°C until needed.

Brevetoxin Affinity Chromatography

1. Synthesize PbTx-3-succinate via the method given in Baden *et al.* (1984a).
2. Wash 5 g Aminohexyl-Sepharose with 3 x 50 mL of dH_2O, followed by 3 x 50 mL rinses with 50% pyridine. Add five mL of the resulting slurry to 300 µmol EDC in 0.5 mL 50% pyridine. Swirl the mixture for 2 h at room temperature.
3. Add 9.9 mg PbTx-3-succinate in 1 mL 50% pyridine, dH_2O, and PBS, pH 7.4 (NO azide!!!). Store in the refrigerator.

Antibody Purification

1. Wash the affinity column with 10 column volumes each of: a) 10 mM Tris, pH 7.5; b) 10 mM glycine, pH 2.5; c) 10 mM Tris, pH 8.8; and d) 10 mM Tris, pH 7.5
2. Load the protein G-purified IgG onto the column in 10 mM Tris, pH 7.5. Recirculate 3 times through the column. Rinse with 10 mM Tris, pH 7.5 until no more protein is eluted (monitored by Bradford assay).

3. Elute the sample using 10 column volumes of 0.1 M glycine, pH 2.5 (3 M NaCl in PBS, pH 7.4 has also worked well). Neutralize eluted IgG with 1 M Tris base, dialyze against 3 changes of PBS overnight and store in 1 mL aliquots at -70°C.
4. Regenerate the column by repeating step 1.

Reagents:
1. Phosphate buffered saline (PBS), pH 7.4 - for 1 L stock solution:
   1.392 g K_2HPO_4
   0.276 g NaH_2PO_4
   8.770 g NaCl
   1 g NaN3 for a 0.1% solution for storage
   Dissolve in 900 mL distilled H_2O and adjust pH to 7.4 using KOH. Bring vol. to 1L.
2. Dextran coated charcoal (100 mL):
   10 g neutral charcoal
   0.25% dextran in PBS *Note: The working mixture is a 1:10 dilution of this stock in PBS. Keep on ice and stir constantly.

Procedure:
1. Add 0.2 mL toxin/sample to 1.5 mL microcentrifuge tubes.
2. Add 0.2 mL [^{3}H]PbTx-3 to centrifuge tubes. Use a serial dilution in triplicate (10, 1, 0.5, 0.25, 0.125, 0.0625 nmol)
3. Add 0.2 mL PBS to centrifuge tubes. Also set up one triplicate sample with 10 µM cold PbTx-3 instead of PBS for determination of non-specific binding.
4. Add 0.1 mL of antibody (1.5 mg protein).
5. Close tubes and invert to mix.
6. Incubate 1 h on ice.
7. Add 0.5 mL dilute charcoal to each tube, then close, invert and mix. Incubate 2.5 min.
8. Centrifuge at maximum speed in the micro-centrifuge for 2.3 min.
9. Assay supernatant solution for radioactivity.
10. Data Analysis

Notes: Free toxin should be bound within the dextran-charcoal matrix. Toxin bound to antibody remains in the supernatant.

APPENDIX III: ENZYME ASSAYS

a) Protein Phosphatase Assay for DSP Toxins and Microcystin-LR

Reagents:
1. rPP1
   0.06 mg/mL recombinant protein phosphatase type-1 in 50% glycerol
2. pNPP (p-nitrophenyl phosphate) substrate (20 mM)
   20 mM $MgCl_2$
   1 mM 2-mercaptoethanol (or 1 mM dithiothreitol)
   50 mM Tris-HCl, adjust pH to 8.5
3. Okadaic acid
   Standard solutions prepared to range from 10 - 1000 µM
4. Microcystin-LR Standard solutions prepared to range from 1 - 1000 nM

Procedure:
1. Place 1 g equivalent of tissue sample extract or aliquot of standard in microtiter plate well.
2. Add 10 µL of rPP1 solution and incubate for 5 minutes.

3. Add 100 µL of pNPP substrate.
4. Read absorbance at 405 nm immediately for use as a blank measurement.
5. Incubate plate for several minutes for sufficient color development.
6. Measure absorbance at 405 nm.

Data Analysis:
1. Subtract blank measurement (step 4 above) from all readings (if not done automatically in step 6).
2. Absorbance will decrease with increasing inhibitor (e.g., OA) concentration. Calculate the relative decrease in absorbance (% activity).
3. Plot %activity vs. toxin standard concentration and analyze using linear regression. Solvefor unknown sample concentration from the resulting linear curve.

APPENDIX IV: NEURORECEPTOR BINDING ASSAYS

a) Synaptosome Binding Assay for PSP Toxin (STX)

This neuroreceptor assay is a competitive binding assay in which radiolabeled STX competes with unlabelled STX and/or its derivatives for a given number of available receptor sites in a preparation of rat brain synaptosomes. The percent reduction in radiolabeled STX binding is directly proportional to the amount of unlabelled toxin present in either a certified reference standard or an unknown sample. Acidic, aqueous shellfish extracts are prepared for testing according to the AOAC (1984) method.

Reagents and Supplies:
1. [^{3}H]STX (Amersham, Arlington Heights, IL, U.S.A.; cat. no. TRK 877)
2. STX Reference Standard (NRC/Inst. for Marine Biosciences, Halifax, N.S., Canada)
3. 75 mM HEPES/140 mM NaCl buffer (35.75 g HEPES + 16.36 g NaCl in 2 L), pH 7.5
4. Rat brain synaptosome preparation (protocol below)
5. Scintiverse liquid scintillation cocktail
6. Multichannel pipette and pipette tips
7. 96 well microtiter filter plate with 0.65 mm Duropore membrane and type C glass fiber filter (Millipore, Bedford, MA, U.S.A.; cat. no. MAFC NOB 50)
8. MultiScreen vacuum manifold (Millipore; cat. no. MAVM 096 01)
9. MultiScreen disposable punch tips (Millipore; cat. no. MADP 196 10)
10. MultiScreen punch kit-B for 4 mL vials (Millipore; cat. no. MAPK 896 0B)

Preparation of Stock Solutions and Standards:
1. [^{3}H]STX stock solution. The following is an *example* for commercially available [^{3}H]STX with this information included on the specification sheet: specific activity - 39 Ci/mmol, volume - 200 mL, amount of radioactivity - 50 mCi.

   50 mCi/(39 mCi/nmol) = 1.28 nmol; STX concentration = 1.28 nmol/0.2 mL = 6.40 mM

   The desired working stock concentration of [^{3}H]STX is 5.0 nM, with a final assay concentration of 0.84 nM; to make up enough of 5.0 nM [^{3}H]STX for one 96 well plate, add 3 mL [^{3}H]STX to 3.84 mL of HEPES/NaCl buffer.

2. Unlabelled STX solutions (i.e., standard curve and inter-assay calibration standard). Make up STX Reference Standard to a concentration of 1.00 x 10^{-4} M. Perform the following dilutions to construct the standard curve:

	Conc. of standard*	Conc. in assay
45 mL 100 mM STX + 705 mL HEPES/NaCl buffer	6.00×10^{-6}	1.0×10^{-6}
50 mL 6.00×10^{-6} solution + 450 mL buffer	6.00×10^{-7}	1.0×10^{-7}
100 mL 6.00×10^{-7} solution + 233 mL buffer	1.80×10^{-7}	3.0×10^{-8}
50 mL 6.00×10^{-7} solution + 450 mL buffer	6.00×10^{-8}	1.0×10^{-8}
50 mL 1.80×10^{-7} solution + 450 mL buffer	1.80×10^{-8}	3.0×10^{-9}
50 mL 6.00×10^{-8} solution + 450 mL buffer	6.00×10^{-9}	1.0×10^{-9}
50 mL 6.00×10^{-9} solution + 450 mL buffer	6.00×10^{-10}	1.0×10^{-10}
Reference	Buffer only	0

*All standards are diluted 1:6 in the assay.

An inter-assay calibration standard containing 3.0×10^{-8} M STX standard (5.0×10^{-9} M STX in assay) made up in 0.05 M acetic acid and stored frozen in 100 mL aliquots should be thawed at the time of the assay and included in *each* analysis (duplicate wells) in order to confirm day-to-day performance.

3. Rat brain synaptosomes: Dilute the stock rat brain synaptosome preparation by 1/4 in ice cold 75 mM HEPES/140 mM NaCl (pH = 7.5) to yield a final protein concentration of about 0.5 mg/mL.

Synaptosome Preparation:

1. Purchase 20 brains of six week old, male Holtzman rats (Harlan Bioproducts, Indianapolis, IN, US; cat. no. BT 403)
2. Remove medulla and discard; place each cerebral cortex in 12.5 mL of 20 mM Tris/140 mM NaCl (pH = 7.1) on ice.
   20 mM Tris/140 mM NaCl:
   2.422 g Tris
   8.18 g NaCl
   1 mL 1.0 M PMSF* (phenylmethylsulfonyl fluoride; Sigma, cat. no. P7626)
   1 L ddH_2O, adjust pH to 7.1
   *dissolve 0.174 g PMSF in 1 mL isopropanol; larger volumes may be made, dispensed into 1 mL aliquots, and stored at -20°C.
3. Homogenize cortices with motorized teflon/glass homogenizer 20 times. It is very *IMPORTANT* to keep each preparation *ON ICE* during the homogenization step. Repeat this procedure for each brain and pool the homogenized tissue.
4. Homogenize the pooled sample with motorized teflon/glass homogenizer 10 times, being careful to keep preparation *ON ICE*.
5. Place pooled homogenate in ultracentrifuge tube(s) always keeping prep *ON ICE*, and centrifuge at 54,000 x *g* for 15 min at 4°C.
6. Pour off supernatant and resuspend pellet(s) with 10 mL ice cold Tris/NaCl buffer per brain (i.e., 200 mL total volume).
7. Pool pellet/buffer suspension in a beaker and homogenize using a probe homogenizer at 70% full speed for 20 s, holding preparation *ON ICE* at all times.
8. Dispense synaptosome preparation into 2 mL aliquots and freeze immediately at -80°C; this preparation is stable for at least six months.

Procedure:

1. Add the following to each of 96 wells *in the order as given*:
   35 mL [^{3}H] STX
   35 mL STX standard or sample
   135 mL synaptosome preparation
   Note: arrange samples vertically in the plate to simplify organization after filters are punched into vials.
2. Incubate for 1 h at 4°C.

3. Filter the entire plate on top of the MultiScreen vacuum manifold.
4. Rinse each well once with 200 mL of ice-cold HEPES buffer using multichannel pipette.
5. Remove the plastic bottom of the 96 well filter plate, blot bottom of plate once on absorbent towel, set plate into the MultiScreen punch, and place the disposable punch tips on top of the plate.
6. Punch wells into the vials pre-filled with about 4 mL Scintiverse liquid scintillant. Put caps on vials and vortex each sample.
7. After allowing vials to sit at room temperature overnight, count in standard liquid scintillation counter (results are more easily tabulated if replicates are counted in a consecutive fashion).
8. Data analysis.

Sample Value Calculations:

The concentration of PSP toxins (in nM STX equivalents) in samples is determined from a competitive binding curve. If the data are graphed as a semi-logarithmic plot of percent total binding of [^{3}H]STX against the concentration of unlabelled STX reference standards, the curve is typically sigmoidal (Fig. 10.5). Thus, it is *essential* to transform the percent total binding data using a logit transformation, and graph the resulting values on a linear y-axis scale against a logarithmic x-axis scale of unlabelled STX concentration. The logit transformation is performed as follows: logit (y) = ln $[(B/B_0)/(1-B/B_0)]$, where B/B_0 = percent total binding; = total counts for a given unlabelled STX standard/total counts for Reference containing no unlabelled STX. Once the data points are plotted, a line of best fit is generated using a simple linear regression. The STX concentrations for the sample unknowns are then obtained by solving the regression equation for x using the logit transformation of B/B_0 for the sample (y).

Note: the displacement curve can also be generated by a computer program such as LIGAND (McPherson, 1985), which will also calculate STX equivalent concentrations for sample unknowns. That part of the curve falling between 30% and 70% total binding provides the most accurate determination of STX concentrations in unknown samples. Those samples yielding percent total binding values outside of this range should be diluted appropriately and re-run. Final concentrations for unknown samples are determined by multiplying the calculated in assay value by 6.0 (i.e., the assay dilution factor) and any sample dilution factors used. The slope of the standard curve should optimally be 1.0. While this is often not achieved, it is *IMPORTANT* that the slope of the regression line fall between 0.8 and 1.2. Otherwise, the sample values obtained will be of questionable reliability.

b) Synaptosome Binding Assay for NSP and CFP Toxins

Note that there are close similarities between this assay and the procedure given above in the STX binding assay. Data analysis for the following methods can be performed with reference to the above procedure for STX binding.

Reagents:

1. Standard binding medium (SBM) 50 mM HEPES (pH 7.4)
   - 130 mM choline chloride
   - 5.5 mM glucose
   - 0.8 mM magnesium albumin
   - 0.01 % Emulphor EL-620 (non-ionic detergent as emulsifier)
   - 1 mM iodoacetimide
   - 0.1 mM PMSF (phenylmethylsulfonyl fluoride)
   - 1 mM 1,10-phenanthroline
   - 1 fM pepstatin A
   - 1 mM EGTA
   - make with and without 1 mg/mL BSA
2. Wash Medium 163 mM choline chloride
   - 5 mM HEPES (to pH 7.4 with Tris base)
   - 1.8 mM $CaCl_2$

0.8 mM $MgSO_4$
1 mg/mL BSA

3. Brain Homogenization Buffer (BHB) 0.32 M sucrose
0.005 M Na_2HPO_4 (pH 7.4 with phosphoric acid)
1 mM iodoacetimide
0.1 mM PMSF
1 mM 1,10-phenanthroline
1 μM pepstatin A

Synaptosome Preparation:

1. Homogenize 2 or 3 Sprague-Dawley rat brains at a time using 5 mL of BHB per brain.
2. Centrifuge homogenate for 10 min. at 3,000 x *g*. Save supernatant on ice and re-homogenize pellet.
3. Centrifuge re-homogenized pellet for 10 min. at 3,000 x *g*.
4. Combine supernatant solutions and centrifuge for 60 min at 12,000 x *g*.
5. Discard supernatant and resuspend pellet in a minimal amount of SBM without BSA.
6. Use microtiter protein assay to determine protein concentration of final solution.
7. Store synaptosomes in -80°C freezer. At this temperature synaptosomes are stable for use in binding assays for months.

Procedure:

1. Add 0.4 mL toxin/sample to 1.5 mL microcentrifuge tubes. Use a serial dilution of standard for a 12 point calibration curve (10,000, 1,000, 500, 250, 100, 50, 25, 10, 5, 2.5, 1, 0 nM)
2. Add 0.5 mL of 2 nM [^{3}H]PbTx-3 to centrifuge tubes.
3. Add 0.4 mL SBM to centrifuge tubes. Also set up one triplicate sample with 10 μM cold PbTx-3 instead of SBM for determination of non-specific binding.
4. Add 0.1 mL of synaptosomes (at 0.5 - 1.0 mg/mL protein to give a final protein concentration of 50 - 100 μg/mL).
5. Close tubes and invert to mix, then incubate 1 h on ice.
6. Centrifuge at maximum speed in the micro-centrifuge.
7. Assay 0.1 mL of supernatant, carefully aspirate the remainder.
8. Rinse pellet with 3 drops of ice-cold wash medium, aspirate.
9. Clip bottom of tube containing the pellet into scintillation vial. Add scintillant, incubate overnight.
10. Vortex vigorously the next day and count in scintillation counter.
11. Data Analysis

APPENDIX V: CYTOTOXICITY ASSAYS

a) Neuroblastoma Assay for PSP Toxins

This functional cellular assay is based on the cytotoxicity of the Na^+ channel blocking toxins, (e.g., PSP toxins and tetrodotoxin). The procedure described here is employed in the **MIST™** kit for routine clinical analysis, but other minor versions are used experimentally in other laboratories. Careful standardization of procedures and meticulous aseptic technique is required, therefore the assay is best performed in a properly-equipped tissue culture laboratory. Some clinical investigators using variants of the neuroblastoma assay have stressed the importance of employing batch-standardized serum and titrating the alkaloids ouabaine and veratridine to achieve maximum reproducibility (S. Gallacher, pers. commun.). The cells are available through Jellett Biotek Ltd. (see Appendix VI) as a kit, which also contains all the other reagents required to perform the bioassay. The **MIST™** Staining Kit may be obtained separately. This eliminates the need for tissue culture expertise and facilities. The cells are shipped on ice, ready

for bioassay, and have a shelf life of 4-5d. Jellett Biotek Ltd. also provides PSP testing services using the **MIST™** Cell Bioassay.

Once revived from cryopreservation, the neuroblastoma cells must be maintained in exponential growth in small tissue culture flasks with frequent nutrient addition and splitting at least once a week. Cells are stable to about 100 splits - after about 70 splits, a new batch of cryopreserved cells should be used and compared with the previous batch. All glassware that comes in contact with the cells should be carefully prepared for tissue culture use by meticulous washing, followed by extensive rinsing to remove any traces of detergent residue and then by autoclaving.

Solutions:

Notes: Solutions 1-4 have limited shelf lives and should be made up only in small amounts as required. Components of solutions 1 and 2 are supplied sterile by manufacturers and must be maintained as such. Most of the following solutions are highly toxic and should be handled with utmost care.

1. Tissue Culture Medium (CM)
   RPMI 1640 with L-glutamine
   10% fetal bovine serum (FBS)
   1% antibiotic solutions (10,000 U/mL PenG, 10,000 µg/mL Streptomycin and 25 µg/mL amphotericin B)
   Small aliquots of FBS and antibiotic solution should be kept frozen and CM should be prepared in small batches upon demand.
2. 0.25% trypsin in physiological saline stored frozen in small aliquots.
3. 10 mM ouabain octahydrate (O) stored at room temperature (dark).
4. 1 mM veratridine (V) in acidic (pH 2) distilled water stored frozen in small aliquots.

Note: Solutions 3 and 4 must be filtered (0.2 µm) into sterile tubes immediately after preparation and before storage.

5. Toxin decontamination solution: 1:1 Javex (sodium hypochorite solution) and 50% NaOH.
6. Phosphate buffered saline (PBS), pH 7.2-7.4, made fresh or autoclaved and stored at 5°C.
7. 10% formalin
8. Gram's crystal violet
9. 33% glacial acetic acid

Equipment:

- 37°C incubator
- microplate reader with filters to allow readings at 595 nm.
- sterile 5 and 10 mL pipettes
- sterile 3-15 mL tubes for solutions
- single channel pipettors (10 to 200 µL). For inoculating the bioassay plates, a repeating multichannel micropipettor is highly recommended.
- sterile tips for the pipettors
- sterile tissue culture flasks and 96-well tissue culture dishes
- peel-and-stick mylar sheets for 96-well dishes
- gloves, biohazard bags

Procedure:

Notes: Since there are many hazardous or toxic components, it is advisable to wear gloves during the entire procedure.The cells are classified as biohazardous because of their cancerous origin, and disposables that come in contact with them should be placed in a biohazard bag, then autoclaved or incinerated. Glassware should be autoclaved prior to washing. Addition of the standards, samples, ouabain and veratridine requires undivided attention. Any excess reagents, containers or tips that have come in contact with these components must be

decontaminated by soaking for at least an hour in toxin decontamination solution, followed by washing or by disposal in a biohazard bag.

1. Dissociate the cells from the tissue culture flasks with 1-2 mL of trypsin solution. Resuspend the cells in CM to a density of 1-5 x 10^5 cells/ mL and inoculate 200 µL of cell suspension into each well of the 96-well dishes.
2. Seal the dishes with peel-and-stick mylar sheets and incubate them at 37°C overnight.
3. The next day, prepare empty sterile 96-well dishes for dilutions of standard and samples. Dilute pure STX with CM to a range of concentrations between 30 to 300 nM with a couple of concentrations beyond the active range on each end. This STX should be diluted just before use; it is not stable stored in the CM. In another dish, dilute the sample with CM to yield a range of 5-8 dilutions. Serial dilutions should be prepared based on the suspected toxicity (ie. 1:2 dilutions if low toxicity is suspected to 1:10 dilutions for extracts with higher suspected toxicity).
4. Once the dilutions of the standard and the samples have been completed, remove the dish(es) containing the growing cells from the incubator and remove the mylar sheet. It is best to keep a sterile lid on the plate of cells between additions, especially if working slowly. Add 10 µL of each dilution of either standard or sample to individual wells in the dish with the cells. Do not use any wells around the outer edge, and do not disturb the cells with the pipettor tips. Leave at least an additional two wells without standard or sample as controls, and mark inoculated wells on the dish and an associated master sheet.
5. Shake the plate gently, then add 10 µL of ouabain stock solution to each well to which either standard or sample dilutions has already been added, and to the two or more controls.
6. Shake the plate gently, then add 10 µL of veratridine stock solution to each control wells.
7. Shake the plate gently, then cover the plate(s) with a new sheet of mylar and incubate for 8h to overnight at 37°C.
8. Remove the plate(s) from the incubator, and observe under an inverted microscope. Cells in the control wells (O + V only) should look swollen, granular and lysing, indicating the plate is ready for development. Remove and discard the mylar sheet. Note: Both the decontamination solution and the formalin give off hazardous fumes, therefore the plate development should be done in a fume hood.
9. Dump the contents of the plate(s) into the toxin decontamination solution and shake out the excess. Do not allow splashing into the dish as this will be instantly lethal to cells.
10. Wash away any lysed or non-adherent cells in PBS.
11. Immerse the plate(s) in 10% formalin and fix for 15 min.
12. Remove the plate(s) from the formalin and shake off the excess.
13. Flood the plate(s) with crystal violet and stain for 5 min.
14. Rinse out the stain using running tap water until no more excess stain is apparent in the rinse water. Shake off the excess water and allow the plate(s) to air dry or dry them in a gentle warming oven.
15. To reconstitute the plate(s), add 100 µL of 33% acetic acid to each well and wait for 1 h.
16. Read the plate(s) spectophotometrically at 595 nm with a microplate reader.
17. To analyse the data, plot the absorbance for each concentration of STX standard and prepare a standard curve by fitting a line to the points using a four-parameter equation. Determine the concentration of STX that effects 50% of the cells (EC_{50}). This should not change from assay to assay (outside the variance limits: µ20%), and will be independent of differences in the initial cell concentration. Find dilutions of each sample which are in the absorbance range of the dynamic part of the standard curve. Use the equation of the fitted standard curve to generate the concentration of STX equivalents in each dilution, then multiply by the inverse of the dilution factor to determine the concentration in the original sample. Average all dilutions which fall within the dynamic range of the standard curve to calculate the final result for each sample (and the standard deviation, if needed).

b) Neuroblastoma Assay for NSP and CFP Toxins

The following procedures (Manger *et al.*, 1993, 1995) are similar to those described above for assaying the Na^+ channel blocking toxins, including use of the same cell line and alkaloids, but the assay is specific for Na^+ channel activators (e.g., PbTx and CTX). Detection is colorimetric, based on the ability of active cells to reduce the tetrazolium dye MTT to a blue colored formazan product.

Reagents:

1. Mouse neuroblastoma cells
   Neuro-2a (ATCC, CCL131)
2. Cell culture medium RPMI 1640 complete medium (Sigma)
   10 % heat inactivated fetal bovine serum (Gibco)
   2 mM glutamine (Sigma)
   1 mM sodium pyruvate (Sigma)
   50 µg/mL streptomycin (Sigma)
   50 units/mL penicillin (Sigma)
3. Ouabain stock
   10 mM ouabain (Sigma) in H_2O, pH 2
4. Veratridine
   1 mM veratridine (Sigma) in H_2O, pH 2
5. Cell harvest solution (CHS)
   0.5% trypsin/0.2% EDTA in PBS
6. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) Stock
   5 mg/mL stock solution in PBS, pH 7.4 - store at 4°C until use.
7. Stop Reagent
   DMSO (dimethylsulfoxide)
8. Brevetoxin (PbTx) standards Dissolved in methanol for stock solution. Dilute 1:100 in cell culture medium prior to analysis. Make serial dilutions from this 1:100 dilute stock.
9. Ciguatoxin (CTX) standards or ciguatoxic fish extract (methanol fraction)

Cell Culture:

Cultures of Neuro-2a are grown in supplemented cell culture medium at 37°C in a humidified air-CO_2 atmosphere (95 + 5). See notes in Appendix V(a) as well.

Procedure:

1. Harvest cells using CHS and seed into a 96-well microtiter plate at a density of 1 x 10^5 in 200 µL growth medium. Incubate for approximately 24 h.
2. Add 10 µL of each solution: sample/standard, ouabain stock, and veratridine stock, to each well. Test samples in triplicate at various dilutions. Process a minimum of 5 wells as ouabain/veratridine controls with no sample addition and 5 wells as untreated controls without sample, ouabain, or veratridine. Assess non-specific interactions with the use of standard without ouabain or veratridine. Add culture medium to make up for solutions not used in various controls. Incubate 4-22 hours at 37°C for detection of Na^+ channel activators.
3. Remove overlaying medium from culture plates. DO NOT WASH the plates.
4. Add 60 µL of a 1:6 dilution of MTT stock to each well. Incubate approximately 15 min at 37°C or until a deposit of reduced dark formazan deposit is observed in control wells (can be as long as 30-45 min).
5. Remove overlaying medium from culture plates. DO NOT WASH the plates.
6. Add 100 µL DMSO to each well.
7. Read absorbance IMMEDIATELY at 570 nm with reference at 630 nm.
8. Data Analysis

APPENDIX VI: AVAILABILITY OF TEST KITS, STANDARDS AND REAGENTS

PSP Toxins (Saxitoxin and derivatives)

The **RIDASCREEN**[R] immunodiagnostic test kit for the detection of PSP toxins is available from R-Biopharm, GmbH, Rösslerstrasse 94, D-1600, Darmstadt, Germany.

The **SAXITOXIN TEST**[R] kit is no longer in commercial production, but information may be obtained from Institut Armand-Frappier, 531 boulevard des Prairies, C.P. 100, Laval, Quebec, Canada.

The **MIST**™ cytotoxicity assay kit is available from JELLETT BIOTEK, Ltd., **MIST**™ Cell Bioassay Laboratory, 47 Wake Up Hill Road, RR1, Chester Basin, Nova Scotia, B0J 1K0, Canada. An alternative contact for further information regarding this technology is via the Nova Scotia Research Foundation Corp., 101 Research Drive, P.O. Box 790, Dartmouth, Nova Scotia, B2Y 3Z7, Canada (Telephone: 1-902-424-8670; fax: 1-902-424-4679; email: jbiotek@fox.nstn.ca).

For PSP toxin standards and reference materials see Chapter 5.

ASP Toxins (Domoic acid and isomers)

For toxin standards and reference materials see Chapter 6.

DSP Toxins (Okadaic acid and derivatives)

Mouse monoclonal antibodies for the ELISA method for DSP toxins presented in Appendix II(b) are available for purchase from Calbiochem:

Anti-okadaic acid Catalog No. 495605

Anti-idiotypic, okadaic acid Catalog No. 495607

ELISA test kits for OA/DTX1 are commercially available from Rougier Bio-Tech, Ltd., 8480 Boulevard St. Laurent, Montreal, PQ, H2P 2M6, Canada. For alternative sources of DSP toxin standards and reference materials see Chapter 6.

Microcystins

Calbiochem, LaJolla, CA (telephone: (800) 854-3417; fax: (800)854-3417):

Microcystin-LR	Catalog No. 475815-S
Microcystin-RR	Catalog No. 475816-S
Microcystin-YR	Catalog No. 475819-S

CFP (ciguatoxins)

Ciguatect solid phase immunobead assay will be commercially available from Hawaii Chemtect Int., Pasadena, CA. Ciguatoxin is not readily commercially available. The typical source of ciguatoxin is suspected ciguatoxic fish tissue.

NSP Toxins (brevetoxins)

NSP toxins may be purchased from Chiral Corporation (1221 Brickell Avenue, Miami, FL 33131; Voice mail and FAX: 305-361-4001) or from the Calbiochem catalogue:

PbTx-1	Catalog No. 203730-S
PbTx-2	Catalog No. 203732-S
PbTx-3	Catalog No. 203734-S
PbTx-6	Catalog No. 203737-S
PbTx-9	Catalog No. 203738-S

GLOSSARY

AFFINITY: Quantitation of the reversible interactions between an antigen or ligand and an antibody or target receptor.
ANTIBODY: A protein (immunoglobulin) produced in an animal as a specific response to the introduction of a substance normally foreign to the organism.
ANTIGEN: A molecular substance, typically a protein or a carbohydrate, capable of provoking the production of antibodies in susceptible animals.
CARRIER: A large immunogenic molecule, usually a protein or synthetic polypeptide, linked to a hapten for induction of antibody production.
CONJUGATE: a chemically-linked hapten-carrier complex.
CROSS-REACTION: The immunological reaction of a specific antibody with different antigenic substances, usually structurally closely related to the original antigen.
DISSOCIATION CONSTANT (K_D): A measure of the tendency of a complex (e.g., a ligand-receptor complex) to dissociate; the lower the value, the tighter the binding.
ENZYME IMMUNOASSAY (EIA): An immunodiagnostic method which incorporates an enzyme label, which can be configured in a variety for formats.
ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA): An immunodiagnostic method whereby antigen adsorption to the antibody linked to an enzyme converts a substrate, generating a coloured reaction product detectable by an increase in optical density.
EPITOPE: The antigenic determinant or antibody binding moiety.
FLUORESCENT IMMUNOASSAY (FIA): An immunodiagnostic method for the detection of antigenic substances, whereby binding to the antibody is detected by a fluorescent label.
HAPTEN: a low molecular weight component with little ability to stimulate an immunogenic response unless linked to a carrier.
IC_{50}: In a radio-receptor assay, the concentration of unlabelled ligand that results in 50% total binding of the radioligand to the available receptor sites.
IMMUNOGLOBULIN (Ig): A high-molecular weight globular protein with four substituent chains which possesses antibody activity.
LIGAND: Any molecule which binds to a specific site on a protein or other molecule; in biotoxin assays, the toxin acts as the ligand.
MONOCLONAL ANTIBODY: An antibody produced from a single line of genetically identical antibody-yielding cells in culture, which are usually fused with tumor cells to ensure continued production of a highly specific antibody.
POLYCLONAL ANTIBODY: An antibody produced from cells of differing (usually undefined) genetic constitution. Such antibodies are typically derived from the serum of animals inoculated with an antigen.
RADIO-IMMUNOASSAY (RIA): An immunodiagnostic method for the detection of antigenic substances, whereby a binding to the antibody is detected by a radioactive tracer.
RECEPTOR: A protein or other molecule which binds a specific extracellular molecule (ligand) and initiates a cellular response; in receptor assays for Na^+ or Ca^{++} channel active toxins, the respective ion conducting channel serves as the receptor.
SYNAPTOSOMES: Membrane-bound sacs produced in homogenates of brain neuronal tissue by cleavage from axon terminals.

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Part B. Mammalian Bioassays

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Although sophisticated physico-chemical methods for phycotoxin analysis have been developed (see Chapters 5, 6, 7 and 8), there is still a paucity of simple, rapid, robust and sensitive procedures that can be used with reliance by regulatory laboratories for public health control of seafood toxicity. This is particularly true for the toxins associated with PSP, DSP, NSP and ciguatera poisoning. In the near future, *in vitro* assays (see Chapter 10.A.) may be applied as routine screening tools for regulatory purposes, but thus far, in spite of increasing ethical considerations, most countries rely primarily or exclusively upon mammalian bioassays. In some countries, regulations against the use of experimental animals complicates effective phycotoxin monitoring, since alternative toxicological procedures are not readily available.

Whole animal bioassays provide a measure of total toxicity based upon the biological response of the animal to the toxins. Although some oral feeding tests have been developed for phycotoxicity screening, and one such technique, the rat bioassay (Kat, 1983), is used in some European countries for DSP testing, the general principle of most mammalian bioassays depends upon intraperitoneal (i.p.) injection of an aqueous or organic shellfish extract. Standardized strains of laboratory mice of defined age, sex and weight are frequently used in phycotoxin bioassays. The choice of extraction solvent depends on the solubility properties of the toxins tested. Following injection of the extract, subsequent observations are made to identify the time- and dose-dependent appearance of typical symptoms (morbidity and mortality) caused by the toxins. Toxicity values are generally interpolated from standard curves, LD_{50} determinations over a fixed time (e.g. 24 h), or are derived from standard toxicity tables relating dosage to death times. Survival time of mice is generally used for the measurement of global toxicity, expressed in mouse units (MU) which are converted into toxin specific units (e.g. µg saxitoxin equivalents [STXeq]) based upon the toxicity response calibrated with reference to toxin standards.

The principal advantage of a well administered and properly calibrated mammalian bioassay, compared to physico-chemical analysis or many *in vitro* methods, is that the toxicity determination is directly relevant to human toxicity effects. Unfortunately, although this is true in theory, it is often difficult to accurately extrapolate results derived from an alternate route of toxin administration (often i.p. injection) to human oral potency. Yet such bioassays can often be rapidly and conveniently conducted without complex equipment or the extensive clean-up procedures often required for more sophisticated analytical methods. Mammalian bioassays also screen inadvertently for the presence of unknown or poorly defined toxic components in the extract matrix, which may ultimately be found to have human health significance. For example, the first indications of the toxicity associated with the ASP syndrome, later related to domoic acid (Wright *et al.*, 1989), were revealed in the course of routine AOAC mouse bioassays for PSP toxicity using acidic aqueous mussel extracts from eastern Canada. Skilled bioassay technicians noted that the aberrant symptoms of ASP were distinguishable from the classic symptomology of PSP intoxication and consequent death.

Whole animal bioassays have numerous inherent and operational deficiencies when applied to the accurate quantitation of phycotoxins. High capital investment and maintenance costs are often associated with the installation and operation of bioassay facilities involving live animals. Mammalian bioassays are labour intensive to perform and they cannot be readily automated. An additional drawback of mammalian bioassays is the high variability among laboratories, due mainly to a number of variables that can affect the results, such as specific animal characteristics (strain, sex, age, weight), general state of health, diet, stress conditions, pH of the injected extracts, etc. Most of these parameters acquire special relevance when sample toxicity levels are near the regulatory limit. For these reasons, careful standardisation

of the assay conditions is required to obtain reproducible and reliable assessment of toxicity and to reduce inconsistencies within and among laboratories.

Mammalian bioassays are susceptible to a host of artifacts and inaccuracies which can bias the validity of the results. False reactions (positive or negative) can occur due to interference by substances coextracted during the sample preparation or to an inappropriate choice of extraction solvents or clean-up method. Many mammalian bioassays for phycotoxins have a poor dynamic range - dilution of the toxic analyte in the sample to achieve death times in the working range of the assay also dilute other components in the matrix and this can lead to non-linearity of the standard dose-response curve. The assays tend to be more reliable for phycotoxins which yield a low LD_{50} and short death times (i.e., high acute toxicity).

Compared to instrumental analytical methods, whole animal bioassays are often much less sensitive (by up to five orders of magnitude) and precise (±20% is typical under optimal conditions). For example, for the AOAC mouse bioassay for PSP toxicity, the acceptable regulatory limit for human consumption of shellfish adopted in many countries (80 µg STXeq/100g soft tissue) is only twice the nominal toxicity detection limit (*ca.* 40 µgSTXeq/100g). This provides little security margin for technical errors. Moreover, no definitive qualitative information is provided on the nature of the toxin components. This is particularly a problem for samples which contain multiple toxin analogues which vary in specific toxicity, or where the co-occurrence of different phycotoxins can lead to synergistic or antagonistic biological responses, e.g. the simultaneous presence of a Na^+ channel activator and a blocker. Nevertheless, despite the numerous technical and ethical problems inherent to mammalian bioassays and the poor information provided on specific toxin composition, such assays are widely employed for phycotoxin monitoring in seafood. Only mammalian bioassays which are in routine use in regulatory programs are presented in detail in this chapter.

PSP MOUSE BIOASSAY

The mouse bioassay for the determination of PSP toxicity was first applied by Sommer and Meyer (1937) to the assay of acidic extracts of mussels from California. In subsequent years, the general procedure has been further standardized and validated in a series of inter-collaborative studies (Association of Official Analytical Chemists; AOAC, 1990). This reference method is the only procedure recognized internationally for quantifying PSP toxicity and it is used worldwide in PSP monitoring programs, albeit with some variation in the acceptable regulatory limit for toxicity (see Chapter 22).

The PSP mouse bioassay for shellfish toxicity involves acidic aqueous extraction of the tissue (whole animal or selected organs) followed by i.p. injection of 1 ml of the extract into each of three standardized mice. The mice are observed for classical PSP symptoms, such as jumping in the early stages, followed by death in <15 minutes by respiratory arrest. The time from initial injection to mouse death is recorded and the toxicity is determined (in mouse units) from Sommer's table (see Appendix I). One mouse unit is refined as the amount of PSP toxin required to kill a 20 g mouse within 15 minutes. The bioassay is only quantitative when the mouse death occurs between 5 and 7 minutes, with great variations to be expected above or below these limits. Several dilutions may be needed to obtain an extract concentration within this range. The precision of this assay is often given as ±20% (C.V.), but this must be regarded as optimal; if a large dilution factor is required, this level of precision is substantially degraded.

After standardization of the mice, mouse units are converted to toxicity units (µg STXeq) per 100g of soft tissue. The tolerance level agreed by most countries is 80 µgSTXeq per 100g tissue. The detailed AOAC procedure is given in Appendix I. Although the method has been extensively validated, there are many caveats to be considered to minimize variability within and among laboratories. The assay sensitivity depends very much upon the mouse strain. For this reason, bioassay standardization and periodic checking of the conversion factor (CF) is crucial. Since mouse calibration is carried out using a STX standard, toxicity

data must be expressed in µgSTXeq per 100 g of soft tissue and not in mouse units, to compare results among different laboratories. The use of male mice is recommended because of the lower variability in their response to the toxin.

Another important parameter influencing the PSP toxin bioassay results is the pH during extraction. In the AOAC (1990) method, extraction in 0.1 M hydrochloric acid followed by heating at 100°C, typically establishes pH ranging between 2 and 4. The PSP assay procedure was initially designed to quantify only STX, but at present about two dozen naturally-occurring PSP analogues have been identified, which vary in toxicity, chemical stability and relative abundance in shellfish and dinoflagellates. Under the hot acidic conditions required in the AOAC protocol a substantial proportion of the labile but low potency N-sulfocarbamoyl toxins (C1-C4, B1, B2) are converted to their respective high toxicity carbamate analogues (approximately 40-60% conversion, depending upon the tissue matrix buffering capacity, A. Cembella, unpub. obs.). Epimerization also occurs with this hot acid treatment, resulting in the conversion of β- to α-epimers, but this usually has a minor effect on net toxicity. The PSP toxins are least stable at alkaline pH, yet heating under strongly acidic conditions (e.g., pH 2) can also lead to chemical transformation, with the degree of conversion depending upon the pH (Nagashima *et al.*, 1991). Between pH 3 to 4, all PSP components are in a range of optimal stability. Low pH of the injected extract can also lead to mouse bioassay artifacts caused by acidosis. A decrease in the acceptable pH range established in the AOAC procedure is highly advisable to reduce variability and improve reproducibility of the assay.

A crucial issue to resolve when applying the mouse bioassay to the analysis of PSP toxin extracts is to determine *a priori* what is to be measured - maximum potential toxicity or actual toxicity. The precautionary principle seems to defend the former approach, despite the possible overestimation of human health risk. In fact, the standard AOAC procedure determines toxicity according to neither of these extremes, but instead yields an intermediate value. In view of the inconsistent and incomplete hydrolysis of N-sulfocarbamoyl toxin derivatives in PSP toxin-containing samples extracted according to the AOAC method (Hall *et al.*, 1990), some authors have recommended a ten-fold increase in the hydrochloric acid concentration to 1.0 M. When followed by heating of the extract, this modification yields toxin values by mouse bioassay which more closely approximate the maximum potential toxicity, but this approach has not been widely adopted since the standard AOAC (1990) procedure is strongly entrenched within regulatory structures.

In early studies, Shantz *et al.* (1958) reported that high amounts of salts (mainly Na^+ ions) dissolved in the extracts can decrease apparent PSP toxicity in the mouse bioassay. The addition of NaOH is sometimes necessary to raise the pH prior to injection, but this should be done carefully and dropwise with stirring to avoid toxin decomposition via localized pH changes.

The presence of high concentrations of certain metals is another potential source of interference with the PSP mouse bioassay. High levels of zinc (McCulloch *et al.*, 1989; Cacho E., 1993), can induce mouse deaths with apparent neurotoxic symptoms. The major difference between zinc toxicity artifacts and symptoms of classic PSP toxicity is that death occurs more than 15 minutes after injection. Depending upon the experience of the assayist this effect could be falsely interpreted as low level PSP activity.

ASP MOUSE BIOASSAY

The AOAC mouse bioassay for PSP toxins can also detect domoic acid at concentrations *ca.* 40 ppm and this procedure was used when ASP toxicity was first identified in Canada in shellfish extracts from eastern Prince Edward Island (Wright *et al.*, 1989). The typical signs of the presence of domoic acid is a unique scratching syndrome of the shoulders by the hind leg, followed by convulsions. The time of observation must be extended from 15 minutes to 4 hours. Mouse deaths associated with mussels containing domoic acid were never observed

after 135 minutes (Quilliam *et al.*, 1989; Todd, 1990). Although the AOAC extraction procedure can yield substantial recovery of domoic acid, the tolerance level established in Canada and subsequently adopted by certain other countries is 20 ppm, therefore the AOAC bioassay procedure is too insensitive to be used with confidence for regulatory purposes to quantify this toxin. For the routine detection of ASP toxins, the AOAC mouse bioassay has been superseded by HPLC methods using diode-array/UV or fluorometric detection (Quilliam *et al.*,1989; Pocklington *et al.*, 1990) which have been proven to be more sensitive and reliable tools (see Chapter 7).

DSP BIOASSAYS

There is no general agreement concerning the appropriate testing procedures to be applied for DSP toxins for regulatory purposes, and standardization of methods represents a difficult administrative and scientific challenge. One of the main problems arises from the different biological activity of the three groups of lipophilic toxins currently included in the DSP toxin complex (Yasumoto, 1990) - 1) okadaic acid (OA) and analogues such as DTX1, DTX2, and acyl-derivatives (DTX3), 2) polyether lactone pectenotoxins (PTX), and 3) yessotoxins (YTX), disulfated polyether compounds. Only toxins belonging to the OA group produce diarrheic effects in mammals (i.e., classic DSP symptoms), however PTX compounds are reported to be hepatotoxic and YTX derivatives cause severe cardiac damage in experimental animals. Although PTX and YTX are acutely toxic to mice upon i.p. injection (Terao *et al.*, 1990), the oral toxicity to humans has not been established. Given that the human health risks of the two latter two groups of DSP toxins are not well elucidated, they should remain included in DSP toxicity monitoring programmes, at least until more toxicological data are available.

Despite the worldwide application of mammalian bioassays for DSP toxicity, there are large differences in the performance of the procedures and in the toxicity criteria employed among countries. Assay selectivity, specificity and DSP toxin recovery depends greatly upon the selection, purity, and ratios of the organic solvents used for extraction and clean-up steps (see also Chapter 6). Most extraction procedures applied to DSP toxins in shellfish samples can also coextract NSP toxins, certain other lipophilic ichthyotoxins, and relatively low-polarity compounds (e.g. fatty acids) which can yield non-specific artifacts in mammalian bioassays. Bioassay procedures as diverse as the oral dosage rat bioassay and the i.p. injection mouse assay are not strictly homologous, because the former technique quantifies only the diarrheagenic effect of certain DSP toxin components, whereas the latter assay yields an estimate of global "DSP toxicity".

Rat bioassay (Kat, 1983)

The rat bioassay is the basis of DSP toxin monitoring in some European countries, including the Netherlands, Germany and Ireland. The presence of DSP toxins is indicated by feeding pre-starved (for 24h) white female Wistar rats, *Rattus norvegicus* (100-120 g), with 10 g of shellfish hepatopancreas. On the following day, the consistency (diarrheic or soft) of the rat faeces is scored: normal (0 or -), normal to soft (1 or +), soft (2 or ++), soft to diarrheic (3 or +++) and diarrheic (4 or ++++). Faecal consistency other than normal suggests the presence of DSP toxins in the sample. If the toxicity score is above 0, shellfish are usually considered unsafe for human consumption.

The rat bioassay simulates the mode of human intoxication, and the symptoms exhibited by rats are similar to those resulting from ingestion of the diarrheagenic DSP toxins by humans. Therefore, the rat bioassay is a biologically "realistic" model of acute toxicity, and

presents the additional advantage of not involving extraction of toxins, thus avoiding the possibility of low recoveries.

There are, however, serious limitations associated with this bioassay procedure. At best the assay is only semi-quantitative and the dynamic range is poor. Due to broad individual variation in sensitivity and symptomology among rats, the precision is also questionable, particularly when few animals are assayed (typically one to three rats). The assay is reasonably diagnostic for the presence of the diarrhegenic DSP components (OA and DTX analogues), but PTX and YTX are not detected in this method. Other diarrheic agents including intestinal pathogens associated with poor animal health could also confound the interpretation of "DSP toxicity", since the assay method is not very specific.

Mouse bioassays

For all of the alternative mouse bioassays described below, a mouse unit is defined as the minimum quantity of toxin needed to kill two of three mice in 24 h after i.p. injection; this corresponds to approximately 3.2 μg DTX1 or 4 μg OA. Although most countries use mouse survival time for the determination of DSP toxicity, there is no international consensus on the appropriate observation period - the acceptable criteria can vary from "two of three mouse deaths in less than five hours" to "two of three mouse deaths in less than 24 hours". Such decisions are both operational and administrative, but they are also somewhat dependent upon the specific procedure for sample preparation prior to mouse bioassay, since various components of differing toxicity may be selectively retained.

Considering the available toxicological studies on the acute toxicity of the DSP toxins, an observation time of five hours might be enough to assure the safety of the tested shellfish if only OA and/or DTX1 were present. For other DSP toxins, the time/dose response may vary and a delay in the appearance of symptoms might occur. In cases where the acyl-derivatives of the OA group are present (Terao *et al.*, 1993), an observation time of 24 h is advisable for monitoring toxicity. Of course, the most stringent criteria should be applied when testing shellfish with an unknown DSP toxin profile.

The mouse bioassay for DSP toxicity is considered to be quantitative, although as the prolonged observation time would suggest, it is less reliable as a determinant of acute toxicity than is the corresponding AOAC mouse assay for PSP toxicity. Furthermore, the precise cause of death in rodents by i.p. administration of DSP toxins is not yet known. Phosphatase inhibition activity is strongly indicated as the toxicity mechanism leading to death, but the results of the i.p. bioassay cannot be simply extrapolated to quantify human oral toxicity or diarrheic symptoms caused by unextracted DSP toxins in a shellfish tissue matrix. One drawback of the procedure is that there may be interference by free fatty acids, which can vary with the particular shellfish species, site of origin, and season. These components may be toxic to mice by i.p. injection but innocuous to humans, yielding false positive reactions for "DSP toxicity" (Takagi *et al.*, 1984).

Method 1 (Yasumoto et al., 1978)

This mouse bioassay procedure is widely applied for routine monitoring of DSP toxicity in shellfish. According to this method, a 20 g sample of homogenized hepatopancreas is extracted with 50 ml of acetone. The solution is filtered into an evaporation flask and the residue is re-extracted twice with two additional volumes of 50 ml of acetone. The solvent is removed by rotary evaporation under reduced pressure and the residue is resuspended in 1% Tween 60 to a final concentration of 5g hepatopancreas/ml Tween 60. The Tween extract is injected intraperitoneally into each of three mice weighting between 18 and 20 g.

A potential pitfall of this bioassay procedure is associated with the aqueous-lipid residue obtained after solvent evaporation. Depending upon factors such as water content of the shellfish, resuspension of the final residue to achieve a specific concentration (1 ml Tween

/5 g hepatopancreas) may be inconsistent, thereby negatively affecting the accuracy of the assay. The high temperatures sometimes required for a total removal of the water may produce low toxin recoveries (Croci *et al.*, 1994).

Interference by low levels of PSP toxins, even at concentrations undetectable by the AOAC (1990) mouse bioassay, can also be a problem. Some of these polar components are coextracted with the DSP toxins by acetone extraction, and they are concentrated during the evaporation procedure, yielding a final extract which can be highly toxic to mice. Short term mouse deaths (minutes to a few hours) with neurotoxic symptomology do not permit the prolonged observations required for DSP toxin detection.

It is also worth mentioning that acetone extracts found to be acutely toxic upon i.p. injection into mice have sometimes been identified in mussels from Canada, France, Norway and Sweden, in the absence of known DSP or PSP toxins (Underdal *et al.*, 1985; Bansard *et al.*, 1995; Stabell *et al.*, 1991). The identity of these components, as well as their toxic effects in humans, are still under study.

Method 2 (Yasumoto et al., 1984)

This modified procedure is now the official Japanese method for surveillance of DSP toxicity and it is also used in some European countries, including Italy and the United Kingdom. In this method, a 20 g sample of homogenized hepatopancreas is extracted thrice with 100 ml of acetone. The extracts are filtered, then the filtrate is collected and the solvent removed by rotary evaporation. The residue is made up to 20 ml with water and the suspension is extracted thrice with 50 ml of diethyl-ether. The combined organic layers are backwashed twice with small quantities of water and evaporated to dryness. As in the original procedure described above, the residue is resuspended in 1% Tween 60 solution to a concentration of 5g hepatopancreas/ml Tween 60 prior to i.p. injection.

The assay is very suitable for use with a wide variety of shellfish tissues. With this procedure, possible PSP interferences are removed in the water layer. High amounts of salts which are concentrated during the evaporation step, and could cause artifactual mouse deaths, can also be removed during the water washing step.

A drawback of this extraction procedure is the poor solubility of YTX in diethyl-ether; depending upon the pH and lipid content of the sample, this toxic component might be lost in the water layer. A substantial improvement is achieved if dichloromethane is used instead of diethyl-ether. Dichloromethane has the advantage of solubilizing all the DSP toxins, including YTX (Yasumoto, pers. comm.).

Method 3 (Lee et al., 1987)

The extraction procedure for this DSP mouse bioassay method was adapted from that applied for the validation of the HPLC-FD (Lee *et al.*, 1987) and it is used extensively for DSP toxin monitoring in Norway (Dahl *et al.*, 1995). In this protocol, homogenized hepatopancreas is extracted with 100% acetone. After evaporation of the solvent, the residue is redissolved in 80% methanol in water which is extracted twice with petroleum ether and then with chloroform. The chloroform phase is evaporated to dryness and the residue is redissolved in 1% Tween 60. Tween extracts (1 ml) are administered by i.p. injection into each of two mice at doses equivalent to 2.5 to 5 g of hepatopancreas. Mouse behaviour and survival time is recorded until 24 hours. If survival time is less than 4 hours, shellfish harvesting and marketing is banned. As in the Le Baut *et al.* (1990) procedure, losses of low polarity DSP toxins can occur during the petroleum ether washing step.

Method 4 (Le Baut et al., 1990)

The following procedure is applied to the monitoring of DSP toxins in shellfish from Portugal and France. Hepatopancreas (20 g) is extracted thrice with acetone. After evaporation of the acetone, the residue is resuspended in 25 ml of methanol:water (80:20). The methanolic phase is washed twice with 25 ml of hexane, then the hexane phases are discarded, and the methanolic layer is evaporated. The resuspension of the residue in 1% Tween 60 and i.p. injection protocols are according to those described for the above methods. Depending on the country, different survival times are considered as DSP toxicity criteria.

One of the main advantages of this assay is that low polarity free fatty acids which could interfere with the assay are removed by the hexane wash. The final residue has a relatively low lipid content and it thus easier to accurately resuspend in Tween 60; the effect is to minimize false positive responses.

The main deficiency of the procedure is that the hexane washing step, besides removing free fatty acids, also preferentially solubilizes the acyl-derivatives of OA and DTX. If these components are present in shellfish samples, substantial loss of toxins can occur (Fernandez *et al.*, in press). Residual PSP toxins and salt interferences remain in this procedure.

NSP MOUSE BIOASSAY

The currently accepted method for the determination of NSP toxins is the American Public Health Association (APHA, 1985) procedure (originally Irwin, 1970), based on a diethyl-ether extraction of shellfish tissue (see Appendix II). The APHA protocol for NSP used extensively in the United States, where the problem of NSP is most acute. After the detection of NSP in New Zealand in 1993, a management strategy to monitor these toxins was developed by the MAF Regulatory Authority. The sample preparation method used for the detection of NSP and DSP toxins was based on acetone extraction of these lipophilic components, followed by partitioning into dichloromethane (Hannah *et al.*, 1995). This method is simpler and more suitable for rapid and quantitative separation of the organic and aqueous phases of the extract than the APHA method, and the extraction efficiency is greater. The procedure is as follows: 300 ml of acetone are added to 100 g of homogenized tissue and homogenized for 20 s. The extract is filtered and the solid residue is returned to the blender jar; 200 ml of acetone are added and the sample is re-homogenized for 20 s. The second extract is filtered and combined into the same flask as the first. The combined extracts are filtered and the acetone is removed by rotary evaporation at 35°C. The aqueous extract is transferred to a 500 ml separatory funnel with 100 ml of dichloromethane. The funnel is gently shaken and the layers are allowed to separate. The lower dichloromethane layer is drained through anhydrous sodium sulfate pre-rinsed with dichloromethane, then 80 ml of dichloromethane are added to the funnel, the mixture is shaken vigorously, and layers are again allowed to separate. The second dichloromethane layer is drained through the same sodium sulfate and washed thoroughly with 20 ml dichloromethane. From the combined filtrates, the dichloromethane is removed by rotary evaporation, and the residue is transferred with a minimum volume of dichloromethane to a 20 ml glass vial. The remaining dichloromethane is evaporated, initially under a stream of nitrogen on a hot plate, and finally by rotary evaporation.

Sample extracts are prepared for mouse injection by suspension in a sterile solution containing 1% Tween 60 to a final sample concentration equivalent to 10 g/ml. The mouse bioassay is conducted by administering 1 ml inoculations, and the bioassay results are calculated in mouse units as defined by Irwin (1970).

The Hannah (1995) procedure is very effective in extracting unknown lipid-soluble toxins from shellfish containing NSP toxins, and the method presents certain advantages compared with the APHA protocol. However, the discovery of a novel bioactive compound

(gymnodimine), produced by the dinoflagellate *Gymnodinium mikimotoi*, a common species in New Zealand waters during neurotoxic events, has led to the local health authorities to return to the diethyl-ether extraction procedure originally established by the APHA (Irwin, 1970). Gymnodimine is not extractable by diethyl-ether, but it causes very rapid mouse deaths when the dichloromethane procedure is used. Since gymnodimine is not considered to present a risk to human health, the monitoring program now employs diethyl-ether extraction as a means of discriminating gymnodimine activity from NSP toxicity.

APPENDIX I: MOUSE BIOASSAY FOR PSP (SAXITOXIN AND DERIVATIVES)

Caution: Rubber gloves should be used when handling materials which may contain PSP toxins.

The following procedure is adopted with only minor modifications from the AOAC (1990) mouse bioassay protocol for PSP toxicity.

Reagents and Materials:

1. Paralytic shellfish poison (STX) standard solution - 100 g/ml (U.S. Food and Drug Administration), as acidified 20% alcohol solution. This standard is stable indefinitely when stored in a cool place.
2. Paralytic shellfish poison (STX) working standard solution - 1 µg/ml. Dilute 1 ml of standard solution to 100 ml with H_2O. This solution is stable for several weeks at 3-4°C.
3. Mice- use healthy mice (19-21 g) from a standard stock colony for routine assays. For mice <19 g or >21 g, apply the correction factor (CF) to obtain the true death time (see Table 10.3). Do not use mice weighing >23 g and do not re-use assay subjects.

Standardization of Bioassay:

Dilute 10 ml aliquots of 1 µg/ml standard STX solution with 10, 15, 20, 25 and 30 ml H_2O, respectively, until i.p. injection of 1 ml doses into a few test mice yields a median death time of 5 to 7 min. The pH of the dilutions should be between 2 to 4 and must not be >4.5. Test additional dilutions in 1 ml increments of H_2O, e.g., if 10 ml standard STX solution diluted with 25 ml H_2O kills mice in 5 to 7 min, test solutions dilutions as 10 + 24 ml and 10 + 26 ml as well.

Inject a group of 10 mice with each of two (or preferably three) dilutions which fall within the median death time of 5 to 7 min. Give a 1 ml dose to each mouse by i.p. injection and determine death time as the time elapsed from completion of injection to the last gasping breath of the mouse. Repeat the assay one or two days later, using dilutions prepared above which differed by 1 ml increments of H_2O. Then repeat the entire test, starting by testing dilutions prepared from fresh working standard solution.

Calculate median death time for each group of 10 mice injected with each dilution. If all groups of 10 mice injected with any one dilution gave median death times <5 or >7 min., disregard results from this dilution in subsequent calculations. However, if any groups of 10 mice injected with a given dilution gave median death times between 5 and 7 min. include all groups of 10 mice assayed at this dilution, even though some of the median death times may be <5 or >7 min. From the median death time for each group of 10 mice at each selected dilution, determine the mouse units/ml (MU/ml) from Sommer's Table (see Table 10.3 below). Divide calculated µgPSP toxin (STX)/ml by MU/ml to obtain the conversion factor (CF), expressed as µgPSP toxin (or µSTXeq) per MU. Calculate the average CF value, and use this as a reference to verify routine assays. Individual CF values may vary significantly even within a given laboratory if the assay techniques and the condition of the mice are not rigidly controlled.

Verification of PSP Toxin Conversion Factor (CF) for Routine Assays:
Check CF value periodically as follows: if shellfish tissues are assayed less than once a week, determine the CF value on each day assays are performed by injecting five mice with the appropriate dilution of working standard. If assays are carried on several days during a given week, only one verification need be made per week using a dilution of standard such that the median death time falls within 5 to 7 min. The CF thus determined should agree with the average value within ±20%. If the CF value does not lie within this range, complete the group of 10 mice by adding five additional specimens to the five already injected, and inject a second group of 10 mice with same dilution of standard. Average the CF determined for the second group with that of the first group - this is the new CF value.

Repeated checks of CF value normally produce results within ±20%. If wider variations are found frequently, the possibility of uncontrolled or unrecognized variables in the technique or in the response of mice to PSP toxin should be investigated before proceeding with routine assays.

Sample Preparation and Extraction:
a) Clams, oysters, and mussels, etc. (whole tissues) - Thoroughly clean the outside of shellfish with fresh H_2O. Open by cutting adductor muscles. Rinse inside with fresh H_2O to remove sand or other foreign material. Remove meat from the shell by separating adductor muscles and tissue connections at the hinge. Do not use heat or anesthetics before opening the shell, and do not cut or damage the soft tissues at this stage. Collect *ca.* 100-150 g shellfish tissue in a glazed dish. As soon as possible, transfer tissues to a No. 10 sieve without layering, and drain for 5 min. Pick out pieces of shell and discard drainings. Grind tissues in household-type grinder with 1/8 - 1/4 " (3-6 mm) holes or in a blender until homogeneous.
b) Scallops, etc. (selected tissues) - Separate edible tissues (e.g., adductor muscle) and apply the extraction procedure to this portion alone. Drain and homogenize as in a).
c) Canned shellfish - Place entire contents of can (tissue and liquid) in a blender and blend until homogenous or grind three times through a meat chopper. For large cans, drain tissues for 2 min on a No. 8-12 sieve and collect all liquid. Determine wet weight of tissue and volume of liquid. Recombine portions of each sample in proportionate amounts and blend (or grind) until homogeneous.

Weigh 100 g of well mixed sample into a tared beaker. Add 100 ml 0.1N HCl, stir thoroughly, and check pH. (pH should be < 4.0, preferably *ca.* 3.0. If necessary, adjust pH as indicated below.) Heat mixture and boil gently for 5 min, then let cool to room temperature. Adjust cooled mixture to pH 2.0-4.0 (never > 4.5) as determined by BHD Universal Indicator, phenol blue, Congo red paper, or by pH meter. To lower pH, add 5N HCl dropwise with stirring; to raise pH, add 0.1N NaOH dropwise with constant stirring to prevent local alkalinization and consequent destruction or conversion of PSP toxins. Transfer mixture to a graduated cylinder and dilute to 200 ml. Return mixture to the beaker, stir to homogeneity, and let settle until a portion of the supernate is translucent and can be decanted free of solid particles large enough to block a 26-gage hypodermic needle. If necessary, centrifuge the mixture or supernate for 5 min at 3,000 rpm or filter through paper. Only enough liquid to perform the bioassay is necessary.

Mouse Assay:
Intraperitoneally inoculate each test mouse with 1 ml of acidic extract. Note the time of inoculation and observe mice carefully for the time of death as indicated by the last gasping breath. Record death time by stopwatch or clock with sweep second hand. One mouse may be used for the initial determination, but two or three are preferred. If death time or median death time of several mice is <5 min, prepare dilutions to obtain death times of 5 to 7 min. If death time of one or two mice injected with the undiluted is >7 min, a total of three mice must be inoculated to establish sample toxicity. If large dilutions are necessary, adjust the pH of the

dilution by dropwise addition of dilute HCl (0.1 or 0.01N) to pH 2.0 to 4.0 (never >4.5). Inoculate three mice with a dilution which gives death times of 5 to 7 min.

Table 10.2. Correction values for weight of mice.

Mouse Weight (g)	Mouse Units (MU)	Mouse Weight (g)	Mouse Units (MU)
10	0.50	17	0.88
10.5	0.53	17.5	0.905
11	0.56	18	0.93
11.5	0.59	18.5	0.95
12	0.62	19	0.97
12.5	0.65	19.5	0.985
13	0.675	20	1.000
13.5	0.70	20.5	1.015
14	0.73	21	1.03
14.5	0.76	21.5	1.04
15	0.785	22	1.05
15.5	0.81	22.5	1.06
16	0.84	23	1.07
16.5	0.86		

Table 10.3. Sommer's table (959.08) relating mouse death times to toxicity units.

Death Time (min:s)	Mouse Units (MU)	Death Time (min:s)	Mouse Units (MU)
1:00	100	5:00	1.92
:10	66.2	:05	1.89
:15	38.3	:10	1.86
:20	26.4	:15	1.83
:25	20.7	:20	1.80
:30	16.5	:30	1.74
:35	13.9	:40	1.69
:40	11.9	:45	1.67
:45	10.4	:50	1.64
:50	9.33		
:55	8.42	6:00	1.60
		:15	1.54
2:00	7.67	:30	1.48
:05	7.04	:45	1.43
:10	6.52		
:15	6.06	7:00	1.39
:20	5.66	:15	1.35

:25	5.32	:30	1.31
:30	5.00	:45	1.28
:35	4.73		
:40	4.48	8:00	1.25
:45	4.26	:15	1.22
:50	4.06	:30	1.20
:55	3.88	:45	1.18
3:00	3.70	9:00	1.16
:05	3.57	:30	1.13
:10	3.43		
:15	3.31	10:00	1.11
:20	3.19	:30	1.09
:25	3.08		
:30	2.98	11:00	1.075
:35	2.88	:30	1.06
:40	2.79		
:45	2.71	12:00	1.05
:50	2.63	13	1.03
:55	2.56	14	1.015
		15	1.000
4:00	2.50	16	0.99
:05	2.44	17	0.98
:10	2.38	18	0.972
:15	2.32	19	0.965
:20	2.26	20	0.96
:25	2.21	21	0.954
:30	2.16	22	0.948
:35	2.12	23	0.942
:40	2.08	24	0.937
:45	2.04	25	0.934
:50	2.00	30	0.917
:55	1.96	40	0.898
		60	0.875

Calculation of Toxicity:
Determine median death times of mice, including survivors, and from Sommer's Table (see below) determine the corresponding toxicity in mouse units (MU). If test animals weigh <19 g or >21 g, apply a correction for each mouse by multiplying MU corresponding to death time by the weight correction factor from Sommer's Table; then determine median number of MU for this group. (Consider death time of survivors as >60 min or equivalent to <0.875 MU in calculating median.) Convert MU to µg PSP toxin (STXeq) by multiplying by the CF value:

µgSTXeq/100g tissue = (µg/ml) X dilution factor X 200

Consider any value >80 µgSTXeq/100g as hazardous and unsafe for human consumption.

APPENDIX II: MOUSE BIOASSAY FOR NSP (BREVETOXINS)

Apparatus:

Electric blender
Analytical balance
Hot plate
Explosion-proof centrifuge with 250 ml centrifuge cups; covered with foil
Explosion-proof chemical hood
1000 ml separatory funnel
400 ml beakers.
Disposable syringes with 26-gauge needles (do not reuse)
Stopwatch, mechanical or electronic, registering to at least 1 s

Reagents:

Hydrochloric acid (HCL), concentrated
Sodium chloride (NaCl)
Cottonseed oil
Diethyl ether, anhydrous, AR (ACS). Use ether only from a previously unopened container- peroxides reduce apparent toxicity.

Test Animals:

Healthy albino male mice (Swiss-Webster strain) weighing 20±1 g are preferable, however, mice weighing between 15 and 25 g may be used. Do not reuse surviving mice. Because mice often eat excessively and may store 1 to 2 g of food in the digestive tract, feed them sufficiently but do not overfeed.

Sample Preparation and Extraction:

Clean, shuck, and drain shellfish. The number of shellfish required for 100 g of homogenate varies from two large clams to eight to 10 small oysters. Homogenize shellfish tissues in an electric blender at high speed for 5 min. Weigh 100 g of the homogenate into a pre-weighed 400 ml beaker and add 5 g NaCl and 1 ml conc. HCL. Stir well. Heat mixture to boiling and cook for 5 min; stir frequently. Let cool to room temperature and transfer to a 1000 ml separatory funnel. Rinse beaker with ether; add rinse to separatory funnel. Perform all subsequent steps under an explosion-proof hood.

Add 100 ml ether to homogenate, stopper, and shake vigorously (venting frequently) for 5 min. Centrifuge at 2000 rpm for 15 min. Carefully decant upper clear yellow ether phase into a 1000 ml separatory funnel, keeping solids in the centrifuge bottle. Repeat extraction three more times until the total amount of ether used is 400 ml. Drain off and discard any bottom layer containing small shellfish pieces and/or water emulsion so that only the ether phase remains. Transfer ether extract to a 400 ml beaker pre-weighed to the nearest 0.01 g. Let ether evaporate in air under the hood until no trace of ether fumes is discernible. An oil residue, which is the crude toxin extract, will remain. Cover tightly and freeze for later bioassay.

Mouse Assay:

Bring weight of crude toxin residue to 9.17 g with cottonseed oil. The volume of oil and toxin mixture represents 10 ml. Thoroughly mix and break up remaining pieces of insoluble matter as possible with a stirring rod.

Slowly fill syringe (with needle in place) with 1 ml of residue-cottonseed oil mixture. Carefully inject (i.p.) 1 ml into each of two weighed mice on the ventral side anterior to the hind leg. If more than one drop of injected mixture leaks from the mouse, reject the mouse and inject another. Record time of injection. If the two mice survive for 2 h, inject three more mice with 1 ml of residue-cottonseed oil mixture or if the two mice die in less than 2 h, make dilutions until the injection solution causes the death of two mice in 2 to 6 h. (Note: the

recommended dilution is 1:1.25, made by adding 2 ml cottonseed oil to the remaining 8 ml of residue-cottonseed oil mixture.) Repeat dilutions if necessary. When the correct dilution is found, inject three more mice.

Observe mice continuously for 6 h. The death time is the time elapsed from injection to the last breath of a mouse. The eyes will darken immediately upon death. If mice survive for 6 h, hold them for a total of 24 h. For a 6 h continual observation period the lower limit of the assay sensitivity is 20 MU/100 g shellfish tissue. Extending the continuous observation period to 15.5 h will increase the assay sensitivity to 10 MU/100 g shellfish. If mice die following continuous observation but within the 24 h period, toxin is present in low quantity.

Death may not occur during an assay, yet physiological signs of NSP toxicity may be observed. The common signs of low NSP toxicity (nonlethal) are weakness of the limbs, imbalance, occasional respiratory spasms, and prolonged lethargy. Acute toxic signs include front and hindquarter paralysis resulting in instability, laboured breathing, prostration, or hyperactivity. To observe nontoxic behavior for comparison, inject two mice with only cottonseed oil to serve as controls.

Toxicity Calculations:

Using Table 3, calculate the corrected MU activity using the following formula: MU X weight correction X dilution = MU/100g shellfish If additional dilutions were not made, use the ten-fold dilution factor - based on the initial addition of cottonseed oil to 10 ml. If additional dilutions were made, multiply these factors also. Interpolate death times and weight corrections that fall between table values. Example: If a 22.3 g mouse died in 390 min, using the original dilution, then the MU = 1.9 X 1.14 X 10 = 21.7.

If mice die after continuous observation, calculate MU as though death occurred at the end of the continuous observation period. Report results as an indeterminate value of "less than"(<), i.e., less than the sensitivity of the test for that time period. If mice survive the 24 h period, assign a value of <10 MU, because the lowest, reproducible sensitivity of the assay is 10 MU/100 g shellfish meat.

Calculate the mean MU if 100% mortality occurs and death times are determinate, or determine the median MU if <100% mortality is observed or if death times are indeterminate. When reporting indeterminate toxicity, note the number of mice that died in 24 h, or if no mice die in 24 h, report toxin as undetectable (<10 MU/100 g shellfish meat). Consider any detectable level of toxin per 100g shellfish tissue as rendering the shellfish potentially unsafe for human consumption.

Table 10.4. Relationship of dose to death time and weight of mice injected with NSP toxins (brevetoxins) extracted from shellfish.

Death time (min) (20 g mice)	Mouse Units (MU/ml)	Mouse Weight (g)	Weight Correction Factor
8	10.0	15	0.69
10	9.0	16	0.75
12	8.0	17	0.81
14	7.0	18	0.87
16	6.0	19	0.94
18	5.0	20	1.00
20	4.5	21	1.06
30	4.0	22	1.12
38	3.8	23	1.18
45	3.6	24	1.24
60	3.4	25	1.30
83	3.2	26	1.36
105	3.0		
140	2.8		
180	2.6		
234	2.4		
300	2.2		
360	2.0		
435	1.8		
540	1.6		
645	1.4		
780	1.2		
930	1.0		

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11. Cyst methodologies

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Many marine phytoplankton species produce dormant cysts or resting spores during their life histories. Alternation between a dormant, benthic stage and a motile, vegetative existence is a complex process that must be considered in our efforts to understand and manage blooms of harmful algal species. Cyst germination provides an inoculum for many blooms, and cyst formation can subsequently remove substantial numbers of cells in later stages. Such cells have other important ecological roles with respect to species dispersal, survival through adverse conditions, and genetic recombination when sexuality is involved in their formation (Wall, 1971). Among the toxic or harmful marine phytoplankton species are many that use this life history strategy. Some of these are listed in Table 11.1, though it should be recognized that many unlisted (non-toxic) species can cause harm due to dense biomass accumulation and anoxia. Here we present a compilation of methodologies useful in laboratory and field studies of phytoplankton resting cysts and spores. Methods for dinoflagellate cysts will dominate the presentation, since most cyst-forming species belong to this group and it has therefore been studied most thoroughly. Information relevant to other algal classes will also be presented when appropriate.

GENERAL CONCEPTS, DEFINITIONS

Cysts

Most toxic or harmful species reproduce by asexual, binary division. Under certain conditions, however, sexuality is induced, involving a series of developmental events that produce morphologically and physiologically distinct cell types called gametes, zygotes, and hypnozygotes (reviewed in Pfiester and Anderson, 1987). The term "cyst" is used to describe a non-motile cell which lacks flagella and an ability to swim. Dinoflagellates form two different types of cysts - temporary cysts and resting cysts (Fig. 11.1). In this manual, the term "cyst" will refer to "resting cyst" or hypnozygote. The terms "germination" and "excystment" will be used synonymously, as will "cyst formation" and "encystment".

Temporary cyst

This non-motile cell is formed when motile, vegetative cells are exposed to unfavorable conditions such as mechanical shock or a sudden change of temperature or salinity. They are typically round or oval-shaped protoplasts liberated by thecal rupture (ecdysis). Initially, cell contents are the same as those of vegetative cells, but through time, starch grains become apparent and pigments break down and change their cellular distribution (Anderson, 1980). Temporary cysts are frequently observed in laboratory cultures, especially in stationary growth phase. They are occasionally observed in natural plankton samples, although it is always difficult to ascertain whether the cysts were present naturally, or were formed by the stresses of the sampling. When conditions become favorable again, temporary cysts quickly re-establish a vegetative, motile existence. The dormancy interval thus allows them to withstand short-term environmental fluctuations. All planktonic species can have a temporary cyst stage, and for most, this stage is unrelated to the reproductive process. However, some species such as

Alexandrium hiranoi and *Peridinium quinquecorne* use this stage for asexual reproduction (i.e. they can complete asexual cell division only through the formation of temporary cysts).

Resting cyst

This thick-walled, highly-resistant stage is occasionally formed in cultures and routinely occurs in natural plankton populations, often towards the end of a bloom (Anderson *et al.*, 1983; Lewis *et al.*, 1979). Resting cyst formation (Fig. 11.1) begins with the sexual fusion of gametes, which produce a swimming zygote (planozygote) that remains in the plankton for several days before falling to the sediment as a non-motile cyst (termed a hypnozygote). Under favorable conditions, cysts can remain viable in sediments for 5-10 years, sometimes even longer.

Dormancy vs. quiescence

It is important to use dormancy terminology with care. The literature on seeds of higher plants defines "dormancy" as the suspension of growth by active endogenous inhibition, and "quiescence" as the suspension of growth by unfavorable environmental (i.e. exogenous) conditions. Thus dormant cysts cannot germinate, even under optimal environmental conditions, while quiescent cysts are competent to germinate, but are inhibited from doing so by some environmental factor. Most cysts must proceed through a mandatory resting period (lasting weeks to months, depending on species) before they are capable of germination. This interval is generally considered a time for physiological "maturation" (Pfiester and Anderson, 1987). The length of this mandatory interval varies considerably among species (12 hrs to 6 months; Pfiester, 1977; Anderson, 1980), and for a single species, can vary with the storage temperature as well. Thus cysts of *A. tamarense* stored at 4°C mature in 4-6 months, whereas storage at warmer temperatures shortens the mandatory interval to 3 months or less (Anderson, 1980). The duration of this process can have a significant effect on the timing of recurrent blooms, as species with a long maturation requirement may only seed one or two blooms per year, whereas those that can germinate in less time may cycle repeatedly between the plankton and the benthos and contribute to multiple blooms in a single season. Recent study, however, suggests that some species such as *Gymnodinium catenatum* and *Pyrodinium bahamense* may not require this maturation period (Blackburn *et al.*, 1989). Once a cyst is mature and the dormancy interval is over, the resting state will continue if external conditions are unfavorable for growth. Thus a quiescent cyst cannot germinate until an applied external constraint (such as cold temperature) is removed. A further complication arises in species which can alternate between dormancy and quiescence through time, due to an endogenous annual "clock" which restricts germination to a particular time of the year (Anderson and Keafer, 1987). Mature, quiescent cysts of *A. tamarense* did not germinate in a consistent manner when exposed to optimal growth conditions throughout the year, but instead showed a variable response depending on the season. An endogenous annual clock was implicated, which might explain the germination of cysts deposited in deep waters where seasonal environmental cues such as temperature or daylength are small or nonexistent.

Factors controlling quiescence

The factors that initiate germination of mature cysts are not known for all species. The primary stimulus for excystment of temperate species is generally accepted to be a shift in temperature to favorable levels, as occurs in seasonal warming or cooling (Huber and Nipkow, 1922, 1923; Anderson and Wall, 1978; Anderson and Morel, 1979). Spontaneous germination of cysts without a change in temperature has been noted on several occasions, however (von Stosch, 1973; Pfiester, 1975, 1977; Binder and Anderson, 1987). Cysts stored at cold temperatures

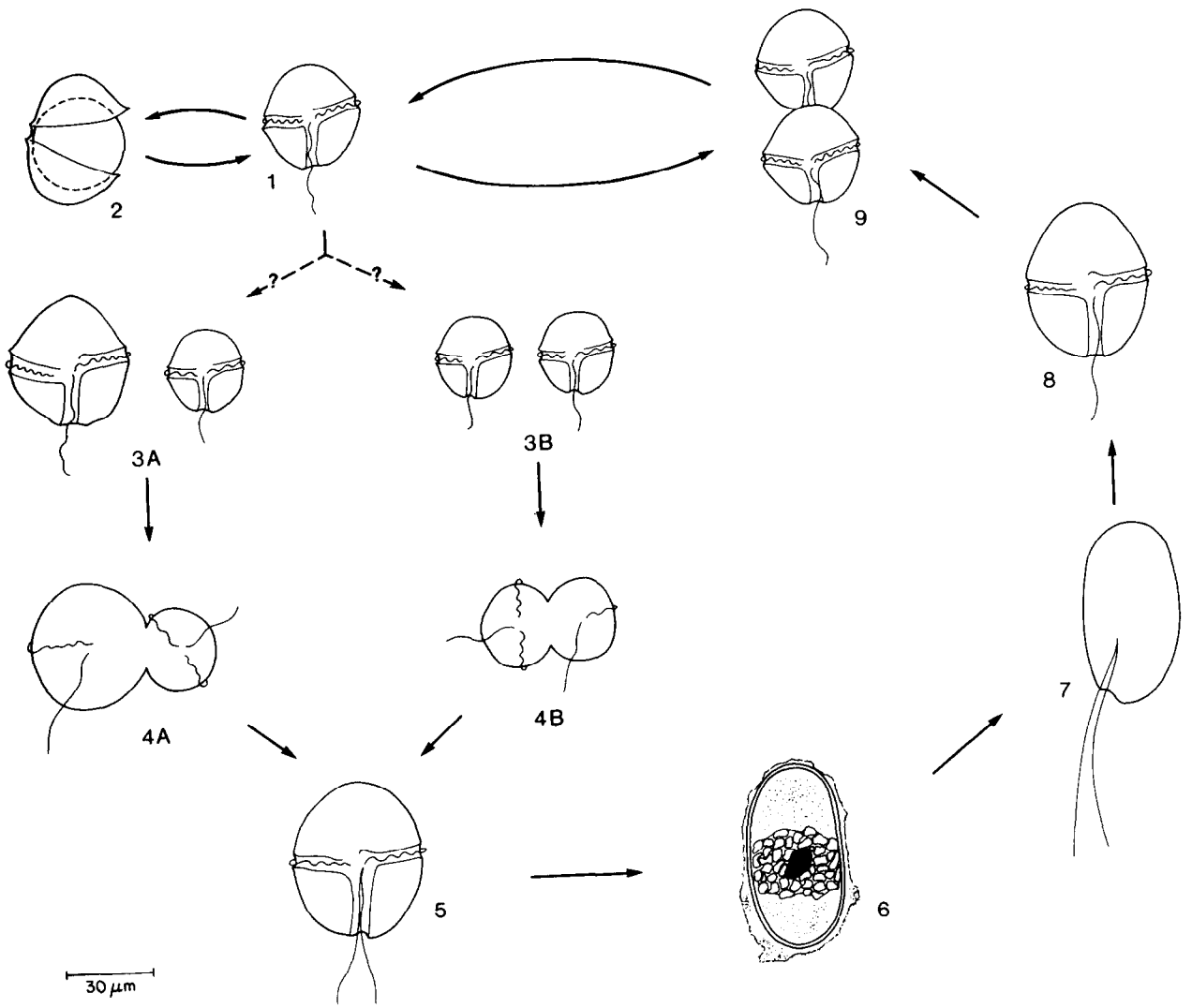

Figure 11.1. Life cycle diagram of a dinoflagellate, using *Alexandrium tamarense* as a model. Two different gamete formation strategies are shown for generality. Stages are identified as follows: (1) vegetative, motile cell; (2)temporary or pellicle cyst; (3A) anisogamous "female" and "male" gametes; (3B) isogamous gametes; (4A & 4B) fusing gametes; (5) swimming zygote or planozygote; (6) resting cyst or hypnozygote; (7&8) motile, germinated cell or planomeiocyte; and (9) pair of vegetative cells following division.

often remain quiescent until the temperature is increased (Huber and Nipkow, 1922; Anderson, 1980). A similar phenomenon has been observed for cysts of *A. tamarense* held at high temperatures, which maintained quiescence for a year and germinated only when temperatures decreased to a favorable level (Anderson and Morel, 1979; Anderson, 1980). This argues for the existence of a permissive temperature "window" within which quiescent cysts will germinate, but outside of which they will continue their resting state (Dale, 1983; Pfiester and Anderson, 1987). In general, temperature can maintain quiescence for extended periods, determine the duration of dormancy after cyst formation, synchronize or entrain cyst populations for more uniform germination, and initiate the excystment process (reviewed in Pfiester and Anderson, 1987). Temperature is thus very important in the dynamics of dormancy, quiescence and germination, though much of the research to date has focused on temperate species. Species from tropical waters where temperature fluctuations are less dramatic might not be as reliant on temperature cues, but this awaits further research.

The effects of other environmental factors on dormancy and excystment are less studied. Nutrient concentrations and other water chemistry variables are not thought to exert significant influence on germination in dinoflagellate cysts, although Binder and Anderson (1987) report that germination of *Scrippsiella trochoidea* cysts was significantly slower in unenriched medium compared to nutrient-replete medium. However, large numbers of cysts often remain in the sediments even though ambient temperatures are suitable for excystment and cell division (Anderson *et al.*, 1983). This is attributed to the burial of many cysts beneath the oxygenated surface layer of sediments. All species tested thus far (Anderson *et al.*, 1987) have an absolute requirement for oxygen during germination. Some species must be exposed to light for either brief (Binder and Anderson, 1986) or prolonged intervals (Anderson *et al.*, 1987) before excystment is possible. Cysts that are buried deep in the sediment can thus remain quiescent for years, their fate either being eventual death if anoxia persists, or germination should they be transported to the sediment surface or overlying water.

Cyst identification

More than 81 marine and 20 freshwater species of modern dinoflagellates are known to produce cysts (Matsuoka *et al.*, 1989a; unpub. data). Of these species, less than a dozen have been known to cause red tides or toxic episodes (Table 11.1). Some cysts are similar to their motile form, but many are completely dissimilar. The important features used in identification of cysts are the shape of the cyst body and ornaments, wall structure and color, and the type of archeopyle (excystment opening) through which the germinated cell emerges. The archeopyle is a very useful criterion for classification to the family and genus levels. It is not visible, however, before excystment, so it is not possible to use this characteristic for identification of living cysts. Furthermore, in comparison with the morphology of motile cells, cysts are usually relatively simple, mostly spherical in shape. As a result, identification of cysts based on a single morphological character is not always reliable, and other characters such as morphology of ornaments, wall structure, color, and paratabulation must be examined. Descriptions and photographs of the cysts of harmful algal species are found elsewhere in this volume. For further details, a diagram of archeopyle types and several keys based on cyst shape and archeopyle type can be found in Matsuoka *et al.* (1989a) and in Chapter 20 of this Manual.

FIELD STUDIES

CYST DISTRIBUTIONS (MAPPING)

Knowledge of the distribution and abundance of cysts can be very useful in ecological and monitoring studies. Historically, such studies have been used to define the geographic range or

bloom dynamics of a particular harmful species (e.g. Anderson *et al.*, 1982a,b; Imai *et al.*, 1991), to identify potential "seedbeds" for bloom initiation (Tyler *et al.*, 1982) or sites for monitoring (Anderson *et al.*, 1982b), or to study the dispersal of an organism from one region to another (e.g. Anderson *et al.*, 1982b; Imai *et al.*, 1991; Tyler *et al.*, 1982). In some cases, it is useful to assess only the presence or absence of a species in a sample, whereas in other situations, a quantitative estimate of the abundance of that species is needed. Clearly, the methods used for these two different determinations will differ. There is probably limited need for quantitative cyst surveys in the initial phases of most projects. Time and effort would be better directed towards large-scale surveys on a presence vs. absence basis rather than quantitative studies over a necessarily much smaller area. Once the baseline surveys are established, it might then be appropriate to monitor cyst population dynamics using quantitative methods.

Table 11.1 Toxic or harmful species producing a resting cyst

MARINE SPECIES	**REFERENCE**
Dinophyceae	
Cochlodinium sp.	Fukuyo (1982)
Cochlodinium sp.	Matsuoka (1985a, 1987)
Gymnodinium catenatum	Anderson, *et al.* (1988), Matsuoka (1987),
Alexandrium catenella	Yoshimatsu (1981)
Alexandrium cohorticula	Fukuyo *et al.* (1990)
Alexandrium minutum	Bolch *et al.* (1991)
Alexandrium monilatum	Walker et Steidinger (1979)
Alexandrium tamarense (= *A. excavatum*)	Dale (1977), Anderson and Wall (1978), Anderson and Wall (1978)
Pyrodinium bahamense var. *compressum*	Steidinger *et al.*(1980); Matsuoka *et al.* (1989b)
Raphidophyceae	
Chattonella antiqua	Imai and Itoh (1988)
Chattonella marina	Imai and Itoh (1988)
Heterosigma carterae (=*akashiwo*)	Imai and Itakura (1991)

SAMPLE COLLECTION

Site Selection

Cysts, as non-motile cells, will settle from the water column and accumulate in areas where lighter sedimentary materials such as silt and clay predominate. High energy environments are characterized by coarse, sandy substrates, which generally have low cyst abundance due to the winnowing away of finer materials. The best sites for cyst collection are thus those where the sediment is muddy rather than sandy. Sediment maps that indicate silt and clay areas can be used to identify good collection sites, or bathymetric maps can be used to identify basins or other depressions where finer materials can accumulate. Protected harbors and embayments are more likely to accumulate cysts than open coastal areas with wave and wind exposure. It is important also to avoid areas exposed to the air at low tide. Living cysts can still be found in such sediments, but viability is better from sites that remain permanently submerged.

Presence vs. absence

The geographic distributions of the cysts of several species have been mapped using qualitative approaches. For example, the simple presence or absence of *A. tamarense* cysts was determined along the coast of southern New England, highlighting regions with the potential for PSP, including several that had no prior history of the problem (Anderson *et al.*, 1982b).

When the absolute abundance of a species is not needed, a variety of sampling methods can be employed to collect sediment and analyze it for the organism of interest. These include commercial coring devices and grab samplers, as well as other devices which can be modified for sediment collection. For example, an old plankton net can be lowered to the sediment surface and slowly dragged across the bottom to collect the surface sediment layer that is often very rich in cysts. In shallower waters, a hand-held, manual bilge pump connected to a garden hose with a flattened funnel taped to the open end can be used to "vacuum" the surface sediment layer into a container. Either of these two approaches can be used in shallow waters from small boats or even while wading. If a grab sampler is used, care must be taken that the surface layer is not lost during retrieval, as it contains cysts formed in the recent past. Deeper in the sediment, cysts might be years or even decades older.

Boats are not always necessary. Careful site selection may permit access from land with chest waders. This is one way to increase the number of stations sampled for a given period of time or for a fixed budget. Another useful non-quantitative sampler is an electric or battery-powered submersible pump that can be lowered to the bottom and used as a vacuum. Be aware that pebbles and debris can clog the pump. SCUBA divers are also useful, but this requires more personnel, is depth-limited, and takes more time.

Clearly there is no standard method for collection. In shallow areas where boats cannot be used, the bilge pump is probably the method of choice. In deeper areas, a plankton net or grab sampler should be used when there is no winch, or when possible, collect several gravity cores and combine surface sediments (i.e. the top few cm that can be swirled and poured off easily).

Relative Abundance Surveys

Another survey approach is to count all cysts in a given sample, with each species then reported as a percentage of the total number. With this approach, it is not necessary to standardize the volume of sediment analyzed in each sample, so sampling and processing methods are considerably simpler than the quantitative approach described below. This method will give a good indication of where potential cyst beds are, especially if the sediments being sampled are fairly consistent with respect to cyst deposition dynamics. For comparisons among vastly different depositional sites over long distances of coastline, relative abundance can provide a useful measure of the relative importance of individual species in the plankton.

Quantitative surveys

Comprehensive, quantitative surveys have been conducted for *Alexandrium* species in the Gulf of Maine (Anderson and Keafer, 1985), the Bay of Fundy (White and Lewis, 1982), and the lower St. Lawrence estuary (Turgeon *et al.*, 1990; Cembella *et al.*, 1988). Other distributional studies include those for *Gyrodinium uncatenum* (Tyler *et al.*, 1982), *Gonyaulax polyedra* (Lewis *et al.*, 1979), and *Chattonella* (Imai and Itoh, 1988). The distribution and abundance of cysts have been shown to correlate with bathymetric features (e.g. basins), with the fine clay and silt sediment fractions, and with the transport pathways of major current systems (Anderson and Keafer, 1985; Cembella *et al.*, 1988).

Collection methods for studies in which the absolute number and distribution of cysts are to be determined place restrictions on the type of sampling to be conducted. The samples must be intact and undisturbed, and this typically calls for gravity or box corers. Some grab

samplers can obtain samples without loss of the surface layer, but this requires special precautions and should be verified before quantitative studies are initiated. Simple gravity corers can be manufactured at low cost, such as the one in Fig. 11.2 developed by the Tokyo University Fisheries Oceanography Laboratory. Some workers prefer to use corers with larger tube diameters than the TUFOL design (e.g. 5 cm rather than 2.2 cm). At the other extreme, a hydraulically damped Craib corer (Baxter *et al.*, 1981) obtains excellent samples (Anderson and

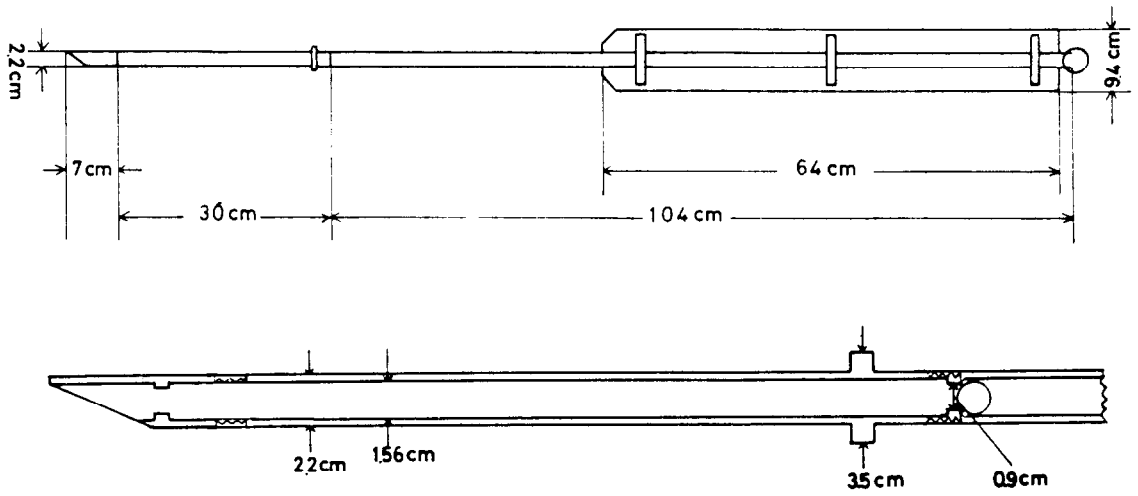

Figure 11.2. Simple gravity coring device designed by the Tokyo University Fisheries Oceanography Laboratory.

Keafer, 1985), but is quite expensive and can only be deployed from larger vessels. Box corers obtain large (0.1 m^2) samples that can be subsampled on deck by manually inserting small core tubes, but again the device is expensive and very heavy, and thus is restricted to larger vessels.

Once core samples are obtained, they should be capped (filled with water above the sediment), and stored in the dark and cold before processing. For temperate species at least, these precautions will minimize germination and bioturbation artifacts, and allow sample processing to be delayed until the samples can be returned to the laboratory.

Fixation

Since it is often possible to store living cysts for years in the laboratory, fixation is not commonly employed. In addition, fixation often alters the appearance of the cyst contents, removing one of the diagnostic criteria that are useful in their identification. Nevertheless, long-term records of cysts are useful, and preservation is therefore needed. Buffered formalin is often used for this purpose. Formalin (37% formaldehyde) saturated with carbonate or borate is added to sediment samples to a final concentration of 5-10%. If the sample is subsequently sieved and processed as described below, additional formalin is needed, though at a lower concentration (2-3%).

Storage

Resting cysts are highly resistant cells that can survive burial in sediments and sometimes ingestion by animals. Cyst longevity, which probably depends most critically on temperature and oxygen, varies considerably between species, but can be at least 5-10 years in natural marine sediments (Keafer *et al.*, 1992). Freshwater species are known to have survived for 17 years in lake sediments (Huber and Nipkow, 1922, 1923). Optimal survival appears to be associated with temperatures that are cold relative to the growth requirements of a particular species. For temperate species, storage at 2-4°C is desired. Storage of tropical species is problematic, as it is not yet clear how well they survive low temperatures. Refrigeration at 4°C may be harmful to tropical forms, so the best approach is probably to maintain the ambient temperature and to rely on anoxia to retard germination in stored samples. Storage of sediment samples in tightly-sealed containers quickly results in anoxic conditions due to organism respiration and the chemical oxygen demand of the sediments. Anoxia is effective in maintaining quiescence without excessive mortality (Anderson *et al.*, 1987). If the sample is not sealed and is instead left open to the atmosphere, oxygenation of surface sediments can lead to germination of cysts in upper sediment layers.

SAMPLE PROCESSING

Two basic procedures are used to process sediment samples for dinoflagellate cysts. One used predominantly for living cysts involves sieving (and sometimes sonication), but no harsh chemicals. This was originally described by Wall and Dale (1968). The other is a chemically rigorous palynological technique which leaves only cyst walls intact (see Dale, 1979). A separate procedure useful for cysts of *Chattonella* and other species is also given below.

Sieving technique (non-quantitative)

1. Prepare a series of sieves of various mesh-sizes. These can either be commercially available metal sieves employed in sediment grain size analysis, or hand-made, inexpensive units (short, open segments of PVC pipe with Nitex mesh epoxied to one end). Useful sizes are 250, 125, 80, 38, and 20 μm. Choose a suitable series of sieves for the sediments being processed and the species of interest. For example, a 250 μm sieve is not needed unless sediments contain many broken pieces of shell or plant tissue. For cysts of small species (i.e. *Alexandrium* or *Scrippsiella)*, a 20 μm sieve is used in the final step to collect the particles of interest; for larger species, a 38 μm sieve is used. Usually, only two sieves are needed, one to remove large debris and the smaller to collect the particles in the size range of interest.

2. Take 5-10 mls of sediment slurry and add filtered seawater (FSW). This slurry can then be sonicated or not, depending on the objective of the study. If cyst enumeration is the only goal, sonication is recommended, as it disaggregates particles and frees cysts from other detritus. Sonication prior to physiological studies is potentially problematic, as it is not known if the ultrasound has effects on cyst physiology. When clean, detritus-free cysts are required for a study, sonication is the only alternative. In such cases, careful temperature control using a water bath during sonication is recommended to eliminate heating effects. Probe sonicators are preferable to bath-type units due to their higher energy, although the latter will work if samples are small and are left in the bath for extended periods (5-10 min, minimum). There are many different brands of sonicators, so the necessary processing time varies. Start with 30 sec to 1 min at a moderate setting with probe units. This can be adjusted depending on results.

3. The sonicated (or unsonicated) sample is then poured slowly through the largest sieve (typically 125 or 80 μm) and the filtrate collected in a beaker. The sieve is rinsed thoroughly with FSW, and all the liquid that was collected in the beaker is poured through the smallest mesh (typically 38 or 20 μm). This liquid will go through more slowly than with the larger mesh, but flow can be increased by tapping the sieve with your fingers from below, blotting on paper towels, or by spraying with FSW. The material on the sieve is then washed into another beaker, Petri dish, or vial. The final dilution of sediment into the vial with the FSW should be light-gray in color. Five to 10 mls of slurry optimally gives about 10 mls of suspension for analysis.

4. There are several methods for separating the cysts from heavier materials. One is to put the final suspension in a small beaker (25-50 ml) and to move the beaker in continuous small circles on the bench top. This creates a vortex in the sample, which collects sand in the center and keeps cysts and lighter material in suspension. A pipette can then be used to remove the bulk of the liquid while the swirling continues, leaving behind a residue of sand to be discarded. Alternatively, if the sample is placed in a Petri dish, FSW can be squirted at one side of the dish using a wash bottle in such a way that the residue is surrounded by the swirling motion of water. Cysts and other light particles will be suspended in the circulating water while heavy sand particles remain at the bottom in the center of the Petri-dish.

Sieving technique (quantitative)

Quantitative methods for cysts in sediment cores are detailed in Anderson *et al.* (1982a) but are summarized below for convenience. The following procedure applies to intact sediment cores.

1. Set-up a ringstand near a sink or source of running water and stabilize with duct tape, C-clamps, or a lead weight at the base. Place a bucket in the sink for the discarded sediment.

2. Place the core tube in the ringstand, remove top core cap, and then carefully remove the overlying water by aspiration. Be sure that the core is undisturbed and that any surface disturbances have settled before aspirating. Suction all the water, removing as little of the flocculent surface layer as possible.

3. Make an extrusion stopper – typically a rubber stopper that is cut horizontally so that it fits snugly inside the core tube. It should be snug enough to support the weight of the overlying sediment without sliding down the core tube, yet loose enough to allow you to push the sediment upwards from below. Place the extrusion stopper on the lab bench.

4. Remove the bottom core cap and very quickly push the bottom of the core tube onto the extrusion stopper while pushing down on the core tube. Place an extruder against the bottom of the core tube and push the sediment up until the top edge of the sediment is even with the top edge of the core tube. The extruder is any cylindrical device that is smaller than the diameter of the core tube that can be used to push the extrusion stopper upwards. A smaller diameter, but longer (capped) core tube works well.

5. Wipe any moisture off the core tube and mark, with a waterproof pen, the intervals to be sectioned. Typically the core is cut into 1 or 2 cm slices, 6-12 cm deep into the core. Start by making the first mark at a convenient and visible reference point on the core tube, usually the interface between the extruder and the bottom of the extrusion stopper. Using a metric rule, mark off the appropriate intervals up to the desired depth or until you reach the top of the core tube.

6. To subsample the first interval, which is usually very liquid, push the fluid sediment up a few millimeters. Place a beaker alongside the top edge of the core tube, then scrape the

sediment into the beaker with the large spatula. Repeat until you have reached the interval mark (i.e. 1-2 cm) as designated by lining up the interval marks on the core tube with the reference point (i.e. the extrusion stopper/extruder interface). Do not push too far at any one time or the sediment will run down the side of the core tube, losing part of the sample.

7. Using a small spatula, mix and then subsample from the sediment in the beaker. Measure the volume of the mud (usually 5 cm^3) in a cut off syringe, eliminating all air pockets. The spoon end of the spatula works well to load the syringe and the flat end is used to scrape off the excess. Dispense the mud into a sample container. Rinse the syringe with FSW into the sample container and then rinse the walls of the sample container. Label the container with sample location, date, depth interval, etc.

8. Rinse the beaker, spatulas and syringe with tap water or seawater to prepare for the next interval. Push the sediment up another 1-2 cm with the extruder. The deeper the core is penetrated, the more compact the sediments become, and therefore less necessary to push the sediment up in small increments to complete the interval. Due to wall friction, the lower sediment layers become contaminated by the upper layers during the coring and extruding process. The outside edge of the sediment core should thus be trimmed and discarded (a few mm). This is best accomplished by slightly loosening the clamps on the ring stand, allowing the core tube to be rotated while cutting the outer edge of the sediment. Scrape the remaining sediment into a dry beaker and subsample as in Step No. 7.

9. Repeat Step No. 8 until all the intervals are subsampled. Again, the sequence of steps is to push the sediment up, trim and discard the outer edge, scrape the sample into a beaker, mix the contents of the beaker, and subsample using the small spatula and the syringe.

10. Be sure that all the samples contain adequate seawater so they will not dry out. Store the samples at an appropriate temperature (usually 2-4°C for temperate species) in the dark.

Cyst concentration

For both techniques mentioned above, one constraint is that sieved material predominantly consists of sediment and detritus. Unless cyst concentrations are high, enumeration and isolation are very time consuming. In addition, it is difficult to state with conviction that a given area is free of cysts since the methods and time constraints typically limit the amount of sediment actually examined to approximately 1 ml. Techniques have thus been developed to concentrate cysts from both sediments and laboratory cultures. The Percoll-seawater density gradient procedure which has been used to separate live meiofauna and microfauna from sediments is not suitable for dinoflagellate cyst concentration because the maximum density of the medium is ~ 1.15 g cm^{-3}, which will not "float" all cysts. The metrizamide gradient method used by Anderson *et al.* (1985) has the correct range of densities for cysts, but is expensive for large-scale studies. A new method was thus developed for separating and concentrating cysts using a non-toxic, aqueous colloidal silica suspension called Nalco 1060 (Schwinghamer *et al.*, 1991). The procedure is summarized as follows.

1. Prepare a "light" and "dense" solution of Nalco. The light solution should be 22.46% (wt/vol) sucrose in distilled water, buffered to pH 8.1 with 0.0125 M Tris plus 0.0125 M Tris HCl (final concentration). The dense solution should be a 50% (wt/wt) suspension of colloidal silica (Nalco 1060; Nalco Chemical Co., Chicago, IL) and aqueous sucrose at a final concentration of 11.23% (wt/wt), buffered to pH 8.1 with 0.0125 M Tris plus 0.0125 M Tris HCl.

2. Prepare either linear gradients (using a gradient mixer) or a step gradient in a 50 ml centrifuge tube using the two solutions. A step gradient is formed by placing a 5 ml cyst

suspension in a 50 ml centrifuge tube. The 20 ml of light solution is carefully injected underneath the sample, thereby displacing it upwards. In the same manner, 20 ml of dense solution is pipetted beneath the light solution.

3. If a linear gradient is used, carefully *underlay* a 5 ml sediment slurry (or culture cyst suspension) below 40 ml of gradient. A pipette works well if care is taken not to disturb the layers.

4. Balance the tubes by adding or removing liquid at the surface.

5. Centrifuge at 3000 rpm (~ 1600 x g) for 30 min at 4°C.

6. With a linear gradient, withdraw centrifuged material in 5.0 ml aliquots using either the device described by Schwinghamer *et al.* (1991) or careful pipetting. One or at most two aliquots should contain a clean suspension of cysts. If a step gradient is used, cysts should be found at the interface between the light and dense solutions.

An alternative to physically concentrating cysts to facilitate detection or enumeration is to make the cysts themselves more visible so that they stand out against a background of detritus and other organisms. One approach recently developed by Yamaguchi *et al.* (1995) relies on the fluorochrome primuline, which binds to the outer wall of *A. tamarense* cysts. When viewed with an epifluorescence microscope, the primuline-labeled cysts exhibit an intense yellow-green fluorescence under blue-light excitation. This makes it easy to find and enumerate *A. tamarense* cysts in a sediment sample. The method also stains other dinoflagellate cysts, but the fluorescence characteristics are sometimes different. The dark walls of *Protoperidinium* cysts, for example, do not fluoresce well following primuline staining. Work is in progress to examine the applicability of this method to a variety of dinoflagellate cysts.

Palynological technique

The technique introduced here is mainly based on standard palynological processing (Matsuoka *et al.*, 1989a). It uses several dangerous chemicals and therefore should be undertaken only with adequate safety precautions.

1. Place 1-2 mls of sediment into a 15 ml polyethylene test tube (do not use glass). Centrifuge and wash with distilled water several times to remove salt.

2. Add 5% hydrochloric acid to the tube to remove calcium carbonate from calcareous nanoplankton, foraminifera and other organisms. The calcareous cyst wall and ornaments such as on *Scrippsiella* and *Ensiculifera* will also be removed at this time, but the inner organic phragma will remain.

3. Centrifuge and wash with distilled water.

4. Add 1% potassium hydroxide solution to the tube and warm to 70°C in a water bath for 3 min. At higher temperature and with longer heating, the relatively thin phragma of *Protoperidinium* and *Alexandrium* sometimes disappear.

5. Centrifuge and wash with distilled water, then add concentrated (25-30%) hydrofluoric acid to the tube to remove silicate materials such as sand, diatoms, and silicoflagellates. Warm in the water bath at 70°C for 2-3 hrs. As the hydrofluoric acid is very dangerous and toxic, this processing should be carried out in a hood with rubber or vinyl gloves. The residue solution containing hydrofluoric acid should be neutralized with calcium carbonate.

6. Centrifuge and wash with distilled water. When cellulosic materials such as plant tissue are abundant in a sample, acetylation may be useful to remove them. The acetylation procedure is as follows:

   a. Add glacial acetic acid to the tube.
   b. Centrifuge and wash using Erdtman's solution, which is a mixture of 9 parts acetic anhydride and one part concentrated sulfuric acid.
   c. Warm the tube in a water bath at 70°C for 15 min.
   d. Remove the Erdtman's solution and add glacial acetic acid again.
   e. Centrifuge and wash with distilled water.

7. Prepare a series of sieves of various mesh-sizes with 250 µm being the upper sieve, 125 µm in the middle and 20 µm at the bottom.

8. Pour all the residue onto the upper sieve and wash it thoroughly. Cysts and other organic particles such as spores and pollen grains will pass through 250 and 125 µm sieves and accumulate on the 20 µm sieve.

9. Wash the remaining material on the 20 µm sieve into a 20 ml vial using distilled water for a final volume of 10 ml.

CYST ENUMERATION

For presence versus absence surveys, the absolute number of cysts of a target species is not as important as the fact that the species is present in a sample. Thus the objective is to scan sufficient material to make this determination, without devoting so much time that the survey becomes unrealistic. To be thorough, 3 mls or more of the final suspension should be examined in a 1 ml capacity Sedgewick Rafter slide or its equivalent before a negative finding is noted. Scanning at 100 or 160X total magnification is most efficient. If a cyst is found, this should be verified by looking for more. Any cyst identification that is unclear should not be counted and the scanning continued. A good quality microscope is very important in this process. Poor objectives or illumination, or even poor sample processing can make many copepod eggs and pollen grains look like cysts. Cysts may even need to be isolated for closer examination under higher power, since the Sedgewick Rafter slide often cannot be used with objectives above 16X magnification. Even those skilled in cyst identification will require 20-30 min to scan a 1 ml slide, therefore 1.5 hrs may be required for one sample (2 hrs if processing is included).

For quantitative studies, the objective is to obtain an accurate estimate of the cyst abundance in a sample. This often requires determination of the horizontal and vertical cyst distribution (Anderson *et al.*, 1982a). Vertical profiles of cysts within the top 6-10 cm of a core are useful in ascertaining the number of cysts that are near the oxygenated surface layer where germination is possible, as well as the total number of living cysts in a sample. Even deeper cores are sometimes used when the long- term history of cyst deposition is sought, such as in studies of species dispersal into an area (Keafer *et al.*, 1992) or of environmental changes in an area. Horizontal cyst maps are useful in delineating the population distribution in an area or in pinpointing potential seedbeds (Tyler *et al.*, 1982; White and Lewis, 1982; Anderson and Keafer, 1985). The most useful approach is an areal contour map, which smoothes out small-scale irregularities in the cyst distribution and provides a good image of the overall cyst distribution in surface sediments. To accomplish this, it is necessary to arbitrarily select a depth interval over which the cyst abundance is to be tabulated. Given that marine sediments are typically anoxic below the top cm, one approach would be to tabulate and plot only those cysts in the oxygenated surface layer. However, this ignores the majority of the living cysts at a station, and does not account for resuspension of deeper cysts by storms, fishing, or animal activity. A compromise approach is to tabulate all cysts in the top 4-6 cm of sediment (Anderson *et al.*, 1982a).

Once the core has been processed as described above, cysts can be enumerated in any counting chamber as long as it holds sufficient volume. A 1 ml Sedgewick Rafter slide is often used, but if cysts are abundant, a 0.1 ml Palmer Maloney slide might suffice. Care should be taken to mix the cyst suspension well before subsampling, and to distribute the sample evenly over the slide. The number of cysts to count will vary with the level of accuracy desired and the time available for the study. Once the number of examined cysts is known in a given volume, the abundance in the original sediment sample can be calculated knowing the volume of sediment processed. Results are usually expressed as cysts cm^{-3} or cysts ml^{-1} of sediment, but it is also possible to dry and weigh the sediment sample so that cysts g^{-1} can be determined. Originally, it was thought that compaction of sediments would make cyst counts inaccurate if normalized to volume rather than mass, but at least in the top 10 cm of sediment, these two units of measure are essentially equivalent so the simpler approach using volume units is often employed.

Most Probable Number Method

This technique was initially introduced for the quantitative analysis of cysts of *Chattonella antiqua*, *C. marina* and resting spores of *Skeletonema costatum* in sediments by Imai *et al.* (1984) under the name "extinction dilution method". The method is useful for these and other cysts that are simple in morphology and small in size, and thus difficult to detect in sediment samples. Later Erard-Le Denn (1992) adopted this technique under yet a different name for the quantitative estimation of *Alexandrium minutum* cysts. Note that numerical data obtained by this technique are not necessarilly equivalent to the number of living cysts in sediments determined by sieving techniques. The MPN data give an estimate of the abundance of cysts that are capable of germinating at that time, but since some cysts might be newly-formed, and thus incapable of germination (i.e. they are immature), the MPN method would underestimate the potential seed population of that species. It is important to understand the dormancy and excystment characteristics of the species being investigated if this method is to be used. The summary of this method given by Imai *et al.* (1984) follows, with minor modifications. Additional discussion of this method is given in chapter 4 of this volume.

1) One gram of sediment is taken from a core or other bottom sample and sieved using 100 µm and 20 µm mesh sizes.
2) The fraction retained on the 20 µm sieve is then resuspended in 10 ml of FSW in order to get a base sample in which sediment density is one-tenth of the original.
3) The base sample is diluted with culture medium as appropriate for a desired dilution series, final volume 10 mls. Dilutions of 1:10 and 1:100 are commonly used, though other options should be considered. Five replicate test tubes are then filled with 1 ml aliquots of the base and the diluted samples, all of which are then incubated under appropriate temperature and light conditions for the species under study.
4) During the incubation, the appearance of vegetative cells in the tubes is checked at four day intervals under a microscope.
5) The number of tubes in which the vegetative cells appear are scored as positives.
6) The most probable number (MPN) of cysts in the sediment sample is then calculated according to the statistical table of Throndsen (1978). See also Chapter 4 of this Manual.

CYST DYNAMICS

Cysts are important during both the initiation and decline phases of blooms, so it is often desirable to study the magnitude and mechanisms of excystment and encystment. Despite the importance of such knowledge, however, it is difficult to obtain more than qualitative information about these processes. This is due to the complex ecological transformations that are occurring and the dynamic nature of the planktonic environment.

Encystment

Several approaches have been used to study the dynamics of encystment. These include the use of sediment traps, frequent observations of sediment samples, and enumeration of planozygotes in bloom populations or the relative proportions of living vs. empty cysts.

Perhaps the best estimates of the flux of cysts to the sediments during a bloom can be obtained using sediment traps (Balch *et al.*, 1983). However, there are no widely-accepted procedures or designs, and it is known that various configurations will collect different quantities of material. Furthermore, material resuspended from the bottom and collected in the trap may complicate the interpretation of sedimentation data. Quantitative analysis of sediment trap data is thus not recommended. It is, however, possible to learn a great deal at a qualitative level about the timing and relative magnitude of cyst formation as a component of phytoplankton population dynamics (Balch *et al.*, 1983). For these purposes, sediment traps need not be elaborate or expensive. A small trap consisting of a 2 liter wide-mouth polyethylene bottle attached to a line between a surface buoy and a bottom weight can be used to collect newly produced cysts. A relatively large trap consisting of three 20-liter polyethylene bottles tethered together can be used to collect cysts for toxin analysis.

Sediment sampling

In theory, it should be possible to repeatedly subsample an area to monitor the changes in cyst abundance as excystment and encystment occur through time. In practice, care must be taken to collect and process sufficient replicate cores at several different stations to account for the patchy distribution of cysts in the sediments and the patchy bloom populations in the overlying waters. Accurate positioning of the vessel is also necessary. In studies of this type, multiple cores are taken at each of several stations. These are sectioned and processed as described above, and the abundance of cysts in the surface layers carefully enumerated. Changes in the abundance of cysts of a species can then be correlated with the dynamics of the overlying motile cell bloom population. Changes in the morphology of the cysts are useful as well, since newly-formed cysts are sometimes easily distinguished from older, mature cysts (Anderson, 1980). For some species (see below), chlorophyll fluorescence can be used to distinguish new (and germinating) cysts from those that are dormant or quiescent.

Another approach is to identify life cycle stages in the plankton that are indicative of sexuality and cyst formation. For many species, gametes are not easily distinguished from asexually dividing vegetative cells, but planozygotes and newly formed cysts are distinctive. Tabulation of these different stages as a percentage of the total population of a species can provide valuable information on the timing and magnitude of cyst formation. The simplest method is to use the large size and distinctive morphology of the planozygotes as diagnostic indicators (e.g. Anderson *et al.*, 1983), but it is also possible to use cytological techniques to stain basal bodies and flagella to provide unequivocal evidence of the life-cycle stage of individual cells (Tyler *et al.*, 1982; Coats *et al.*, 1984).

Lewis *et al.* (1979) demonstrated the utility of monitoring the relative proportions of living vs. empty cysts of *Gonyaulax polyedra* in studies of the dynamics of that species. Samples were collected and processed as described above, but empty cyst walls were counted as well as living, viable cysts. As cysts were formed and deposited in surface sediments, the percentage of empty cysts decreased, providing a useful indication of the timing and magnitude of cyst formation. This approach is useful only for those species that have resistant and distinctive cyst walls. Species such as *Alexandrium*, which produce non-descript cysts, would be difficult to study in this manner since the cyst walls would be difficult to distinguish from other detritus in a sediment sample.

EXCYSTMENT

Several of the methods used for studies of encystment dynamics can also be applied to excystment studies. Frequent sampling of sediments to monitor cyst abundance and vertical distribution can sometimes reveal changes associated with excystment, though again, the patchiness of cyst accumulations makes this approach difficult. Monitoring of life cycle stage in the plankton can also be attempted, in this case the newly germinated planomeiocyte being of special interest. In dinoflagellates, planomeiocytes can be distinguished by their large size and trailing, "ski track" flagella, but such features are not always discernible without special scrutiny or cytological stains. One useful procedure is to use a viscous medium such as glycerol in FSW to immobilize living cells and to make their dual longitudinal flagella visible. This procedure is obviously difficult to apply to multiple, fresh field samples. An alternative but complex approach is to use protargol staining to highlight basal bodies as diagnostic features (Coats *et al.*, 1984).

Another useful technique that can provide i ıformation on the germination dynamics of some species involves monitoring the chlorophyll .luorescence of cysts. In *A. tamarense*, for example, quiescent cysts show no red fluorescence when excited with blue light until they begin to alter their physiology in preparation for germination. Cysts in surface sediments can thus be examined through time using an epifluorescence microscope to document the temporal change in the proportion of the population that is synthesizing chlorophyll. Anderson and Keafer (1985) used this approach to demonstrate the rapid germination cycle of *A. tamarense* in shallow waters, compared to the long, gradual germination process in deeper coastal waters.

LABORATORY STUDIES

ISOLATION AND GERMINATION

Laboratory studies of cyst-forming species require that cultures be established. This can either be accomplished by isolating individual vegetative cells from plankton samples, or by isolating cysts and germinating them to obtain the initial cells for cultures. Isolation procedures employ micropipettes of various types. Pasteur pipettes whose tips have been drawn out over a flame (50-100 µm diameter) are commonly used, either connected to a tube which the user sucks on to draw cells into the tip, or used alone such that capillary action draws the cells up. Another method uses capillary tubes (1 mm ID, non-heparinized) which are drawn into very fine, hollow strands over a flame and snapped off to produce a narrow tip. These are attached to thin tubing (such as that used on many nutrient autoanalyzers). The thin tubing and very fine capillary tip restrict the amount of material that is drawn into the tube with mouth suction.

A small amount (~ 0.5 ml) of the sieved cyst sample is placed onto a large counting chamber such as a Sedgewick Rafter slide. Filtered seawater is then added to 1 ml, and the cysts isolated by micropipette. If necessary, a small needle (insect pin) attached to the tip of a glass tube can be used to clear the background around a cyst on the slide.

Isolated cells or cysts are placed into culture medium suitable for the species of interest. They can be incubated in culture tubes, in the wells of tissue culture plates, in Petri dishes, or in individual slides. The 96-well tissue culture plates are useful because they can be monitored using a dissecting or an inverted microscope to determine the success of the isolations. The wells are half-filled with medium, individual cysts are isolated and deposited in the well, the cover replaced, and the entire chamber sealed with plastic tape for incubation. Plates can be easily scanned with an inverted or stereo microscope, but this does not allow cysts to be easily pinpointed for individual observation. It does, however, make it very easy to quantify cyst germination as long as the emerging cells swim or divide.

If accurate germination statistics are needed, the Palmer-Maloney slide method of Wall and Dale (1968) can be used. The cyst (or cysts - no more than 10) are isolated from a sediment

sample and placed in the center of the Palmer-Maloney chamber with a small drop of medium, and a ring of Vaseline is placed around the outer edge of the raised gray surface. A large coverslip is placed over this and pressed down so that the drop of medium becomes a flattened disc that remains in the center of the slide, not touching the edges of the well or the Vaseline. No appreciable evaporation will occur for at least one week. Individual cysts are then located and their coordinates noted using the Vernier scales found on many microscope stages. Use of the coordinates allows the user to return to each specific location to look for empty cyst walls. This becomes important when pennate diatoms and other algae overgrow the slide as contaminants. [NOTE: Palmer-Maloney slides are surprisingly expensive. One alternative is to cut one or two sheets of Parafilm to the size of a microscope slide. These are placed on the slide and a well is cut out of the center.]

Some workers use 36 mm Petri dishes as a convenient and reliable vessel for germination. Filled with 2-3 mls of medium and sealed with Parafilm or similar coverings, they do not lose medium to evaporation. The entire dish can be examined under a dissecting microscope, and cysts and vegetative cells easily recognized against the dark background. The base of the dish is sufficiently clear that examination and photography with an inverted microscope is possible as well. Once germination has occurred, cells can be isolated or left to divide for relatively long periods before transfer to larger culture vessels.

The tubes, Petri dishes, tissue culture plates, or slides are placed at a suitable growth temperature under lights, with germination expected in 2-5 days. If the original cyst processing and isolation was clean and thorough, it is possible to establish a culture directly from the swimming cells in the tissue culture wells, either through individual isolations or using many cells at once (if other algal contaminants are not present). It is generally desirable to establish true clonal cultures, so isolation of individual vegetative cells (one cell per culture tube or well) is usually necessary. Note that if multiple cells produced by a cyst germination are used to start a single culture, the result will not be clonal, in a strict sense of the word. This is because most cysts are the result of mating between male and female gametes, and the germinated cyst will produce these two genetically different mating types. A true clonal culture of a heterothallic organism should not be able to produce cysts unless it is combined with a culture of an opposite mating type.

ENCYSTMENT STUDIES

The ability to form cysts in laboratory cultures is a major asset to any research program on a particular organism. Examples for marine species are found in Watanabe *et al.* (1982), Anderson *et al.* (1984), Blackburn *et al.* (1989), and Nakamura *et al.* (1990). Unfortunately, some species that respond reasonably well to culturing do not form cysts as easily. It is thus often useful to start a culture collection of numerous isolates of the species of interest, in hopes that some of them will produce cysts in sufficient quantities for laboratory study.

In laboratory cultures, depletion of either nitrogen (nitrate or ammonium) or phosphorus will often induce sexuality (e.g. Pfiester, 1975; Turpin *et al.*, 1978; Anderson *et al.*, 1984). This needs to be carefully controlled, however, since cessation of growth in batch cultures due to over-crowding or carbon limitation does not generally induce cyst formation. If f/2 medium (Guillard and Ryther, 1962) is the standard, nutrient-replete growth medium for a species, reduction of phosphorus or nitrogen levels to f/40 or f/80 will often result in limitation and sexuality leading to cyst formation (Anderson *et al.*, 1984).

A problem arises in efforts to determine encystment rates using laboratory cultures, as nutrients are exhausted so quickly in batch cultures that some cells begin the process of cyst formation, but do not complete it due to unfavorable conditions (Anderson *et al.*, 1985; Anderson and Lindquist, 1985). The low yield of cysts (10-20%) reported in such studies is deceptive, since a large percentage of the motile population can be planozygotes that presumably would have formed cysts had conditions been suitable.

Nutrient limitation has also been implicated in sexual induction in natural populations, though no direct measurements have yet proven this inference. In fact, cyst production has been

observed when external nutrients were at or above concentrations that previously supported only vegetative growth (Anderson and Morel, 1979; Anderson *et al.*, 1983). The precise set of environmental cues that stimulate encystment is not well-defined, and recent studies indicate that factors other than macronutrient availability (e.g. iron stress) may be involved (Doucette and Harrison, 1989). Furthermore, given the discovery of endogenous control of cyst germination for *A. tamarense* (Anderson and Keafer, 1987), the possibility of endogenous or "clock"-regulated sexuality must also be considered.

If organisms are heterothallic (or if their mating characteristics are unknown), crosses between cultures of different isolates are necessary. In such cases, individual cultures of the strains to be crossed are grown in reduced nutrient medium and then combined. Multiple isolates crossed with each other and self-crossed will generate a matrix of successful matings that can be used to define the mating type of each isolate (e.g. Yoshimatsu 1984). For some fastidious species, extra precautions are necessary in medium preparation. Anderson *et al.* (1984) found that cyst yield of *A. tamarense* could be increased by minimizing precipitates and chemical contaminants during medium preparation and sterilization. Although tedious and somewhat expensive, the precautions described by those authors for glassware cleaning and medium preparation are recommended for optimal growth and cyst production.

EXCYSTMENT STUDIES

A number of basic physiological characteristics should be determined for the cyst of a species of interest. With respect to excystment, these include the length of the mandatory dormancy interval, and the factors that regulate quiescence or that trigger germination for that species. These studies are easiest when cysts can be formed in culture, but when laboratory cultures are not available for certain species, it is still possible to obtain relatively "clean" cyst preparations using field populations. One approach that has been successfully used on *Gymnodinium catenatum* (Anderson *et al.*, 1988) and numerous other dinoflagellate species (unpub. data) involves collection of a mixed plankton assemblage using a plankton net, and the resuspension of that material in filtered seawater from the study site which has been enriched with f/2 levels of vitamins, metals, and EDTA. Sometimes, f/80 levels of major nutrients are added as well. The assemblage is incubated in the laboratory at the temperature of the ambient water at an appropriate daylength and light intensity. Within a few days, cysts will be produced by many of the species in the sample. These are of a known age, and can readily be isolated by micropipette to study morphology or germination characteristics.

Cysts of a known age can also be collected in a sediment trap (deployed underneath a bloom but sufficiently far above the bottom to avoid resuspension of "old" cysts). The least attractive option, but one which will work if a recent bloom has produced abundant new cysts, is to collect and work with surface sediments directly. Cysts can be isolated individually for germination trials, or an alternative procedure can be attempted that utilizes unprocessed sediment. This involves the preparation of a large sample of sediment "slurry" in FSW, which is then subdivided into numerous subsamples. The cysts of interest in 5-10 of these aliquots are enumerated to obtain a statistically sound estimate of initial concentrations. The remainder of the subsamples are then incubated experimentally. The difference between the initial counts and the number of cysts remaining after incubation provides a good measure of germination success (Anderson *et al.*, 1987).

Once cyst suspensions are obtained using one of the above methods, a variety of experimental manipulations are possible. Determination of the mandatory dormancy interval for cysts or spores requires that cysts of known age be stored under different temperatures and periodically exposed to optimal growth conditions to assess germination success (Anderson, 1980; Binder and Anderson, 1987; Blackburn *et al.*, 1989). The permissive temperature "window" for germination (e.g. Anderson *et al.*, 1985) can be determined by incubating quiescent cysts across a range of temperatures (in the light) and assessing the percentage of germination after an arbitrary interval. Since germination will be slow at low temperatures, a month or more of incubation is sometimes needed. Different temperatures can be provided by

the use of multiple incubators, or through the use of a temperature gradient bar (Watras *et al.*, 1982) which is heated at one end and cooled at the other, resulting in a continuous gradient in temperature. Light requirements for germination require special handling of the cyst suspensions, as even brief exposure to low levels of light can trigger excystment in some species (Binder and Anderson, 1986). Samples can be processed in near darkness, or using a red, photographic light, though in both cases, some exposure is unavoidable. Tubes can then be wrapped with different layers of screening or shaded with neutral density filters to provide the needed light variation (Anderson *et al.*, 1987).

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12. Methods of Nutrient Analysis

J.C. Valderrama

The methods hereby described are manual. These, as much as the recommended equipment, intend to be used not only ashore, but also aboard a research vessel with a minumum of facilities. They permit to run a reasonable amount of samples, within an acceptable lapse of time, with technical personal who will be able to learn and manage these methods after a short period of training. The automated techniques, although more advantageous, are very much expensive and demand a higher qualified personnel. Beside this there are many methods for using these techniques depending of the many different manufacturers.

The most used principle on which the analysis of the nutrients in seawater is based, is the transformation, through a chemical reaction of the substance to be analyzed, to an other compound which can be measured colorimetrically within the wavelength range of the visible spectrum. Such compounds should yield an absorbance which can be related to its concentration within the interval where the Lambert Beer's law prevails. When manual methods are used the chemical reactions are allowed to run until an equilibrium point is reached.Note that the concentration µmoles/L referred, for example, to NH_3-N, means nitrogen coming from ammonia and not the concentration of ammonia itself. The same applies to NO_2-N, NO_3-N, etc.The reagents to be used have to be of high quality e.g. pro analysi (p.a.). When no other quality is pointed out (i.e. puriss, tech., etc.) it means that the reagent is at least p.a. Observe that analytical reagents have a storage limit beyond which they should not be used.

It is a good practice to keep the standard stock solutions (SSS) no longer than six months and when a new batch is prepared to check on the new SSS against the old one.All glassware should be used clean and dry. The use of phosphate-free detergents is recommnded. The rinsing should be done immediately after using, first with tap water and then, at least three times with deionized or bidistilled water (BdW). It has to be pointed out that the deionizing process excludes ions but not micro-organisms, which sometimes may produce contaminations. The glassware for measuring volumes, i.e. volumetric flask, pipettes, burettes, etc. should be of class-A type.

For adding reagent solutions, automatic syringe pipettes are recommended (Fig. 12.1), but actually most kind of automatic dispensers can be used. The reagent solutions must be calibrated at least every time they are exchanged for a new batch. It is important to emphasize that the most exact analytical work, the most sophisticated techniques and instrumentation, the highest quality of the reagents, cannot give precise and reliable results if something is wrong with the sample. It may have been altered by changes in composition before it reached the laboratory or it may have been contaminated during the sampling, etc. Therefore, it must be stressed that the sampling procedure is the most important step in analytical work.The analyst should realize that the constituents in his sample have a three-dimensional pattern and this pattern, especially in the marine environment in contrast to others, is influenced by chemical, physical and biological processes which may result in a more or less pronounced variability in the distribution at the sampling moment. Normally, only the main features of this distribution are known before the sampling and one has not control over it. In sea-water sampling procedures, the errors cannot be completely eliminated, but they can be diminished by applying improved sampling methods, by taking a large number of samples or by using large samples.

The analyses of samples should be commenced without delay. If it is necessary the samples should be stored in a dark and cold place but not more than 6 hours. The quick freezing of samples to -20°C, in order to store them for longer periods may be applied with some reservations. Description of the nutrient analyses refers here only to those called macronutrients (or major micronutrients), i.e. nitrogen, phosphorus and silicon compounds, as distinct from the micro-nutrients (or minor micronutrients), basically trace metals (e.g. cobalt,

iron, manganese, copper, molybdenum), that occur in extremely low levels in most ocean waters. Analysis of micronutrients demands highly sophisticated methods and equipment, such as Atomic Absorption Spectrophotometry (AAS), Neutron Activation, or Anodic Stripping Voltammetry (ASV). Besides, the handling of such samples demands high cost installations in order to avoid the contaminations that very easily can affect the analyses. A brief account of methods for micronutrients is provided in the chapter 13 by Haraldsson and Graneli.

DETERMINATION OF AMMONIA

Scope and field of application

The method is specific for ammonia and applicable to all kinds of natural waters. "Ammonia" here refers to the sum of ammonia and ammonium ions, because the original proportions of them in a water sample are pH dependent. The detection limit of the method is about 0.10 µM (in 50 mm cells). The Lambert-Beer's Law is followed up to about 40 µM. Interferences from amino acids and urea can be neglected. To compensate for the influence of salinity on the developed colour a correction salt factor has to be applied.

Principle

In a weakly alkaline solution, ammonia reacts with hypochlorite to form monochloroamine which in the presence of phenol, catalytic amounts of nitroprusside ions and an excess of hypochlorite yields indophenol blue. The reaction is quantitative in the pH range between 10.8 and 11.4. The reaction mechanism is complicated and not yet fully understood (Koroleff, 1983a).The precipitation of Mg and Ca hydroxides in the sample solution, when pH is higher than 9.6, is avoided by addition of a complexing buffer reagent with citrate ions which keeps the Mg and Ca ions in solution.

Reagents

"Ammonia-free" water (AFW): There is no standard procedure for the preparation of water with a very low ammonia content. Deionized water may sometimes be used without subsequent destillation, but it must be remembered that some ion exchange resins may leach out ammonia containing organic substances. If the ammonia blank concentrations are higher than 0.3 µM the water should be distilled. In this second step, 2 ml of concentrated sulphuric acid (96%) and 1 g potassium peroxodisulphate, $K_2S_2O_8$, are added per liter. The solution should be boiled for 10 minutes (without the condenser) to remove ammonia and then distilled until a residue of about 150 ml. AFW should be stored in a tightly sealed plastic container with thick walls (IOC, 1983).

Citrate buffer solution: 67 g trisodium citrate dihydrate, $Na_3C_6H_5O_7 \cdot 2H_2O$, 34 g boric acid, H_3BO_3, 19 g citric acid dihydrate, $C_6H_8O_7 \cdot 2H_2O$, and 30 g sodium hydroxide, NaOH, are dissolved in AFW and diluted to 1 L. The solution is stable and should be stored in a well stoppered glass bottle at room temperature.

Reagent A, phenol-nitroprusside solution: 35 g phenol, C_6H_5OH, (a slightly pink quality may be accepted) and 0.4 g of sodium nitroprusside dihydrate, $Na_2Fe(CN)_5NO \cdot 2H_2O$, are dissolved in AFW and diluted to 1 L. Stored in a tightly closed dark glass bottle in a refrigerator, the solution is stable for several months.

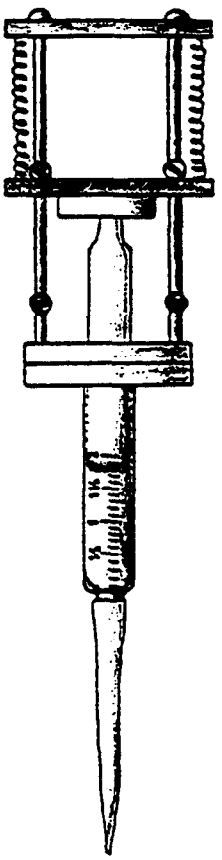

Figure 12.1
Automatic syringe pipette.

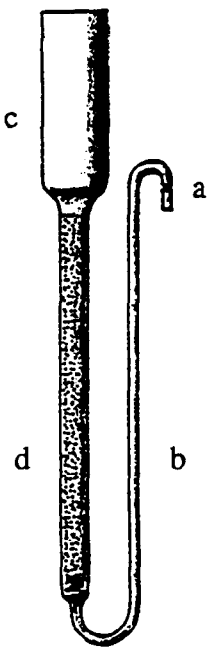

Figure 12.3 (scale 1:4)
Reductor column for nitrate.
Capillary tubing:
a O.D.= 6 mm I.D.= 1 mm
b O.D.= 8 mm I.D.= 2 mm
Ordinary tubing:
c I.D.= 32 mm
d I.D.= 10 mm

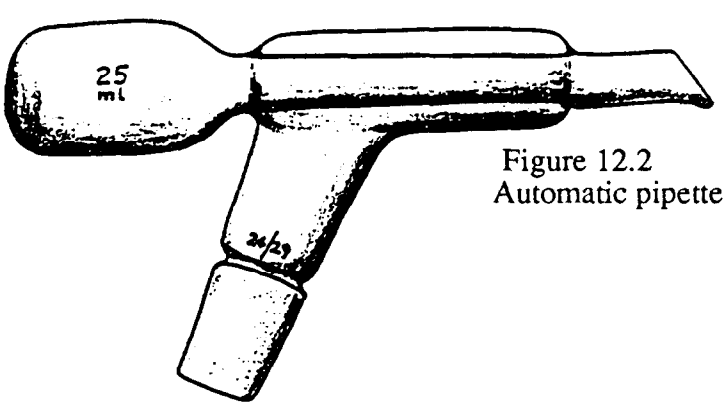

Figure 12.2
Automatic pipette (Vogel type) 25 ml.

Reagent B, hypochlorite solution: 4 g sodium salt of the dichloro-isocyanuric acid (Merck 110888 or Aldrich, catalogue-nr 21, 892-8) and 15 g NaOH are dissolved in AFW and diluted up to 1 L. Stored in a tightly closed dark glass bottle in a refrigerator, the solution is stable for several weeks.

Ammonia Standard Stock Solution (NH_3-N SSS): 0.13373 g ammonium chloride, NH_4Cl (MW: 13.49), dried at 110°C to constant weight, is dissolved in AFW and diluted to 250 ml. Stored in a tightly closed dark bottle with some drops of chloroform, in a refrigerator. The solution contains 10 µmoles/ml.

Apparatus and equipment

25 ml test tubes with ground glass stoppers.
Automatic syringe pipettes of 1 and 2 ml.
25 ml automatic pipettes (Vogel type, Fig. 12.2).
Spectrophotometer, preferably with digital display.
Photometric cells of 10, 50 or 100 mm length as required.

Sampling

Even if the analysis of ammonia should be commenced without delay, the samples could be cooled in a refrigerator but should be analysed within about 3 hours. A preservation for longer periods is not recommended. Filtration of samples should be avoided, besides that it is nearly impossible to obtain filters free of ammonia, it may also evaporate the ammonia contained in the sample. Polluted waters containing high concentrations of ammonia are often turbid and may be diluted before the analysis. Any residual turbidity can be compensated with a similarly diluted sample (without reagents) in the reference cell.

Analytical procedures

All work with ammonia analysis must be carried out where smoking is not permitted, in order to avoid contaminations. Before the test tubes can be used for calibration or analysis, they must be carefully cleaned according the following procedure: every tube is filled with about 25 ml water (not necessarily AFW) and reagents are added as described later. All ammonia contained in the tubes (dissolved in the water or adhered to the glass walls) will react. Then they are rinsed with AFW and stored filled with AFW. The tubes should be kept stoppered when not used. They should not be washed between the different sets of calibrations or analyses, but just rinsed with AFW (IOC, 1983).

Calibration. In order to avoid disturbances from variations in pH and salinity of the samples, the calibration can be carried out in either of two ways. For work in true oceanic areas, where the salinity variations are small, the working standard solutions (WSS) are diluted with "ammonia-free seawater" i.e. surface seawater from an open sea area, preferably collected shortly after a plankton bloom. For work in e.g. estuaries, where the brackish water displays large salinity variations, a calibration in AFW, followed by corrections for the salinity (see Table 12.2) of each sample, is preferred.A series of (WSS) from the NH_3-N SSS is prepared by dilution with AFW or "ammonia-free seawater", using volumetric flask. Table 12.1 below may be used.

Table 12.1. Working standard solutions for ammonia

5.0 ml NH_3-N SSS	to	1 L	=	0.05 µmoles/ml (D)
20.0 ml D	to	250 ml	=	4.0 µM NH_3-N
5.0 ml D	to	250 ml	=	1.0 µM "
5.0 ml D	to	500 ml	=	0.5 µM "
2.0 ml D	to	500 ml	=	0.2 µM "

From each of the WSS above, 25 ml triplicates are transferred to the test tubes. In addition, two sets of "blank samples" are prepared, also in triplicate, but only with AFW. To all the tubes the reagents are added as described later, but to one of the blank sets a double volume of reagents is added.The blank samples here correct for the absorbance caused by the residual ammonia impurities in the AFW. The second set of blanks, those with double volume of reagents, corrects for the ammonia impurities in the reagents only. The linear regression of the absorbances measured in the spectrophotometer versus the concentrations of the WSS (including absorbances of the first set of blanks, concentration = 0) gives the Calibration Factor (CF). The product of CF and the absorbance of the second sets of blanks will be a constant (K) which is deduced from the results obtained with the samples.Using a 50 mm cell one obtains currently a CF of approx. 11.

Analysis of the samples. With the automatic pipette, 25 ml of samples are transferred to the test tubes and 1.5 ml of citrate buffer and 0.7 ml of each of reagents A and B, in this order, are added, by means of the automatic syringe pipettes. Then the tubes are stoppered, shaken and kept in a dark place during the reaction period (at least 8 hours). Absorbance remains unaltered during a maximum of 48 hours. The absorbance (A_S) is measured in the spectrophotometer using wavelength of 630 nm and glass cells of suitable length depending of the colour intensity of the sample. As reference AFW is used.

$$\text{Concentration } NH_3\text{-N} = CF \bullet A_S - K \ [\mu M]$$

If the WSS were diluted with AFW and the samples are sea-water, results are corrected using the correction factors given in the Table 12.2, depending the salinity of the sample:

Table 12.2. Salinity correction factors (cf) for ammonia analysis (Koroleff, 1983a)

Sal. (psu)	<8	11	14	17	20	23	27	30	33	36
pH	0.8	10.6	10.5	10.4	10.3	10.2	10.0	9.95	9.90	9.80
cf	1.00	1.01	1.02	1.03	1.04	1.05	1.06	1.07	1.08	1.09

Accuracy and precision

Good accuracy and precision are difficult to obtain in the analyses of ammonia and are highly dependent on how successfully contamination is avoided during the analytical steps. At concentration levels of about 5 µM the coefficient of variation (reproducibility) is ± 2% (under ideal circumstances).

DETERMINATION OF NITRITE

Scope and field of application

The method is specific for nitrite ions and applicable to all types of marine waters. It is not appreciably affected by salinity, small changes in reagent concentrations, or by temperature (Grasshoff, 1983).Using 50 mm cells, the detection limit of the method is about 0.02 µM and it shows a linearity up to about 10 µM.

Principle

The determination is based on the reaction of nitrite ions with an aromatic amine (sulphanilamide) which leads (at pH 1.5-2.0) to the formation of a diazonium compound, which is coupled with a second aromatic amine (N-(1-naphtyl)-ethyl-endiamine) to form a highly pink coloured azo dye with a molar absorptivity of about 46,000 at 543 nm (Bendschneider *et al.*, 1952).

Reagents

Sulfanilamide (SAN): 10 g sulphanilamide, $NH_2C_6H_4SO_2NH_2$, is dissolved in a mixture of 100 ml concentrated hydrochloric acid, HCl (36%), and 600 ml bidistilled water (BdW). After cooling the solution is diluted to 1 L. At room temperature, stored in glass bottles, the reagent is stable for several months.

Naphtylamine solution (NED): 1 g N-(1-naphtyl)-ethylene-diamine dihydrochloride, $C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$, is dissolved in BdW and diluted to 1 L. The solution should be stored in a dark glass bottle in a refrigerator, and must be renewed as soon as it develops a brown colour (usually stable for 1-2 months).

Nitrite Standard Stock Solution (NO_2-N SSS): 0.17250 g sodium nitrite, $NaNO_2$ (MW: 69.00), dried at 110°C to constant weight, is dissolved in BdW and diluted to 250 ml. The solution should be stored in a tightly closed dark bottle, with 3-4 drops of a saturated $HgCl_2$ solution in a refrigerator (Kirkwood, 1992). The solution contains 10 µmoles/ml.

Note: Aged solid reagent, even if it is of analytical grade, may contain less than 100% $NaNO_2$ (it is unstable in air) and should, therefore, not be used for the preparation of SSS.

Apparatus and equipment

25 ml test tubes with glass or plastic stoppers.
Automatic syringe pipettes of 1 ml.
25 ml automatic pipette (Vogel type).
Spectrophotometer, preferably with digital display.
Photometric cells of 50 mm length.

Sampling

Nitrite is an intermediate compound in the simplified redox chain ammonia <-> nitrite <-> nitrate, and the samples, therefore, cannot be properly preserved. Filtration of samples should, if possible, be avoided. If they are slightly turbid and contain no other disturbing substances,

such as samples arised from nearshore areas, they may be analyzed together with turbidity blanks instead of being filtrated.

Analytical procedures

Calibration. As there is no salinity effect in the formation of the azo dye, the calibration can be done in solutions made with BdW. A series of WSS from the NO_2-N SSS is prepared by dilution. Table 12.3 below may be used.

From each of the WSS above 25 ml triplicates are transferred to the test tubes. In addition, one set triplicates of "blank samples" with BdW is prepared. Reagents are added to all the tubes as described later.The linear regression of the absorbances measured in the spectrophotometer versus the concentrations of the WSS (including absorbances of the blank samples, concentration = 0) gives the Calibration Factor (CF). Using a 50 mm cell one obtains currently a CF of approx. 4.

Table 12.3. Working standard solutions for nitrite

5.0 ml NO_2-N SSS	to	1 L	=	0.05 µmoles/ml (D)
5.0 ml D	to	250 ml	=	1.0 µM NO_2-N
5.0 ml D	to	500 ml	=	0.5 µM "
1.0 ml D	to	200 ml	=	0.25 µM "
1.0 ml D	to	500 ml	=	0.1 µM "
1.0 ml D	to	1000 ml	=	0.05 µM "

Analysis of the samples. 25 ml of samples are transferred to the test tubes. If the turbidity has to be analyzed, another 25 ml of sample is transferred to a second tube. With an automatic syringe pipette 0.5 ml of SAN is added to the tubes and after not less than two minutes, but no longer than eight minutes, 0.5 ml of NED is added only to one of the tubes. The tubes are stoppered and shaken. After not less than 8 minutes, the absorbances (A_S) are measured using the second tube, that with SAN only, as reference, at a wavelength of 543 nm and using a 50 mm cell. The colour intensity is stable for two hours.The concentration of nitrite in the samples is found by multiplying their absorbances by the CF:

$$\text{Concentration } NO_2\text{-N} = CF \bullet A_S \quad [\mu M]$$

Accuracy and precision

At a concentration level of about 0.5 µM the coefficient of variation is ± 0.2%. As turbidity introduces a considerable systematic error, it is recommendable in routine analytical works to compensate for it according to the method already described.

DETERMINATION OF NITRATE

Scope and field of application

The method generally applied for the determination of nitrate ions is based on its reduction to nitrite, which is then determinated as described previously. The method determines the sum of nitrite and nitrate ions, therefore, a separate determination of nitrite must be conducted, and its concentration subtracted from that obtained with this method. At concentrations higher than about 20 µM NO_3-N calibration factors for a low and high range must be established.

Principle

Nitrates are reduced to nitrites almost quantitatively by amalgamated granulated cadmium. The reduction is carried out at a pH of about 8.5. Ammonium chloride buffer is used to control the pH and to complex the liberated cadmium ions (Carlberg, 1972).

Reagents

SAN and NED reagent solutions are the same used as in the nitrite determinations.

Buffer solutions: 25% Stock buffer, 250 g ammonium chloride, NH_4Cl, is dissolved in BdW and 25 ml concentrated ammonium hydroxide (25%) is added. Dilute to 1 L.
2.5% work buffer (WB), 100 ml of stock buffer is diluted with BdW to 1 L.
Wash buffer solution (WbS), 20 ml of 2.5% WB is diluted with BdW to 1 L.

Hydrochloric acid 2M: 165 ml of concentrated commercial HCl (37%) is diluted with BdW to 1 L.

Mercuric chloride solution 1%: 5 g mercuric chloride, $HgCl_2$, is dissolved in 500 ml BdW.

Synthetic sea water (SSW): 36 g sodium chloride, NaCl, 12 g magnesium sulphate heptahydrate, $MgSO_4.7H_2O$ and 0.25 g sodium bicarbonate, $NaHCO_3$, are dissolved with BdW and diluted to 1 L. For analytical purposes it is equivalent to a salinity of 40 psu. For calibration work it may be diluted to the desired salinity.

Nitrate Standard Stock Solution (NO_3-N SSS): 0.25278 g potassium nitrate, KNO_3 (MW: 101.11), dried at 110°C to constant weight, is dissolved in BdW and diluted to 250 ml. Stored in a tightly closed dark bottle with 2-3 drops of a saturated $HgCl_2$ solution in a refrigerator (Kirkwood, 1992). The solution contains 10 µmoles/ml.

Cadmium coarse pulver: commercially available granulated cadmium is sieved and the fraction between 35 and 40 mesh, i.e. around 0.5 to 0.42 mm, is retained and used.

Warning: Cadmium is a poisonous metal. It should, therefore be handled with great care. All operations on the dry metal, particularly the granules, must be done in a well ventilated area e.g. a fume cupboard. *Never inhale the dust!*

Amalgamated cadmium: The required amount of cadmium metal is about 35 g per reductor column (see Fig. 12.3). The sieved granules are rinsed from oxides by washing with 2M HCl. Then they are washed with plenty of water to eliminate all HCl.All the washed metal is transferred to a round botton flask that is filled with 1% $HgCl_2$ solution. The flask is closed with a glass stopper. After this step all contact between air and the metal should be avoided.

The flask is rotated for 90 minutes in a horizontal position or shaken with suitable equipment. Finally the flask is opened and the turbid sublimate solution is rinsed out with BdW.

The used $HgCl_2$ solution must not be poured into the sewer! When a suitable volume of $HgCl_2$ is collected, 25 ml concentrated HCl is added per liter and then precipitated with hydrogen sulphide, H_2S, or sodium sulphide, Na_2S. The liquid is filtered and the precipitate stored, discarding the clear filtrate.

Apparatus and equipment

All those recommended for nitrite determination.
Test tubes graduated or marked at the 25 ml volume.
500 ml round botton flask.
Reduction columns (RC) (Fig. 12.3).
Photometric cells of 10 mm length.

Sampling

The samples should preferably be analyzed immediately after the sampling. If a storage time longer than 12 hours is necessary, quick freezing of samples to -20°C is recommended.

Analytical procedures

Preparation of the reduction columns: A small ball of thin copper wire is placed at the bottom of the RC and above the wire a small burl of glass wool. The RC is filled entirely with water. With a small plastic spoon the metal granules are poured into the RC, making sure that no cavities are formed in the reductor (the RC is gently knocked with, e.g. a rubber stopper) and filled to about 1 cm below the reservoir.The amalgamated metal is activated by passing through about 150 mL WbS containing about 100 µM NO_3-N. The packed RC is then rinsed thoroughly with WbS only, before to be used for analysis. A newly prepared RC reduces nitrate with an efficiency of 95-100 %.

Calibration: There is a significant salinity effect in the calibration of nitrate measurements by manual methods using Hg-Cd reductors. Freshly amalgamated RC show a salinity effect of less than 10%, while the same RC, after several weeks use, show a higher discrepancy, up to 30%, when calibration against WSS made up from BdW is compared with standards in SSW of 35 psu, therefore WSS should be made from SSW or the magnitude of the salinity effect should be recorded frequently, whereafter proper correction of the data should be made (Fogelqvist *et al.*, 1990). A series of WSS is prepared from the NO_3-N SSS by dilutions with BdW (or SSW) using volumetric flasks. Table 12.4 below may be used.

Table 12.4. Working standard solution for nitrate

5.0 mL NO_3-N SSS	to	250 ml	= 0.20 µmoles/ml (D)
25.0 ml D	to	500 ml	= 10.0 µM NO_3-N
25.0 ml D	to	1000 ml	= 5.0 µM "
5.0 ml D	to	1000 ml	= 1.0 µM "

Triplicates of WSS and blank samples with BdW are analyzed as described below. Each RC should be calibrated using blanks and calibration solutions.The linear regression of the absorbances measured in the spectrophotometer versus the concentrations of the WSS (including absorbances of the blank samples, concentration = 0) gives the CF.

Using a 50 mm cell one obtains current ly a CF of approx. 4.3.

Analysis of the samples: 25 ml of sample is poured in the reservoir, immediately add 1 ml of WB usig an automatic syringe pipette, followed by other 25 ml of sample. This is passed through the amalgamated metal and drops in a test tube. The first 25 ml is discarded, it is used as washer. The second 25 ml is handled as nitrite sample. Now the turbidity reference samples are unnecessary. 50 ml should flow through the RC in 15 to 20 minutes. The RC is now ready to receive the next sample. After every analytical batch the RC must be flushed with WbS. It should never be left to dry.The concentration of nitrate in the samples is calculated by multiplying their absorbances (A_S) by the CF:

$$\text{Concentration } NO_3\text{-N} = CF \bullet A_S \ [\mu M]$$

Control of the reduction efficiency: The reduction efficiency of each RC must be controlled from time to time, preferably for every analytical batch. Duplicates of WSS for nitrite are analyzed, followed by WSS for nitrate of the same concentration:

$$\text{Reduction efficiency} = \frac{\text{Abs. of the nitrate WSS} \bullet 100}{\text{Abs. of the nitrite WSS}} [\%]$$

If the reduction efficiency decreases below 85 %, the RC is emptied, the fillings washed quickly with 2M HCl and rinsed very thoroughly with water. The fillings are again dried, sieved and reamalgamated as described above.

Accuracy and precision

The precision (standard deviation) of the method when the samples are analyzed with one and the same RC is ± 0.1 µM in the range 0-5 µM, ± 0.2 µM in the range 5-10 µM and ± 0.5 µM at higher concentrations. Precision of results from different RC depend on the reduction efficiency of the RC.

DETERMINATION OF DISSOLVED INORGANIC PHOSPHATE

Scope and field of application

The method described here, a modification of the Murphy and Riley's (1962), uses two different solutions that make the reagents more stable. It shows no measurable effects from salinity but relatively high concentrations of silicate, arsenate or hydrogen sulphide may interfere.The relationship between concentration and absorbance is linear up to about 25 µM when measured in a 10 mm cell.

Principle

The phosphate ions in the sample react in acidic solution with ammonium molybdate to yield a phosphomolybdate heteropoly acid complex, which is reduced by ascorbic acid to a blue-

coloured complex (with molar absorptivity of about 22,700) and displaying a maximum absorbance at 882 nm. With the addition of trivalent antimony ions as catalyst the reduction proceeds swiftly. In order to compensate for the varying turbidity it is necesary to measure the samples of seawater against water from the same depths. These samples are used as references and should be of the same acidity as a sample.To obtain a rapid colour development and to depress the interference of silicate, it is important that the final pH is less than 1, with a $[H^+]$: [Mo] ratio between 60 and 80 (Sucheng Pai, 1990).To reduce interferences from hydrogen sulphide from anoxic water samples, the acidified ascorbic acid reagent is added even to the reference samples.

Reagents

Sulphuric acid 4.5 M: 250 ml concentrated acid (96%) are slowly added, under constant cooling and mixing, to about 700 ml BiD, finally the solution is diluted to 1 L.

Ammonium heptamolybdate reagent: 25 g ammonium heptamolybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$, and 1 g potassiumantimonyltartrate, $K(SbO)C_4H_4O_6$, are dissolved in 300 ml BiD and the volume is adjusted to 1 L with 4.5 M H_2SO_4. If stored in a dark glass bottle, at room temperature, the reagent is stable for several months.

Ascorbic acid reagent: 50 g ascorbic acid is dissolved in 250 ml BdW and the volume is adjusted to 500 ml with 4.5 M H_2SO_4. If stored cold, in a dark bottle, the reagent is stable at least a week (as long as it remains colourless).

Phosphate Standard Stock Solution (PO_4-P SSS): 0.34023 g potassium dihydrogen phosphate, KH_2PO_4 (MW: 136.09), dried at 110°C to constant weight, is dissolved in BdW and diluted to 250 ml. The solution should be stored in a tightly closed dark bottle, with 3-4 drops of a saturated $HgCL_2$ solution and in a refrigerator (Kirkwood, 1992). The solution contains 10 µmoles/ml.

Apparatus and equipment

25 ml test tubes with glass or plastic stoppers.
Automatic syringe pipettes of 1 ml.
25 ml automatic pipette (Vogel type).
Spectrophotometer, preferably with digital display.
Photometric cells of 10 and 50 mm.

Sampling

The analysis should be commenced as soon as possible after sampling, preferably within half an hour. Samples should be kept in a cool dark place and not warmed to room temperature until the time of analysis. On short trips it may possible to add the reagents to the samples and perform the measurements ashore. No entirely satisfactory preservation method is known, but freezing in a dry ice/acetone bath may use as emergency alternative (IOC, 1983).

Analytical procedures

All glassware to be used should be used only for the analysis of phosphate. A solution of the water containing the detergent used for cleaning must be tested for phosphate content. This is the only safe way to avoid unnecessary complications.

Calibration: A series of WSS from the PO_4-P SSS is prepared by dilutions with BdW using volumetric flask. Table 12.5 below may be used.

Table 12.5. Working standard solutions for phosphate

5.0 ml PO_4-P SSS	to	500 ml	= 0.10 µmoles/ml (D)
5.0 ml D	to	250 ml	= 2.0 µM PO_4-P
2.0 ml D	to	200 ml	= 1.0 µM "
1.0 ml D	to	200 ml	= 0.5 µM "
1.0 ml D	to	500 ml	= 0.2 µM "

From each of the WSS above 25 ml triplicates are transferred to test tubes. In addition, one set of blank samples is prepared with BdW, also in triplicate. The reagent solutions are added to all the tubes as is described later. For the calibration no turbidity blanks are needed.The linear regression of the absorbances measured in the spectrophotometer versus the concentrations of the WSS (including absorbances of the blank samples, concentration = 0) gives the Calibration Factor (CF). Using a 50 mm cell one obtains currently a CF of approx. 9.

Analysis of the samples: From each sample two 25 ml portions are transferred to test tubes. Add 0.5 ml of the Ascorbic acid reagent to both tubes and 0.5 ml of the Molybdate-Antimonyl reagent to only one. The tubes are stoppered and shaken and after 5 minutes each sample is measured against its turbidity blank, used as reference, in the spectrophotometer.

The colour of the samples is stable for at least 24 hours, however, if arsenates are present, the reduction of the arsenate molybdate complex is complete after one hour. For this reason, if the presence of arsenates is suspected measurements must be done as soon as possible after 5 minutes.The concentration of phosphate in the samples is found by multiplying their absorbances by the CF:

$$\text{Concentration } PO_4\text{-P} = CF \bullet \text{Abs. of the sample } [\mu M]$$

Accuracy and precision

At a concentration level of about 0.2 µM, the coefficient of variation is ± 15% and ± 2% at a high level (3 µM).

DETERMINATION OF REACTIVE SILICATE

Scope and field of aplication

The method is based on the reaction between dissolved silicon compounds and a molybdate solution. But as only silicic acid and its dimer react with molybdate at any appreciable speed, the method gives only the amount of "reactive" silicate, which is probably a reasonable measure of the silicate available to growing phytoplankton, though a small fraction of the measured silicate could not be taken up by diatoms (Koroleff, 1983c). The method has a concentration

range up to 120 µmoles/L, but samples with higher concentrations can be diluted with BdW. The detection limit is about 0.1 µM (with 50 mm cells).

Principle

Determination of dissolved silicon compounds is based on the formation of a heteropoly acid when the sample is treated with a molybdate solution. The two yellow isomeric forms, in which this silicomolybdate complex occurs, depending on pH, differ only in their hydration. But besides the higher molar absorbance displayed by the ß-silicomolybdic acid its stability is less than the α-form. Using ascorbic acid as reductor and maintaining a pH <2 one obtains an intensely blue coloured complex. The addition of oxalic acid avoids the reduction of any excess molybdate reagent and eliminate the influence of any phosphate present.In seawater, the complex exhibits a molar absorptivity of about 19,000. The colour is formed within 30 minutes after the addition of reagents and is stable for at least several hours and shows a maximum absorbance at 810 nm.

Reagents

All the reagent solutions should be stored in plastic bottles, on account of the silicon content of glass.

Molybdate reagent: 99 g ammonium heptamolybdate is dissolved in 700 ml BdW, to this solution 200 ml concentrated H_2SO_4 (96%) is carefully poured, under constant cooling and mixing. After cooling, the solution is diluted to 1 L. The reagent stored at room temperature is stable for several months.

Oxalic acid solution.: 90 g oxalic acid, $(COOH)_2 \cdot 2H_2O$, is dissolved in BdW and diluted to 1 L. At room temperature the reagent is stable indefinitely.

Ascorbic acid solution.: 17 g ascorbic acid, puriss, is dissolved in BdW and diluted to 1 L. Stored cold in a brown bottle (plastic!) the reagent is stable for weeks.

Silicate Standard Stock Solution (Si SSS): 0.47015 g disodium hexafluorosilicate, Na_2SiO_6 (MW: 188.06), dried at 110°C to constant weight, is dissolved in BdW (in a plastic beaker) and diluted to 250 ml. The solution is immediately transferred to a polyethylene bottle, with 2-3 drops of a saturated $HgCl_2$, in a refrigerator. The solution contains 10 µmoles/ml.

Apparatus and equipment

25 ml stoppered plastic tubes.
Automatic syringe pipettes of 1 ml.
25 ml automatic pipettes (Vogel type).
Spectrophotometer with digital display.
Photometric cells of 10 and 50 mm.

Sampling

The subsampling for silicate analysis should be performed with plastic bottles only (polyethylene, polypropylene or PVC).The samples should be stored in a cool dark place and not longer than a day until analysis. When silicate samples are stored frozen, special problems may arise from its tendency to polymerisize in these conditions. This phenomenon is favoured

more in less saline waters. When frozen samples are analyzed they must be allowed to stand for at least three hours at room temperature after thawing (MacDonald *et al.*, 1982).

Analytical procedures

Calibration: A series of WSS from the Si SSS is prepared by dilution with BdW or SSW using volumetric flask and the the solution is transferred inmediately to plastic bottles. Table 12.6 below may be used.

From each of the WSS above 25 ml triplicates are transferred to test tubes. In addition, one set of blank samples is prepared, also in triplicate. To all the tubes the reagent solutions are added as described later.The linear regression of the absorbances measured in the spectrophotometer versus the concentration of WSS (including absorbances of the blank samples, concentration = 0) gives the Calibration Factor (CF).The dissolved salts of seawater reduce the final colour intensity to some extent and this variation is a linear function of the salinity. Thus, if the CF was determined using SSW diluted with BdW, a correction factor is applied: 1 + 0.0045 **s**, where s correspond to the salinity expressed in psu (Koroleff, 1983c).For routine analysis in seawater it is normally sufficient to prepare WSS in SSW of a salinity representing a mean value of the salinities in the working area. However, for the greatest accuracy, it should be more convenient to calibrate in BdW and then correct for the salt error. This is of particular importance in areas where the silicate concentrations in surface water may become very low as a result of phytoplankton primary production.Using a 10 mm cell one obtains currently a CF of approx. 50.

Table 12.6. Working standard solutions for silicate

5.0 ml Si SSS to 200 ml	=	0.25 µmoles/ml (D)
40.0 ml D to 200 ml	=	50.0 µM Si
20.0 ml D to 200 ml	=	25.0 µM "
10.0 ml D to 250 ml	=	10.0 µM "
5.0 ml D to 250 ml	=	5.0 µM "

Analysis of the samples: 25 ml of sample is transferred to test tubes, and 0.7 ml of Molybdate Reagent is added. After 20 minutes (salinity <10 psu) or 10 minutes (salinity >10 psu) 0.7 ml of the oxalic acid solution is added, followed immediately by 0.7 ml of the ascorbic acid solution. Swirl gently after the reagent additions.After not less than 30 minutes, the absorbances are measured using BdW as reference and cells of suitable length at a wavelength of 810 nm.The absorbances obtained from the WSS should be corrected for the disturbances produced by the silicate content of the reagents and BdW. For this purpose prepare a "reagent blank" using two sets of 25 ml, one set determinated as sample (A1) and the other set only with Molybdate Reagent added, in this case measure the absorbance after one hour (A2).

Absorbance of Reagent Blank = A1 - A2

The concentration of reactive silicate in the samples is found by multiplying their absorbances by the CF:

Concentration Si = CF .Abs. of the samples [µM]

Accuracy and precision

Several international intercalibration studies have reported the blue silicomolybdic acid method as a very accurate procedure with a rather low tendency for systematic errors. At a concentration level of about 5 μM the coefficient of variation is ± 4% and ± 2% at 50 μM.

SIMULTANEOUS DETERMINATION OF TOTAL PHOSPHORUS AND TOTAL NITROGEN

Scope and field of application

The method describes a simultaneous oxidation by persulphate for the determination of the total concentrations of nitrogen and phosphorus in the same seawater sample (Koroleff, 1983d).The yields obtained by oxidation of various nitrogen compounds depend on the form of nitrogen linkage and structure of the organic material. Thus, for example, nitrite, nitrate, ammonia, urea, some aliphatic aminoacids and some proteins give yields over 92%. Pronouncedly poor yields have been obtained from compounds containing N - N bonds, while a double bond between nitrogen atoms seems to prevent their oxidation to nitrate entirely (Nydahl, 1978). On the other hand, organic phosphorus compounds are easily broken down to inorganic compounds by treating them with peroxodisulphate solution. Hydrogen sulphide produces poor yields in the total phosphorus measurements caused by the coprecipitation of phosphate with the colloidal hydrated iron(II) oxide produced from iron in anoxic waters, or the formation of iron (III) phosphate during the oxidation process (Ichinose *et al.*, 1984).The digestion time, a function of the autodecomposition of peroxodisulphate, needs to be no longer than 30 minutes, provided that the temperature attained in the autoclave is 115 - 120°C (1.5 - 2 atm).

Principle

To allow nitrogen compounds to become oxidized, it is necessary to use an alkaline medium, otherwise nitrate is not produced in quantifiable amounts. Conversely, the oxidation of phosphorus compounds must be performed on an acidified medium. In the simultaneous oxidation the reaction starts at pH 9.7 and finishes at pH 5-6. These conditions are obtained by a boric acid - sodium hydroxide system. In seawater samples no precipitate is formed when the oxidation reagent is added. At elevated temperature (110 - 120°C) a precipitate is formed which, however, almost dissolves as oxidation proceeds. After the oxidation the remaining small amount dissolves upon swirling. The free chlorine, which is formed in seawater samples, evanesces if the samples are allowed to stand a couple of days before the analysis, or is reduced when ascorbic acid is added before the molybdate reagent (Valderrama, 1981).After oxidation, the nitrogen compounds are determined as nitrate and the phosphorus compounds as inorganic phosphate as was described formerly.

Reagents

In addition to the reagents required for determination of disolved inorganic phosphate and nitrate, the following items are needed.

Sodium hydroxide 1 M: 40 g sodium hydroxide, NaOH, is dissolved in AFW and diluted to 1 L.

Oxidation reagent (OR): 50 g sodium peroxodisulphate, $K_2S_2O_8$, and 30 g boric acid, H_3BO_3, are dissolved in 425 mL 1M NaOH and made up to 1 L with AFW.The peroxodisulphate has to have a very low nitrogen content. A suitable reagent is the product from Merck (No. 5092) with a maximum content of 0.001% N. If OR is stored in dark glass bottles and protected from direct light in room temperature it is stable for several months.

Total P-Total N Standard Stock Solution (TPN SSS): 1.12600 g dried glycine, H_2NCH_2COOH (MW:75.09). and 0.34023 g potassium dihydrogen phosphate previously dried at 110°C to constant weight are dissolved in AFW and diluted to 250 ml. Stored in a tightly closed dark bottle, with 2-3 drops of a saturated $HgCl_2$ solution, in a refrigerator. The solution contains 10 µmoles-P/ml and 60 µmoles-N/ml.

Apparatus and equipment

In addition to the apparatus and equipment required for the determination of phosphates and nitrates, the following items are needed.

30 ml automatic pipette (Vogel type).

50 ml bottles of alkali resistent glass, with screw caps of non nitrogen containing material, suitable caps are those of polypropylene from Nalgene Labware.Stainless steel autoclave or a pressure cooker that will maintain an internal pressure of 1.5 - 2 atm.

50 ml graduated glass cylinder.

Sampling and pretreatment

Soon after the sampling, e.g. in connection with the analysis of the other nutrient parameters, 30 ml portions of the samples are transferred to the oxidation bottles. To each bottle 4 ml of OR is added with an automatic syringe pipette and the bottles tightly stoppered. The samples are boiled for 30 minutes at 1.5 - 2 atm in suittable batches to fill the autoclave. Together with each batch boil two reagent blank samples consisting of 4 ml OR and no extra AFW. The autoclave should be used with 200-300 ml AFW. After boiling, the autoclave should be left to reach the room temperature before opening it. The samples should be gently swirled to promote dissolution of the precipitate that may have formed. If samples are boiled with OR soon after sampling they may be stored for at least three months before the determination is performed.

Analytical procedures

Calibration: A series of WSS from the TPN SSS is prepared by dilution with AFW using volumetric flask. Table 12.7 below may be used.

Table 12.7. Working standard solutions for TPN

1.0 ml TPN SSS to 100 ml = {	0.10 µmoles P/ml 0.60 µmoles N/ml	(D)	
10.0 ml D	to 200 ml	=	5.0 µM P and 30.0 µM N
6.0 ml D	to 200 ml	=	3.0 µM P and 18.0 µM N
2.0 ml D	to 200 ml	=	1.0 µM P and 6.0 µM N

From each of the WSS above 30 ml triplicates are transferred to the oxidation bottles, in addition, one set of blank samples is prepared, also in triplicate, with 30 ml AFW. 4 ml OR is added to all the bottles. Continue according the former description. The blank samples will compensate for any phosphorus and nitrogen present in AFW and OR. The analysis proceeds as described later.CF for phosphorus (CF_P) and nitrogen (CF_N) are obtained as for phosphate and nitrate. When 50 mm cells are used, CF of 12 and 27 for P and N respectively, can be considered as representative.

Analysis of the samples.: The content of the oxidation bottle is adjusted to 40 ml with AFW, then 10 ml is pipetted into a 50 ml graduated cylinder in order to determine nitrate and the remaining 30 ml is used to determine dissolved inorganic phosphate.With each batch of samples two blanks, made with 4 ml of OR added to oxidation bottles, are run in duplicate. These blanks are handled just as the samples. They are used to correct the disturbances introduced by traces of nitrogen or/and phosphorus present in the OR. The absorbances measured from these blanks are averaged and deducted from those obtained from the samples. To the 10 ml pipetted into the graduated cylinder 1 ml working buffer for nitrate is added and volume adjusted to 50 ml with BdW. Then continue just as for determination of nitrate.The remaining 30 ml in the oxidation bottle, are treated as a phosphate sample, but now 0.7 ml of reagent are added instead of 0.5 ml and the molybdate-antimonyl reagent is added 2 minutes after the ascorbic acid.

$$\text{Concentration of TP} = CF_P\,(A_{SP} - A_{BP}) \quad [\mu\text{M-P}]$$

$$\text{Concentration of TN} = CF_N\,(A_{SN} - A_{BN}) \quad [\mu\text{M-N}]$$

Where A_{SP}, A_{SN}, A_{BP} and A_{BN} are the absorbances of the samples and the blanks, for P and N, respectively.

Accuracy and precision

The coefficient of variation of the method can be considered as being about 4% for both components. At a concentration level of about 1 µM-P, the relative error is 2% and at 30 µM-N is 4%.

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13. Trace metals as nutrients

C. Haraldsson and E. Graneli

The major elements regulating phytoplankton growth and species composition are usually thought to be macronutrients such as phosphorus, nitrogen and for the diatoms also silicon. For a long time it has been known that phytoplankton also requires small amounts of substances (micronutrients) usually found in trace amounts (1 pM to 1 nM) in the open ocean waters (Hudson and Morel, 1993). Among the micronutrients are found important metals *e.g.* Fe, Mn, Co, Ni, Cu and Zn; metalloids such as Se and non-metals such as iodine. These trace nutrients are required in metabolic processes for both marine and fresh water phytoplankton (Edvardsen *et al.*, 1990; Brand, 1991; Bruland *et al.*, 1991). The absence of one of these elements may limit (Brand *et al.*, 1983) and the excess might inhibit phytoplankton growth (Anderson and Morel, 1978; Brand *et al.*, 1983; Graneli *et al.*, 1986; Sunda, 1989). Trace metals as nutrients in the sea have gained a renewed interest in the last years. Some recent studies have suggested that the absence of high phytoplankton biomass in waters rich otherwise with macronutrients, as *e.g.* Antarctic and the southern part of the North Pacific might be due to the lack of essential trace metals such as *e.g.* Fe (Martin and Fitzwater, 1988; Martin and Gordon, 1988; Martin *et al.*, 1989, 1990, 1991). A suggestion has even been made by Martin *et al.* (1990) that it would be possible to increase the primary production in the Antarctic water by fertilizing them with iron since this will consequently increase the uptake of carbon dioxide which might offset the continuing increase in atmospheric CO_2.

Several harmful algal blooms have been correlated not only to the increase in the discharge of nitrogen and phosphorus to coastal waters from land but there is evidence that trace metals might also have been involved in the development of certain harmful species (Okaichi, 1975; Prakash, 1975; Provasoli, 1979; Nakamura *et al.*, 1988, 1989; Wells *et al.*, 1991). Ono and Takano (1980) and later Honjo (1993) have correlated the two decades of occurring blooms of the flagellates *Chattonella antiqua* and *Heterosigma carterae* in the Seto inland Sea to the very high concentrations of vitamin B_{12} found in these waters. Cobalt is the central atom of this vitamin. In their case these authors explain this high level of vitamin B_{12} in these waters by the increase in industrial discharge containing high metal loads. The concentrations and ratios of trace metals can already at "natural" concentrations affect the species composition and succession in the local plankton community (Bruland *et al.*, 1991). With this in mind it is relevant to consider the possible connection between trace metals and phytoplankton blooms not only in the open seas but also in the coastal waters where their concentrations are much higher.

Cobalt is an important component in the formation of vitamin B_{12}, and as such has been correlated to the positive effect it has on certain algal species. (Holm-Hansen *et al.*, 1954; Swift, 1981). Cobalt seems to have other functions in the cell metabolism of algae other than the central atom in cobalamine. Holm-Hansen *et al.* (1954) found Co to be an essential element for blue-green algae; Iswaran and Sundara Rao (1964) found Co to be required in the nitrogen fixation process for bacteria or blue-green algae. Furthermore, it has also been pointed out that Co can have other functions for algal production *e.g.* replacing zinc as a nutrient (Price and Morel, 1991).

CASE STUDY: COBALT AND THE *CHRYSOCHROMULINA POLYLEPIS* BLOOM

In Scandinavian waters during May-June 1988 a bloom of the prymnesiophyte

Chrysochromulina polylepis swept the waters of the Kattegat and Skagerrak killing seaweeds, caged and natural fish stocks (Rosenberg *et al.*, 1988; Lindahl and Rosenberg, 1989; Dahl *et al.*, 1989; Nielsen *et al.*, 1990; Kaas *et al.*, 1991). This species produced a toxin that affected a wide range of animals and algae. This toxin enabled the algae to increase without competition for nutrients with other phytoplankton species and losses to grazing (Edvardsen *et al.*, 1990; Nielsen *et al.*, 1990; Maestrini and Granéli, 1991).

RESPONSE OF DIFFERENT ALGAL GROUPS TO COBALT ADDITIONS

Experiments were performed to check if cobalt additions would have different effects on *Chrysochromulina polylepis* (Graneli and Risinger, 1994) and two other algae *Ditylum brightwellii* (diatom) and *Prorocentrum minimum* (dinoflagellate) (Graneli and Haraldsson, 1993; Segatto and Graneli (in prep.)). The three algae responded quite differently to cobalt additions. *C. polylepis* after some days of incubation increased the biomass well above the control bottles (without cobalt additions) in all the cobalt treatments. This biomass stimulation was both found as an increase in chlorophyll *a* and in cell numbers (Fig. 13.1). The dinoflagellate *Prorocentrum minimum* neither showed a stimulation nor an inhibition of biomass accumulation to cobalt additions (Fig. 13.1). The diatom *Ditylum brightwellii* was however inhibited by cobalt additions producing lower chlorophyll and lower cell numbers (Fig. 13. 1) in the bottles where cobalt was added compared to the control bottles. The stimulation by Co addition on *Chrysochromulina polylepis* indicates that this metal might have been involved in the bloom of this alga in 1988.

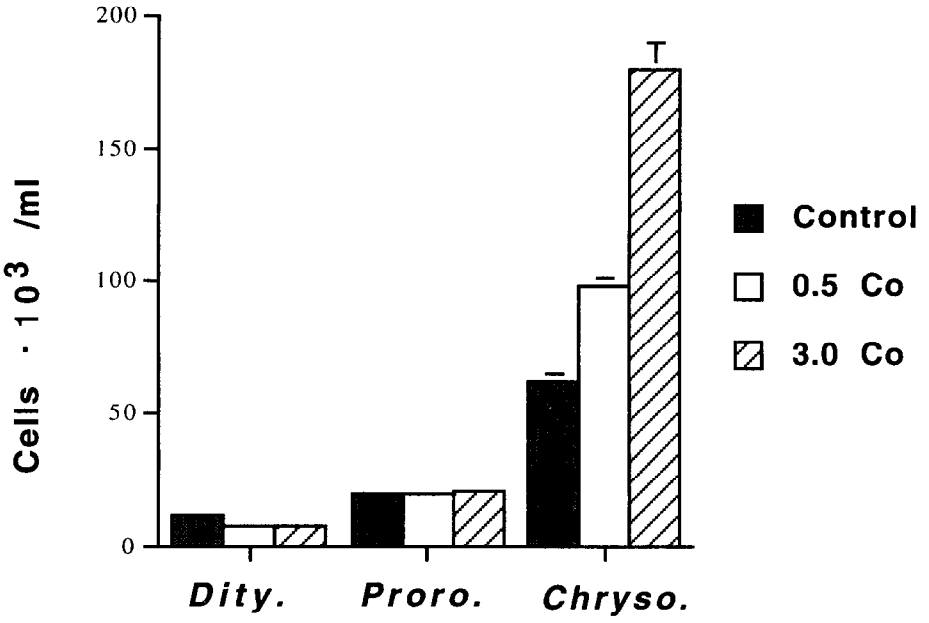

Fig. 13.1. Maximum chlorophyll *a* values for *Chrysochromulina polylepis, Ditylum brightwellii* and *Prorocentrum minimum* cells growing in 26‰ diluted Atlantic sea water with additions of nitrogen and phosphorus with or without different cobalt additions. Control= no extra cobalt added. n = 4 bottles for each treatment. (Redrawn from Graneli and Haraldsson, 1993).

ACID PRECIPITATION AND METALS IN COASTAL AREAS

The increase in the transport of trace metal nutrients to the coastal areas has until recently been related to industrial, sewage and agriculture discharge. However, acid precipitation on soils has also been shown to increase the discharge of some very important metals for phytoplankton growth in coastal areas. Thus acid precipitation might have been indirectly involved in the *C. polylepis* bloom in Scandinavian waters in 1988 by increasing the mobility of Co from soils. Graneli and Haraldsson (1993) found that the concentration of some metals in streams (*e.g.* Co) is clearly dependent upon pH. Although there is a signifcant amount of scatter in the data, the cobalt concentration approximately follows a straight line where:

$$[Co_{(nM)}] = 19.3 - 2.3*pH$$

DETERMINATION OF TRACE ELEMENTS

Examples of the concentration of trace metals in sea water are presented in Table 13.1 where samples taken from the Antarctic are compared with water from the north-east Atlantic and coastal seawater from the Swedish west coast. Due to the low concentrations in sea water, the study of trace elements involves a number of problems. The fundamental problems in inorganic trace analysis are addressed in several books (Howard and Statham, 1993; Vandecasteele and Block, 1993; Van Loon, 1985). The determination of trace elements in seawater requires highly specialised procedures for sampling, cleaning of sample containers and sample work up (Bruland, 1983). Although many methods for trace element determination in seawater can be found in literature, only relatively few have been proven to perform well. To be able to obtain satisfactory results as quickly as possible after entering the field of trace metal analysis, it is recommended to choose a method that fulfils one or more of the criteria listed below.

- The method has performed well in several intercalibration exercises (e.g. Bewers *et al.*, 1986).
- The method is in widespread use.
- The method can be found among those used in the preparation of certified reference materials (see below).

Table 13.1. Concentrations found for some trace metals in sea water. The results for Antarctic water come from the Weddell Sea (Westerlund and Öhman, 1991), the samples from the Atlantic ocean come from the North-East Atlantic (Danielsson *et al.*, 1985). The concentrations of trace in the coastal waters from the Swedish West Coast were determined at the Dept of Analytical and Marine Chemistry, Univ. of Göteborg by the method described by Danielsson *et al.* (1982).

	Antarctic	Atlantic ocean	Coastal Water
Cd (nM)	0.5-0.8	0.1-0.3	0.1-0.3
Cu (nM)	2.0-2.9	1-2	10-20
Co (nM)	0.02-0.04	-	0.3-5
Fe (nM)	1-10	2-50	40-100
Ni (nM)	6-7	3-10	10-20
Pb (nM)	0.01	-	0.2-0.5
Zn (nM)	3-7	1-3	10-50

A few examples of methods that have been used successfully for trace element determination are given below.

Sampling and sample storage

Sampling is a critical step in the study of trace elements in seawater (Bruland, 1983). The main problem being contamination; this can be caused by a number of reasons including dirty samplers, metal from hydrowires, metal containing dust particles from paint. It is of importance to use a well cleaned sampler that has preferably been used earlier, new samplers frequently cause problems. The sampler and hydrowire should be of a type especially designed for trace metal studies. Presently the most common sampler is the Go-Flo sampler (General Oceanics) which is hung on a Kevlar hydroline.

In order to avoid losses of trace elements by processes involving precipitation, coprecipitation or adsorption to container walls it is common practice to add acid to the samples *e.g.* suprapure nitric acid (1-2 ml/l). Sample containers are normally made of polyethylene and cleaned extensively before use (*e.g.* 3 weeks in 6M HCL followed by 3 weeks in 6M HNO_3). Before use the bottles are stored at least a month in 0.1 % HNO_3.

Instrumental methods

Instrumental methods used in trace element determination in sea water include graphite furnace atomic absorption spectroscopy (GFAAS), total reflection X-ray fluorescence (Friedman, 1989) and electrochemical methods. The most commonly used method is GFAAS. With most instrumental methods it necessary to use a preconcentration step, an exception to this is with electrochemical methods (Mart *et al.*, 1983). The signal in electrochemical analysis depends on the chemical form of the element to be determined. This can be a problem when determining the total concentration of an element where the methods must include a pre-treatment to destroy the organic material, an example of this is irradiation with UV-light. That the signal is dependent on the chemical form of the metal can be used to obtain valuable information about reactivity or bioavailability of an element (Nürnberg *et al.*, 1983). This is perhaps the most interesting property of electrochemical methods.

Pre-concentration methods

Whith most instrumental methods a sample preconcentration step is necessary. This is done for two reasons, firstly the concentrations of many elements are so low that it is near or below the detection limits for most instruments. Secondly the high salt content of sea water causes interference. Examples of preconcentration methods are: Liquid extraction methods where the elements of interest form water insoluble complexes with a complexing agent. The complexed metal is then extracted by an organic solvent. The complex forming agent should not form complexes with sodium, potassium or calcium. A common group of complexing agents are dithiocarbamates. These form complexes with *e.g.* cobalt, cadmium, copper and iron. Analytical methods based on liquid extraction have been described by *e.g.* Bruland *et al.* (1979) and Danielsson *et al.* (1982).

A number of other methods exist *e.g.* adsorption of dithocarbamate complexes on chromatography columns (Friedman, 1989) and coprecipitation with $Mg(OH)_2$. Regardless of method used, accurate and precise determinations requires strict procedures for control of reagents and laboratory equipment. The use of a clean room laboratory is generally necessary for the determination of trace metals in sea water.

Standard materials

In order to evaluate laboratory practices and the accuracy of a method, certified reference materials are of high importance. The, at present, most commonly used reference materials, are those produced by National Research Council of Canada. The reference sea water Nass-3 is certified for more than 10 elements.

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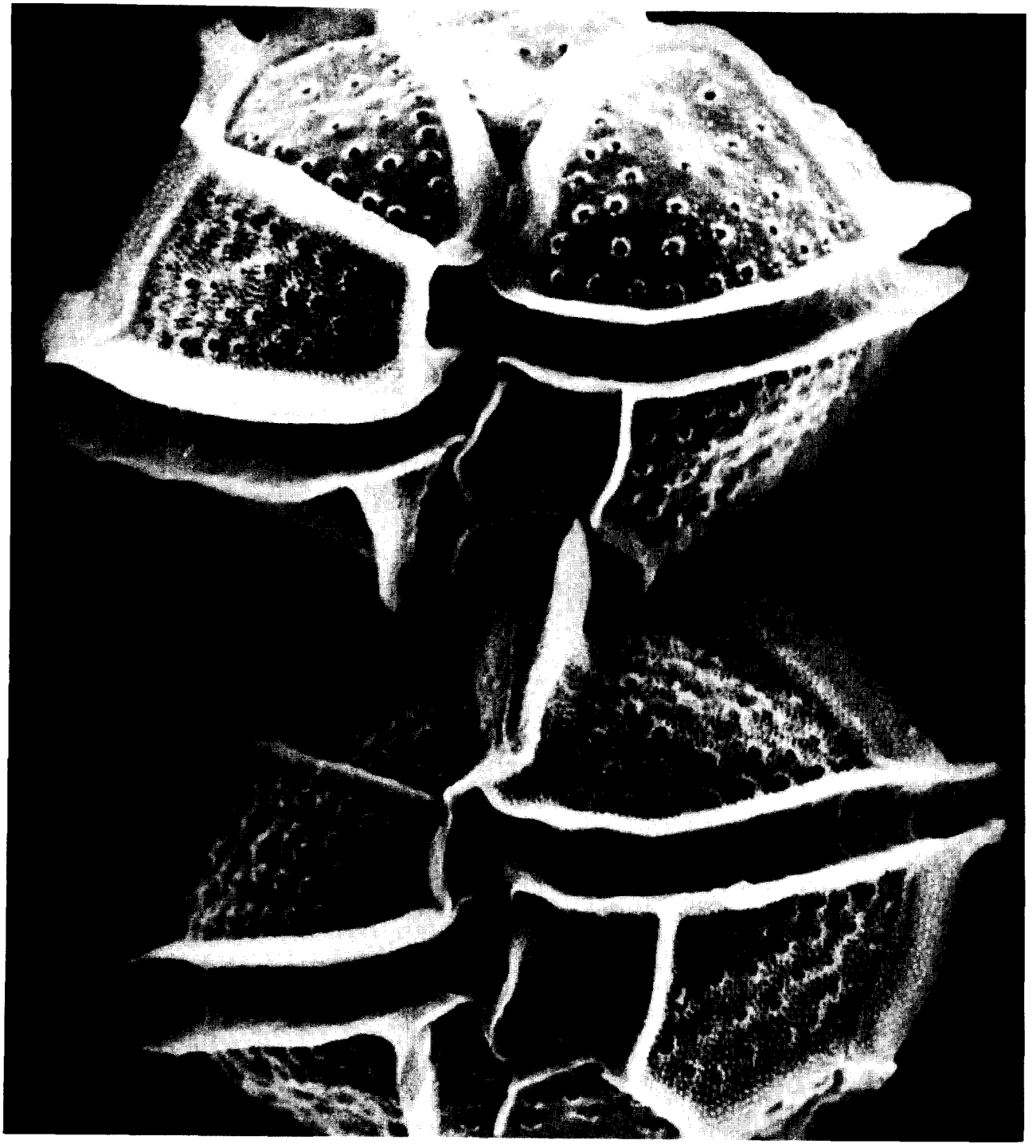
Part II

Taxonomy



14. Taxonomic Principles

G.M. Hallegraeff

Harmful algal blooms are often almost monospecific events. Correctly assessing the precise taxonomic identity of the causative organism thus becomes crucial in deciding whether knowledge on toxicology, physiology and ecology gained from similar blooms can be reliably applied to the species at hand. Resolution of the species concept in harmful algae has become a profound issue of discussion at conferences dealing with toxic phytoplankton.

WHAT IS A SPECIES?

While genera are more or less subjective taxonomic units that attempt to reflect close relationships, species are supposed to be evolving biological units (see Taylor 1993, for a discussion of the history of development of this concept). This has been the basis for the Biological Species Concept with species boundaries defined according to the ability of organisms to interbreed and produce viable offspring. While readily applicable to Metazoan animals, there are problems with plants where some apparently distinct species successfully hybridise (often with weakly viable offspring) and in particular with protists for which sexual fusion is infrequent or apparently absent. For dinoflagellates, for example, sexuality has been documented for only about 10% of species (see Chapters 11 and 20) and accordingly very few attempts have been made to use sexual interbreeding for strain and species definition (see Beam & Himes 1982 for *Crypthecodinium cohnii*; Blackburn *et al.* 1989 for *Gymnodinium catenatum*; Anderson *et al.* 1994 for *Alexandrium tamarense*). These studies have revealed the existence of morphologically similar natural populations that are reproductively isolated, analogous to the "sibling species" concept known from animal studies. For most harmful algal species, however, the usual mode of proliferation is by asexual fission and sexuality in addition to genetic recombination serves a special purpose in the life cycle of these organisms (cyst formation and survival in dinoflagellates; auxospore formation and cell enlargement in diatoms). For permanently asexual organisms, only discontinuities in morphological or biochemical characters can be used to constitute species boundaries.

TAXONOMIC CRITERIA USED FOR IDENTIFICATION AND CLASSIFICATION

Morphology of an organism is the complex expression of its genotype, subject to phenotypical change due to the environment, life cycle transformations and other influences. Morphological traits and biogeographical distributions of organisms continue to be considered as the primary means for traditional species classification. Through examination of thousands of individuals one needs to develop an understanding of what are conservative characters useful for taxonomy and what are highly variable characters. Cultured cells can have more variable morphology than field material, and considerable care should be exercised in basing new species descriptions exclusively on cultured material. For diatoms, useful conservative taxonomic features include strutted/ labiate/occluded process patterns and diatom valve markings (number of striae; areolae in 10 µm) (Chapter 17). For armoured dinoflagellates thecal plate patterns are the most diagnostic, with hypothecal characters being more conservative than epithecal ones (Chapter 15). For less ornate unarmoured dinoflagellates and especially nanoplanktonic and pico planktonic taxa (such as *Aureococcus anophagefferens*) ultrastructure of chloroplasts,

pyrenoids, flagellar roots etc. have become indispensable morphological adjuncts. In addition, non-morphological characters such as lipid, pigment and toxin biochemistry, immuno cytological traits, chromosome number and DNA content (e.g. for *Gymnodinium* cf. *nagasakiense*; Partensky *et al.* 1988), and more recently nuclear or plastid DNA sequences are now being increasingly used to aid in species recognition. While marine phytoplankton species have remained morphologically conservative they can have accumulated significant genetic variability. Looking alike does not necessarily mean genetically identical, and looking different does not mean genetically isolated (Taylor 1993). Morphospecies designations therefore sometimes can be of limited use for ecological purposes. The dinoflagellate *Alexandrium tamarense* is known to exist as toxic and non-toxic strains, bioluminescent and non-bioluminescent populations, and cold-water and warm-water forms. In some cases the use of biochemical, molecular, and physiological data has corroborated morphotaxonomy (e.g. arguments for the synonomy of *A. minutum* and *A. lusitanicum*; Costas *et al.* 1995), while in other cases apparent conflicts have inspired a revaluation of traditional species discrimination (e.g. the *Phaeocystis pouchetii* complex; Medlin *et al.* 1994; the elevation of *Pseudo-nitzschia multiseries* to species level; Manhart *et al.* 1995) or provoked further debate on the species concept in unicellular algae. While the presence of a ventral pore in the first apical plate of the dinoflagellate *Alexandrium tamarense* has been widely accepted as a stable taxonomic character (Balech 1995) discriminating this species from *A. fundyense*, Anderson *et al.* (1994) demonstrated that cultured clones of the two taxa were sexually compatible. Furthermore, Scholin and Anderson (1994) working with the same *A. tamarense* "species complex" found that ribosomal RNA sequences of isolates clustered more logically on the basis of geographic origin than morphotaxonomy.

GENERAL COMMENTS ABOUT TAXONOMIC NOMENCLATURE

The science of taxonomy seeks to delimit stable groups of individuals that share common traits. The correct name for a phytoplankton species should fulfil stringent requirements as spelled out by the International Code of Botanical Nomenclature (ICBN; most widely used for algae), International Code of Zoological Nomenclature (ICZN; sometimes used for dinoflagellates and euglenoids which have a large proportion of colourless species) or the International Code of Nomenclature of Bacteria (used for cyanobacteria).The aim of these codes is to produce nomenclatural stability. Under the ICBN, for species descriptions after 1 Jan.1958 there is a requirement for a written description of the essential characters, an illustration, a Latin diagnosis and a designation of type material. The name must not have been used previously in that rank for a member of the Plant Kingdom (if it had it would be referred to as a homonym) and it must have priority, being published before any other name applied within the same rank to the same organism (others being referred to as synonyms). More and more, the practice of lodging permanent mounts in a museum or herbarium is being replaced by the designation of light or electron micrographs as holotypes, while with (cyano)bacteria pure cultures can serve as types (Stanier *et al.* 1978). The recommendation by the ICBN that names above the level of genus should be typified (e.g. the class Prymnesiophyceae based on the genus *Prymnesium*) has not been generally accepted (i.e. leaving the name Haptophyceae as a valid alternative, Chapter 16). When new combinations result from transferring species from one genus to another, the species name should be retained, unless it has already been used with the genus to which the species is brought, in which case a new species name must be provided. After 1 Jan. 1953, the basionym (the combination under which the species first appeared) must be cited, with publication details provided. An author's name in parentheses means that since the original description of the taxon its name has been changed. There are important procedural differences between the ICBN and ICZN (e.g. requirement or not for a Latin diagnosis), and neither the ICBN nor ICZN recognize as homonyms those names proposed under one code but preoccupied under the other. This can lead to absurd situations in which a scientist declaring him/herself a zoologist can be precluded from using names a botanist can use (Patterson and

Larsen 1992). A proposal (Taylor *et al.* 1987) to resolve these nomenclatural problems was not accepted at the Berlin Botanical Congress. There are also differences between botanists and zoologists in the recognition of infraspecific categories. Botanists use the terms variety (often conceived as small differences in genotype) and form (a response of an organism with the same genotype to a different environment), although the meaning of these terms is not agreed upon, while zoologists only recognize the term subspecies, which carries with it a notion of geographic isolation and lack of interbreeding which is difficult to apply to marine phytoplankton with virtually unlimited dispersal options. Palaeontologists working with fossil dinoflagellate cysts by consensus have chosen to treat them as form genera under the ICBN. When a dinoflagellate taxon has both a fossil (e.g. *Polysphaeridium zoharyi*) and modern representation (*Pyrodinium bahamense*; Chapter 20), then the name-carrier should preferably be the living organism, since this provides the most complete information (Wall & Dale 1968). This would necessitate, however, the official conservation of e.g. the genus *Gonyaulax* Diesing 1866 against the older equivalent cyst name *Spiniferites* Mantell 1850. An attempt to produce a unified classification of living motile dinoflagellates and fossil dinoflagellate cyst taxa has been prepared by Fensome *et al.* (1993).

NAME CHANGES

Name changes always cause concern and confusion to non-specialists, but reflect the ever developing scientific understanding of natural relationships among organisms. Two examples are provided to illustrate the taxonomic principles described above. The diatom *Pseudo-nitzschia australis* Frenguelli 1939 was classified as a section within the genus *Nitzschia* by Hasle (1965). Since the name *N. australis* was preoccupied, the new name *Nitzschia pseudoseriata* Hasle was introduced. Recently *Pseudo-nitzschia* was revived for species with step-wise overlapping colonies (Chapter 17) thus necessitating a return to the name *P. australis*. Another example is the toxic dinoflagellate *Gonyaulax tamarensis* Lebour 1925, which once it was recognised that its thecal plate pattern (4 apicals, no intercalaries) did not fit in the genus *Gonyaulax*, was relegated for a number of years to either the poorly defined genus *Alexandrium* Halim 1960, or *Gessnerium* Halim 1967 (based on an erroneously optically reversed *Alexandrium*) or *Protogonyaulax* (based on absence of contact between the first apical plate homologue [1'] with the apical pore complex [Po]; Taylor 1979). This confusion was eventually resolved by a reexamination of material of the type species *Alexandrium minutum* from the type locality in Alexandria Harbour, Egypt (Balech 1989), which revealed the variable nature of the contact between 1' and Po and thus indicated that *Protogonyaulax* could not be maintained (Chapter 15). The dinoflagellate organism in question now should be called *Alexandrium tamarense* (Lebour) Balech. To alleviate the problems of ever-changing taxonomy of harmful phytoplankton, it is recommended: (1) to study type-material or, if this is not available, collect and reexamine material from the type locality; (2) establish and curate type specimen collections using permanent mounts and/or photomicrographs (which is required by the codes), but also include video tapes and preferably living cultures; and (3) incorporate life cycle features e.g. cysts in species descriptions (Steidinger 1990). Original names as far as possible should be retained until complete information is available on the existing available and valid genera.

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15. Taxonomy of Harmful Dinoflagellates

F.J.R. Taylor, Y. Fukuyo and J. Larsen

WHAT ARE DINOFLAGELLATES?

This group of unicellular eukaryotic organisms are termed "flagellates" because the majority, including the harmful species, can swim by means of a pair of whip-like flagella. They rotate as they swim, but so do most flagellates. They occur in both salt and freshwater and can be both planktonic (up in the water) or benthic (associated with the bottom). Approximately 50% of the species are photosynthetic (Taylor, 1987). The group is characterised by form (grooves associated with the flagella: see below) but exceptions are recognised by features such as their nucleus or flagellar details (see Spector, 1984 and Taylor, 1987 for general references dealing with the group as a whole). Recently the classification of the group, including its rich fossil representation, has been revised (Fensome *et al.*, 1993).

GENERAL COMMENTS ABOUT HARMFUL DINOFLAGELLATE SPECIES

As is evident from a glance at this chapter, dinoflagellates are the protist group with the largest number of harmful species. The great majority are photosynthetic (*Noctiluca* and some species of *Dinophysis* are the notable exceptions), for no obvious reason other than that they are more likely to be abundant than non-photosynthetic species and their behaviour makes them amenable to physical aggregation. Both thecate and athecate species can be harmful. Most are marine. Chain formers seem to be over-represented for no obvious reason and quite a few are closely related (Taylor, 1985, 1993).

Identificatory publications dealing with harmful dinoflagellates

The need for assistance in identifying potentially harmful species has led to the creation of documents, of which this is the latest. Since many of these species are cosmopolitan within their latitudinal range, both Northern and Southern hemisphere (Taylor, 1987), aids created for one area may be useful in another. An early source created specially for harmful species recognition by aquaculturists is that by Gaines & Taylor (1986) but more lavishly published and illustrated versions have been produced by Larsen & Moestrup (1989), Fukuyo *et al.* (1990) building on the species sheets issued by a Japanese working group on red tides since the late 1970s), and Hallegraeff (1991). Tropical benthic species potentially involved in ciguatera were described by Fukuyo (1981). Balech has contributed many recent descriptions, notably those in his most recent monograph on *Alexandrium* (Balech, 1995). These works should be consulted for further detail in descriptions, illustrations, reference citations and regional information. General taxonomic matters are discussed in chapter 14.

Taxonomic criteria used in harmful dinoflagellate identification and classification

The following is a brief guide to the criteria and terminology used in dinoflagellate description. Cell size and shape are obvious features used. Cell contents have not been used much since all

harmful species recognised so far, with the exception of *Noctiluca*, are photosynthetic. Surface ornamentation (pores, spines, ridges, etc.) are also used if present.

Essentially we can distinguish between those that lack a readily visible, multiplate cellulosic wall, the **theca** (hence called **athecate** dinoflagellates) from those that possess a theca (**thecate** dinoflagellates). The terminology relating to each is described below. Another distinction that can be made is between the great majority of dinoflagellates which have distinctive flagella arising from the (ventral) side of the cell, referred to as the **dinokont** condition, from the one harmful genus, *Prorocentrum,* in which they both arise from the anterior end, referred to as **desmokont**.

In describing dinoflagellates orientational terminology is used. The end which is forward when the cells move is named the **apical** pole and the opposite is the **antapical.** Desmokonts are considered to be laterally flattened. In *Prorocentrum*, which is thecate, the flagella arise from a field of tiny platelets that have not been used much in taxonomy so far. This **periflagellar area** is asymmetrically excavated out of one of the large lateral plates, the **right valve**, the other being the **left valve**.

In dinokonts the side that the flagella arise from is the **ventral** side and the opposite side is **dorsal,** with the organism's **left** and **right** following zoological convention. These terms are also used in describing the side that the cell is being viewed from. The most characteristic view, except in highly flattened species, is usually the ventral view. When sending a photograph or drawing to an expert this view should not be omitted. The cells can usually be rolled over by carefully tapping on the coverslip or using a glass needle.

Other features used in descriptions include the position of the girdle (= cingulum) and whether it is **displaced** or not: i.e. whether the proximal and distal ends meet or are offset. In the latter case, if the left (proximal) side is more anterior the displacement is **left-handed**, or it is **right-handed** if the opposite is true. The former is much more common. The degree of offset is given in girdle widths. In *Alexandrium*, for example, the girdle has a left-handed displacement of approximately one girdle width.

In thecate species the plate pattern, **tabulation**, is extremely important. Due to extensive study its degree of reliability is well established (some features are more conservative than others: see Taylor, 1987). The description of new species or any critical taxonomy requires complete elucidation of the plate pattern, which can be difficult, requiring special techniques. Fortunately, for most routine identifications this is not necessary, identification relying on general form, size and one or two key characters. The descriptions below focus on these.

All the above features are morphological. Such morphologically defined species are termed morphospecies (Taylor, 1992, 1993). In general there is agreement between morphological and other cell features but there is increasing recognition of genetic variability within morphospecies. For example, toxicity cannot always be assumed for a particular morphospecies: in some all strains tested have been found to be toxic, e.g. *Alexandrium catenella* and possibly some *Dinophysis* spp.. In others non-toxic strains are known, e.g. *Alexandrium tamarense* and *Gambierdiscus toxicus* or toxin production may be environmentally inducible.

Life cycle features are also very important and all stages, when known, play a part in recognition of species. In this publication the use of cysts (dormant benthic stages) in identification is dealt with in chapter 20 by Matsuoka & Fukuyo.

The basic types of dinoflagellates involved in harmful blooms

Dinoflagellates show a great range of forms but most of this diversity can be resolved to five basic types (Taylor, 1980) and the harmful species are dealt with under these categories below.

GYMNODINIOIDS AND NOCTILUCOIDS

Amphidinium Claparède et Lachmann

The girdle is strongly dislocated towards the anterior end of the cell, so the episome is small, often tongue-shaped or appearing as a small cap on the top of the cell. The genus is not clearly delimited from *Gymnodinium* and assignment of species is often arbitrary. *Amphidinium* comprises about 100 photo- or heterotrophic species. It is found mostly in salt or brackish water habitats, and many species are benthic.

Amphidinium carterae Hulburt Fig. 15.1

Synonyms: See comments.

More or less oval cells, flattened dorso-ventrally. The episome is small, crescent or tongue-shaped, deflected to the left describing a descending spiral which is displaced 2-3 girdle widths. One, widely branched and usually peripheral chloroplast with a large central pyrenoid. The nucleus is crescent-shaped and located in the hyposome. Length 12-18 µm, width 8-10 µm.

Distribution: *Amphidinium carterae* belongs to a group of species which includes *A. operculatum* Claparede et Lachmann which is the type species of the genus, and *A. klebsii* Kofoid et Swezy. *A.carterae* and *A. klebsii* are the most often reported species of this complex, but cannot always be clearly distinguished. According to Taylor (1971), they can be distinguised by the chloroplast structure. The size of the cells should also be considered; *A. carterae* appears to be smaller (Hulburt (1957) indicated a length of 12-15 µm) than *A. klebsii* (Kofoid & Swezy (1921) indicated a length of 46 µm). A detailed examination of the type species is not available, and is needed before the taxonomic problems in this group can be addressed. *A. carterae* can produce hemolytic compounds and may be implicated in ciguatera.

Amphidinium klebsii Kofoid et Swezy Fig. 15.2

Synonyms: *A. operculatum* Claparède et Lachmann, sensu Klebs (1884).

This species is similar to *A. carterae* in having more or less oval cells with a small tongue-shaped, leftwards deflected episome. It contains numerous long, slender chloroplasts attached to a large pyrenoid located in the central part of the hyposome. Length: 20-46 µm, width: 14-30 µm.

Distribution: *Amphidinium klebsii* has on several occasions been reported from the same sites and localities as *A. carterae*. It is not at present possible to establish the precise distribution of the two species, see comments on *A. carterae*.

Comments: This species may be difficult to distinguish from *A. carterae*, but can be identified by the larger size and by the numerous slender chloroplasts deployed around the large pyrenoid (Taylor, 1971), see also comments on *A. carterae*. This species can produce hemolytic compounds.

Cochlodinium Schütt

Cochlodinium is characterized by a descending girdle which performs 1.5 up to 4 turns around the cell. The genus comprises about 40 species most of which are heterotrophic (Larsen and Sournia, 1991). Many are poorly known and in need of reexamination.

Cochlodinium catenaum Okamura, *C. citron* Kofoid et Swezy as well as several unidentified species have been associated with harmful events, but only *C. polykrikoides* is included here as this species causes the most serious problems.

Cochlodinium polykrikoides Margalef Fig. 15.3

Synonyms: *C. heterolobatum* Silva, *C.* type '78 Yatsushiro

The cells often form short chains consisting of rarely more than 8 cells. The individual cells are more or less oval, only slightly flattened and with a girdle making 1.8-1.9 turns around the cell, notched at the antapex. An apical groove is present. A red stigma is located on the dorsal side of the episome. There are several band-shaped chloroplasts. The nucleus is located in the episome. Length 30-40 µm, width 20-30 µm.

Distribution: *Cochlodinium polykrikoides* has been reported from the American east coast, Japan where it is widely distributed and often forms harmful blooms, Korea and Puerto Rico.

Comments: This species may be toxic to juvenile fish, but the toxic principles are unknown.

Gymnodinium Stein

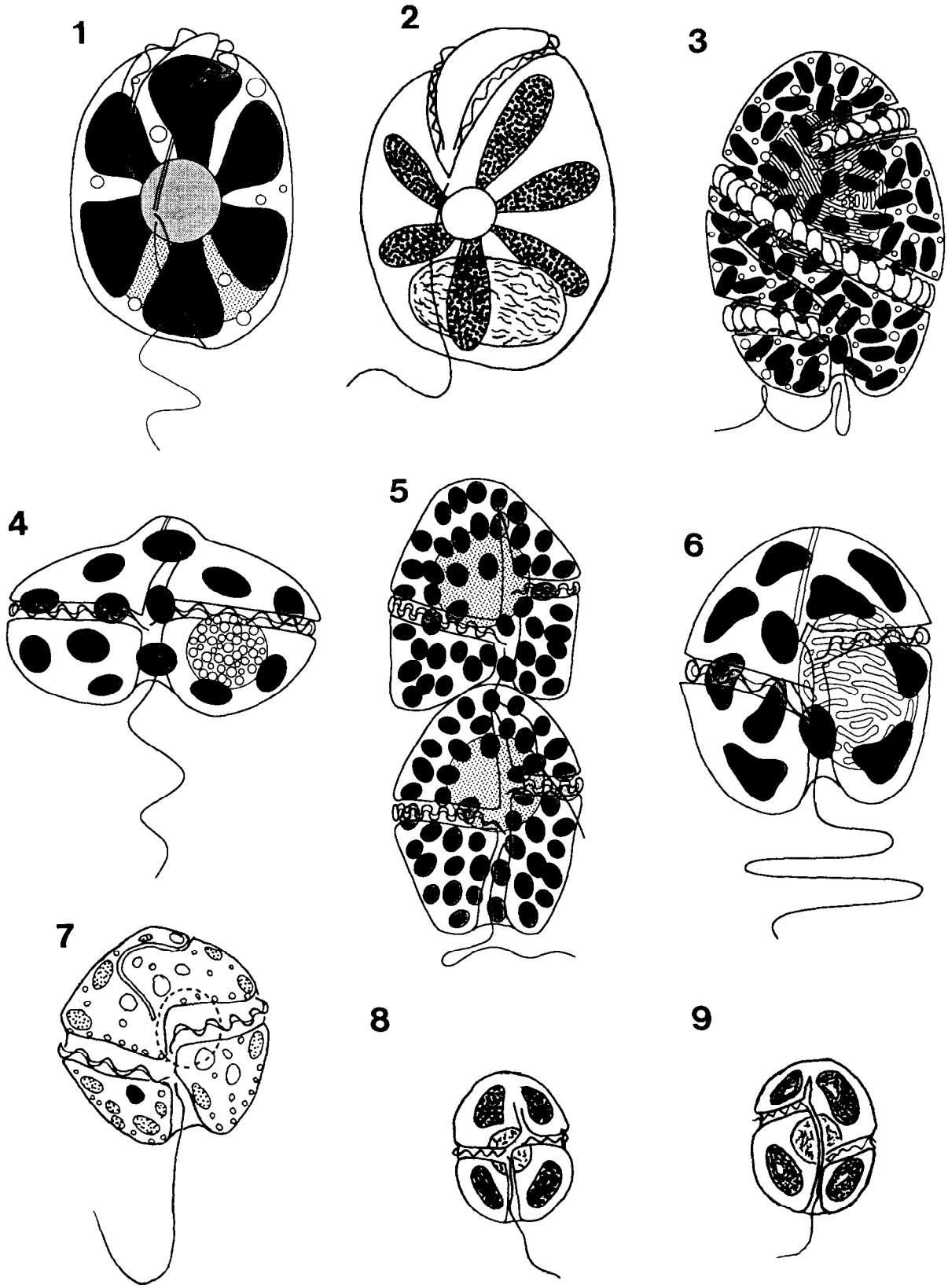
The girdle in species of *Gymnodinium* is located in the equatorial region of the cell, and shows little (less than one fifth of the cell length) or no displacement. Species with a girdle displacement of one fifth of the cell length or larger are referred to *Gyrodinium* (Kofoid and Swezy, 1921). However, generic assignment is often arbitrary, as several species have a girdle displacement of about one fifth of the cell length. *Gymnodinium* comprises more than 200 photo- or heterotrophic species. Most species occur in marine environments. Several toxic, phototrophic species of *Gymnodinium* including *G.breve, G.mikimotoi* and presumably *G.veneficum* do not contain peridinin, but fucoxanthin or fucoxanthin derivatives as major accessory pigments (Riley and Wilson, 1967; Jeffrey *et al.*, 1975; Tangen and Bjornland, 1981; Rowan, 1989).

Gymnodinium breve Davis Fig. 15.4

Synonyms: *Ptychodiscus brevis* (Davis) Steidinger

The cells appear almost square in outline, but with a prominent apical process; strongly flattened dorso-ventrally. The girdle is not or only slightly displaced and then describing a descending spiral; three vertical ridges are present near the distal end of the girdle. The sulcus intrudes onto the episome. An apical groove extends from near the sulcal extention on the episome across the apical process and onto the dorsal side of the cell. There are numerous chloroplasts with pyrenoids.

Distribution: *Gymnodinium breve* occurs regularly in the Gulf of Mexico, but *G. breve* or *G. breve*- like species have also been reported from the West Atlantic, Spain, Greece, Japan, and New Zealand.



Figures 15.1-9. Gymnodinioid dinoflagellates: Fig. 1. *Amphidinium carterae*; Fig. 2. *A. klebsii*; Fig. 3.*Cochlodinium polykrikoides*; Fig. 4. *Gymnodinium breve*; Fig. 5. *G. catenatum*; Fig 6. *G. mikimotoi*; Fig. 7. G. *pulchellum*; Fig. 8. *G. veneficum*; Fig. 9. *Gyrodinium galatheanum*. [Figs. 1, 3, 4, 5. 6 by H.Takayama; Fig. 2. after D.Taylor 1971; Fig. 7. by K. Matsuoka; Fig. 8. after D.Ballentine 1956; Fig. 9 by J. Larsen].

Comments: It is uncertain whether the *G. breve*-like species reported to occur outside the Gulf of Mexico and the Western Atlantic region should be assigned to *G. breve* or if they represent different, closely related, species (Steidinger *et al.* 1989). The toxins are responsible for neurotoxic shellfish poisoning (NSP) which may be harmful to both fish and mammals, while aerosolization of the toxins may be responsible for asthma-like symptoms. NSP has been restricted so far to the southeastern part of the USA, particularly the Gulf of Mexico and most recently New Zealand.

Gymnodinium catenatum Graham Figs 15.5, 15.10 i-j

The cells form chains of usually 4, 8, or 16 cells, occasionally longer. Unfavourable conditions may cause the chains to break up into single cells, and non-chain forming clones possibly also exist. The girdle describes a descending spiral which is displaced up to one fifth of the cell length. The sulcus extends from the antapex to the apex which is also surrounded by a semi-circular apical ring. The cells contain numerous chloroplasts with conspicuous pyrenoids. The nucleus is located in the central part of the cell. The reported size ranges are for solitary cells: 34-65 µm long and 27-43 µm wide; for chain-forming cells 23-60 µm long and 27-43 µm wide, with the terminal cells being about the size of solitary cells.

Distribution: It was first reported from California (USA) and has subsequently been reported from several places including Argentina, Australia (Tasmania), Italy, Japan, Mexico, Philippines, Portugal, Spain and Venezuela. According to Hallegraeff and Bolch (1992), dinoflagellate cysts may travel considerable distances in ships' ballast water, and *G. catenatum* could have been introduced into Australian waters in this way. Live cysts have recently been found in Danish sediments (Ellegaard *et al.*, 1994), but vegetative cells have never been reported from Danish waters.

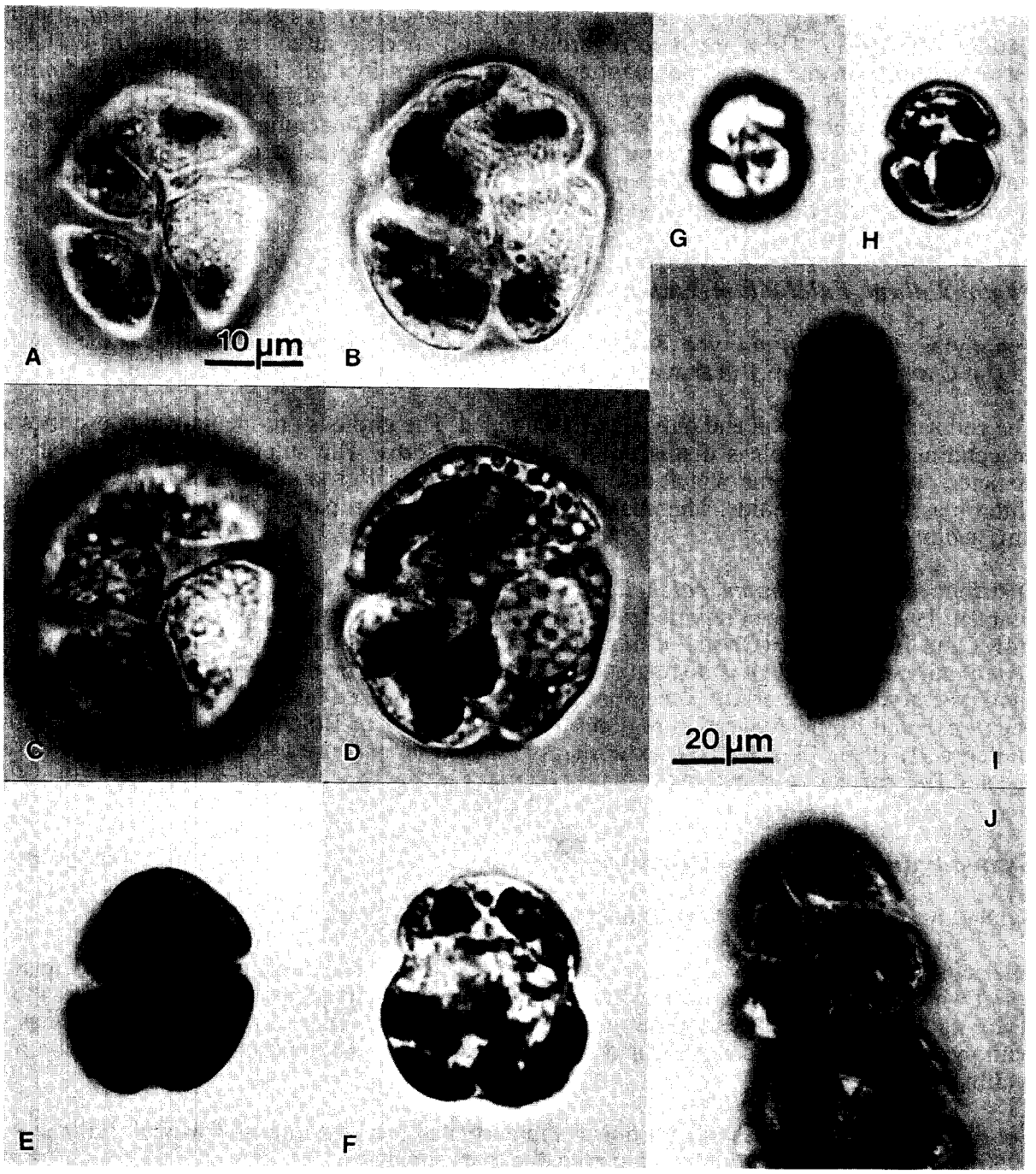
Comments: *Gymnodinium catenatum* is the only unarmoured dinoflagellate known to produce PSP-toxins. It is distinguished from other species of *Gymnodinium* by its chain-forming habit. Single cells are best identified by the large pyrenoids and the conspicuous sulcus. Recent observations by Fraga *et al.* (1995) suggest that another closely related species, *Gyrodinium impudicum,* may exist. The life cycle of *G. catenatum* was described by Blackburn *et al.* (1989) (hologamy, heterothally) resulting in a round (42-52 µm diameter) brown resting cyst (hypnozygote) with microscopic reticulations.

Gymnodinium mikimotoi Miyake et Kominami ex Oda Figs 15.6; 15.10 c-f

Synonyms: *Gymnodinium nagasakiense* Takayama et Adachi, *Gymnodinium* sp.1 (Adachi, 1972), *Gymnodinium* type-'65 (Iizuka and Irie, 1969), *Gyrodinium aureolum* Hulburt, sensu Braarud and Heimdal (1970).

The cell outline is variable, ovate to almost round, flattened dorso-ventrally. The girdle is wide and describes a descending spiral which is displaced about one fifth of the cell length. The sulcus continues for a short distance onto the episome, where an apical groove extends in a straight line from near the sulcal intrusion across the apex and a short distance down on the dorsal side of the cell. There are several more or less oval chloroplasts, each with a conspicuous pyrenoid. The nucleus is located in the left side of the hyposome. Length: 24-40 µm, width: 20-32 µm.

Distribution: *Gymnodinium mikimotoi* is widely distributed and has formed blooms in Australia, Denmark, Ireland, Japan, Korea, Norway and Scotland.



Figures 15.10 a-j. Light micrographs of gymnodinioid dinoflagellates. Figs a, b. *Gymnodinium pulchellum;* c-f. *Gymnodinium mikimotoi* (c-d. live cells; e. lugol-fixed; f. formaldehyde- fixed); g-h. *Gyrodinium galatheanum*; i-j. *Gymnodinium catenatum*. Scalebars in Figs a-h, j, = 10 µm, Fig, i. = 20 µm.

Comments: The morphological similarity between the Pacific *G. mikimotoi* and the European *Gyrodinium aureolum* has been noted by several authors (Taylor, 1985; Fukuyo *et al.*, 1990; Hallegraeff, 1991). They are here regarded as con-specific although genetic differences between European and Pacific populations do exist (Partensky *et al.* 1988). Thus, *Gyrodinium aureolum* Hulburt (1957) is considered a different species which differs from *G. mikimotoi* in several details such as position of the nucleus, dorsoventral flattening of the cell, number of chloroplasts and pyrenoids, and posssibly by lacking an apical groove. *Gymnnodinium mikimotoi* produces both hemolytic and ichthyotoxins, and has caused damage in fish farms in Australia, Northern Europe, Japan and New Zealand.

Gymnodinium pulchellum Larsen Figs 15.7, 15.10 a-b

Synonyms: *Gymnodinium* type '84K (Onoue *et al.*, 1985; Fukuyo *et al.*, 1990; Hallegraeff, 1991); *Gymnodinium* sp.1 (Takayama, 1985).

The cells are broadly oval and only slightly flattened. The girdle is pre-median and describes a descending spiral which is displaced 1-1.5 girdle widths. The sulcus intrudes for a short distance onto the episome which also has a sigmoid apical groove. It has several irregular, chloroplasts with pyrenoids. The nucleus is located in the left part of the cell. Length: 16-25 µm, width: 11-16 µm.

Distribution: *Gymnodinium pulchellum* was described from the Melbourne area, Australia (Larsen, 1994). It has been reported also from Tasmanian waters (Hallegraeff, 1991 as *G.* type '84K) and from Japan (Onoue *et al.*, 1985 as *G.* type '84K; Takayama, 1985 as *G.* sp.1). *Gymnodinium pulchellum* is distinguised from *G. mikimotoi* by the sigmoid apical groove. It should be noted, however, that other, presumably closely related but undescribed, species have been observed in the Australian region (*Gyrodinium* sp.1 of Bolch and Hallegraeff, 1990); Hallegraeff, 1991: Figs 8I,J), so extreme care should be exercised when dealing with these species.This species is ichthyotoxic.

Gymnodinium veneficum Ballantine Fig. 15.8

Synonyms: *Gymnodinium vitiligo* Ballantine

The cells are more or less oval, not or only slightly flattened. The girdle describes a descending spiral which is displaced 1-2 girdle widths. The sulcus is slightly sigmoid being deflected in the intercingular region, with a short protusion onto the episome. There are usually 4 chloroplasts with pyrenoids. The nucleus is located in the central part of the hyposome. Length: 9-18 µm, width: 7-14 µm.

Distribution: It was first described from a culture isolated from the English Channel (Ballantine, 1956), but is presumably widely unreported due to its small size.

Comments: *Gymnodinium veneficum* is not easily distinguished from *G. micrum*, but it usually has 4 chloroplasts and a less pronounced sulcal deflection in the inter-cingular region as compared to *G. micrum.*. *Gymnodinium veneficum* is toxic to a wide variety of invertebrates and particularly to fish.

Gyrodinium Kofoid and Swezy

The girdle in species of *Gyrodinium* is located in the equatorial region of the cell, and is displaced more than one fifth of the cell length (Kofoid and Swezy, 1921). The genus is not

clearly delimited from *Gymnodinium* (see above). It comprises about 100 photo- or heterotrophic species. Most species are marine.

Gyrodinium galatheanum (Braarud) Taylor Figs 15.9, 15.10 g-h

Synonyms: *Gymnodinium micrum* (Leadbeater et Dodge) Loeblich III, *Woloszynskia micra* Leadbeater et Dodge, *Gymnodinium galatheanum* Braarud.

The cell shape is oval to round. The girdle is deeply incised, describing a descending spiral which is displaced almost one fifth of the cell length. The sulcus is strongly deflected in the inter-cingular region and extends onto the episome The cell contains usually 2 chloroplasts, one in the episome and one in the hyposome which are visible by LM. The nucleus is located in the central part of the cell. Length: 9-17 µm, width: 8-14 µm.

Distribution: This species has been reported from the North and South Atlantic. It is presumably wide-spread, but easily overlooked because of its small size.

Comments: The gross morphology of *G. galatheanum* is shared by several small species of unarmoured dinoflagellates including *G. micrum* and *G. veneficum* Ballantine and the taxonomic status of these species is uncertain. It does not contain peridinin, but a fucoxanthin derivative as accessory pigment (Bjornland and Tangen, 1979).This species is toxic to mussels and juvenile cod (*Gadus morhua*).

Noctiluca scintillans (Macartney) Ehrenberg Fig. 15.11

Synonyms: *N. miliaris* Suriray

The cells are large and more or less balloon-shaped, with a striated tentacle. It is phagotrophic, and the cells are usually colourless. In certain tropical area it may attain a green colour because of the presence of endosymbioitic flagellates (Sweeney, 1976). Diameter 200-2000 µm.

Distribution: *Noctiluca scintillans* is widely distributed and it has formed blooms in both temperate, subtropical, and tropical waters.

Comments: Blooms of *N. scintillans* may cause a strong red discolouration of the water. It may be bioluminescent except in parts of the northern and eastern Pacific Ocean. It accummulates large amounts of ammonia in the vacuole which may be toxic to fish.

Pfiesteria piscicida Steidinger et Burkholder Fig. 15.12

The ichthyotoxic "phantom" dinoflagellate *Pfiesteria piscicida* was first recognised in North Carolina in 1991 (Burkholder *et al.*, 1992). Its ephemeral presence (cysts germinate in the presence of life fish, and encyst again after fish death) may explain many mysterious fish kills along the southeast coast of the United States.The organism was described as a new species and genus within the order Dinamoebales (Steidinger and Burkholder, in press; Steidinger *et al*, 1995). Multiphasic life cycle with polymorphic and multiphasic bi-and triflagellated, amoeboid, and nonmotile cyst stages with transitional forms between all stages. All stages are unicellular. Bi-and triflagellate stages are typically planktonic and ephemeral and resemble gymnodinioid forms but are actually small cryptic peridinioids with a plate formula of Po, cp, X, 4', 1a, 5'', 6c, 4s, 5''' and 2''''. Biflagellate stages are of two size classes, 5-8 µm and 10-18 µm, both regarded as haploid. Triflagellate stages, 25-60 µm, with one transverse and two longitudinal flagella are regarded as planozygotes. Amoeboid stages vary in size (5 -250 mm), shape and

type of podia. Filose and lobose amoeboid stages are thecate with a honeycomb-like surface structure of repeated coalesced units similar to *Heterocapsa* or *Katodinium* organic scales. Cyst stages, 25-33 µm, range from spherical testate forms with a honeycomb surface pattern to forms resembling scaled chrysophytes. The cells are heterotrophic and mixotrophic, capable of phagocytosis, and with cleptochloroplasts present.

PERIDINOIDS

This thecate group is one of two in which the plates are arranged in the classic five-horizontal-plate series, the other being gonyaulacoids (below, see Fensome *et al.*, 1993 for a full discussion of this). Many are non-photosynthetic although the harmful species below is photosynthetic.

Peridinium, "Glenodinium"

The older literature is confusing in that all marine *Protoperidinium* species were included in the genus *Peridinium,* but the latter is now restricted to species with five or six cingular plates (instead of three plus a small transitional in *Protoperidinium).* This action also resulted in the marine species of the latter genus all being non-photosynthetic whereas it appears that the *Peridinium* species are all photosynthetic. *Glenodinium* has been used variously in the past but, if one uses the type species as an indication of its character, the species below is clearly not a *Glenodinium.*

Peridinium polonicum Woloszynska Fig. 15.13

Syn.: *Peridiniopsis polonicum* (Woloszynska) Bourrelly, *Glenodinium gymnodinium* Penard?

Ovoid cells, somewhat flattened dorso-ventrally. The girdle is slightly indented, lacking lists, median with a left-handed displacement of one girdle width. The sulcus is straight, slightly wider than the girdle only indenting the posterior profile very slightly. The first apical plate has four main sides ("ortho") and it has two small, unequal anterior intercalaries (sometimes only one and thence put in the genus *Peridiniopsis*). The two antapicals are also unequal, the second being bigger than the first. Length: 34-43 µm, transdiameter: 28-38 µm.

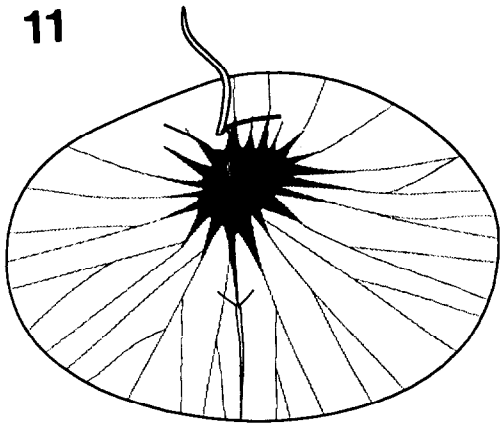
Distribution: First described from Europe; its harmful effects have occurred in Japan.

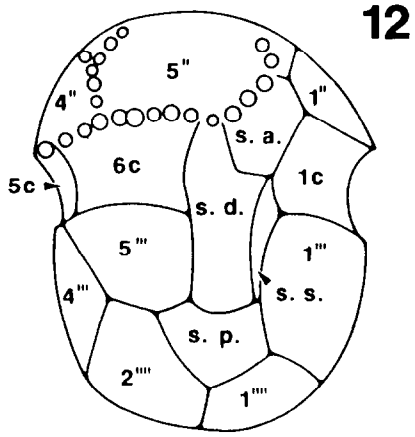
Comments: The only freshwater dinoflagellate to be positively linked to harmful blooms, being linked to fish kills.

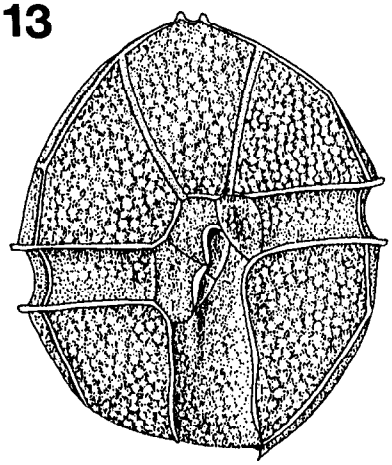
GONYAULACOIDS

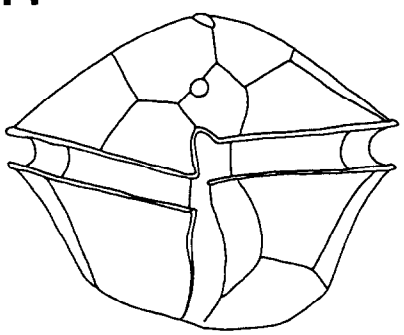
These are similar to the peridinioids but differ in the symmetry of their thecal plates (see Taylor, 1980, 1987; Evitt, 1985; Fensome *et al.*, 1993). They are predominantly photosynthetic and the majority of toxic species belong to this assemblage.

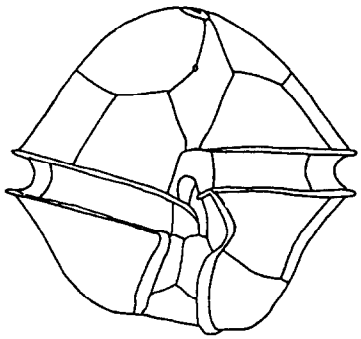
An important feature used in generic distinctions is the shape and relationships of the homologue of the traditional "rhomb" plate (Kofoidean first apical). Within gonyaulacoids it may extend all the way to the apical pore complex (APC), in which it is a typical first apical

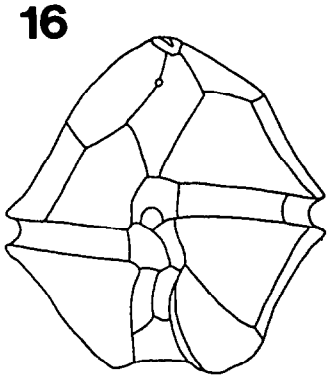












Figures 15.11. *Noctiluca scintillans*; Fig. 15.12. *Pfiesteria piscicida*; Fig. 15.13. *Peridinium polonicum*; Figs. 15.14-16. Different *Alexandrium* morphotypes: Fig. 15.14. *A. hiranoi,* with the cingulum above the middle; Fig. 15.15. Round cell type of *A.catenella, A minutum, A.ostenfeldii, A.tamarense*, with the cingulum in the middle; Fig. 15.16. Angular cell type of *A. acatenella, A. angustitabulatum, A. cohorticula, A. tamiya-yanichi*, with the cingulum in the middle. [Fig. 11. by H.Takayama; Figure12. by K.Steidinger; Figs.13-16. by H. Inoue].

plate. If not to varying degrees, in which it would then be, strictly speaking, a first post-cingular plate. Evitt (1985) recognised three states, named as insert, metasert and exsert

Alexandrium, Goniodoma Figs 15.14-16,. 15.38 c-d

Syn.: *Gessnerium* Halim; *Protogonyaulax* Taylor

This gonyaulacoid genus has the most species producing paralytic shellfish poisons although not all members are toxic. For a number of years *Alexandrium* and *Protogonyaulax* were held separate (*Gessnerium* being an erroneously optically inverted *Alexandrium)*, with those species in which the first apical plate contacted the APC ("insert"). The majority of toxic species, assigned to *Protogonyaulax,* and those in which they did not contact one another ("metasert") assigned to *Alexandrium* even though it was evident that they were closely related (Taylor, 1979, 1985). Variability of this condition in *A. minutum*, the type species of *Alexandrium*, has led to an abandonment of this position. The genus has been reviewed in outline by Balech (1985) and in detail by Balech (1995). A number of the species recognised in these reviews are considered to be infraspecific variants here and elsewhere (Cembella & Taylor, 1986; Taylor, 1992a,b) as the result of culture-based studies.

Generic characterisation:

Thecate gonyaulacoid cells with the Kofoidean plate formula: P, 4', 6", 6c, 9-11s, 5"', 1p, 1"" (or 2"" if the 1p is interpreted as in Balech & Tangen, 1985). The girdle has a left-handed displacement of 1 girdle width. Horns and spines are absent. Girdle margins have very low ridges. The APC has a triangular outer plate (Po) and a comma-shaped inner opening. An accessory pore may be present on Po and on the posterior sulcal when the cells are in pairs or chains. The first apical plate (1u in the Taylor-Evitt system), or its homologue when not in contact with the APC, is usually five-sided and insert or metasert. Plate surfaces usually lack ornamenation other than pores. The resting cyst is usually smooth and ovoid to round and has a chasmic (slit-like) archeopyle.

The descriptions here are limited to those known or strongly suspected to be toxic. However, as so many species of this genus are toxic and there is variability in toxicity in some of them, a bloom of any member of this genus should be viewed with concern for possible PSP in local shellfish (for other species such as *A. andersoni, A. balechii, A. compressum, A. fraterculus, A. insuetum, A. pseudogonyaulax, A. taylori,* etc., see Balech, 1995).

Alexandrium acatenella (Whedon & Kofoid) Balech Fig. 15.17

Syn.: *Gonyaulax acatenella* Whedon & Kofoid, *Protogonyaulax acatenella* (Whedon & Kofoid) Taylor

Cells single, medium sized, longer than wide with a slightly angular outline when seen in ventral view. The epitheca is longer than the hypotheca (roughly the same length as the latter plus the girdle). In some individuals the APC may project slightly. It is narrowly rectangular in shape.The suture between the second postcingular and the conventional posterior intercalary may be ridge-like in British Columbia. The surface is clearly porulated. A ventral pore is present. Balech reports a pore on the posterior sulcal plate in Japanese material. Length: 35-51 µm (most near 40), transdiameter: 26-35 µm.

Distribution: West coast of North America, Japan, Argentina, possibly Chile.

Comments: This species is only dubiously separated from *A. tamarense* by the proportions of the epitheca to hypotheca. Balech notes that it has a wider posterior sulcal plate. A population

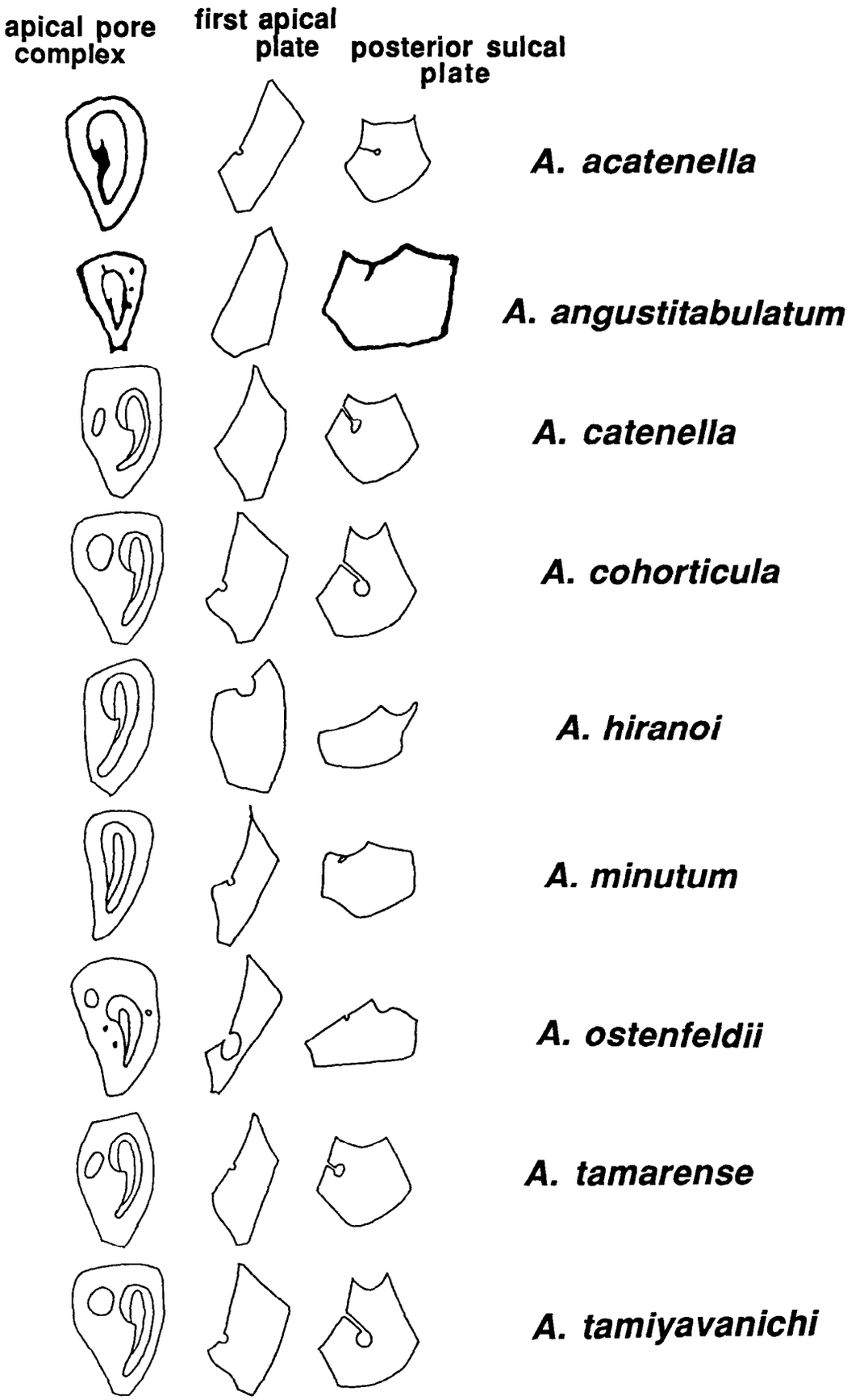


Figure 15.17. Shapes of the three key diagnostic plates (apical pore complex, first apical plate, posterior sulcal plate) of (in alphabetical order) *Alexandrium acatenella A. angustitabulatum, A. catenella, A. cohorticula, A. hiranoi, A. minutum, A. ostenfeldii, A. tamarense* and *A. tamiyavanichi.* [by Y. Fukuyo and H.Inoue; *A. acatenella* and *A. angustitabulatum* after Balech 1995].

closely resembling the original description was responsible for human illness and a fatality in British Columbia in 1965 (Prakash & Taylor, 1966).

Alexandrium angustitabulatum Taylor in Balech Fig. 15.17

Another small species resembling *A. minutum*. The ventral view is ovoid and can appear to have a symmetrical posterior indentation if slightly tilted. The epitheca is slightly larger than the hypotheca. The APC is almost rectangular. The first apical plate usually contacts the APC. It lacks a ventral pore. Its two longest margins are parallel and the suture with the sixth precingular plate is short. The sixth precingular is narrow. The anterior sulcal is subequal in length and breadth or slightly wider. Length: 17-25 µm, transdiameter: 14-24 µm.

Distribution: This species was isolated from mixed material from Whangarei Harbour, New Zealand sent to Canada in 1983.

Comments: A culture of this species has been shown to produce toxins. The shape of the first apical plate and lack of a ventral pore distinguish the species from *A. minutum*.

Alexandrium catenella (Whedon & Kofoid) Balech Fig. 15.17

Syn.: *Gonyaulax catenella* Whedon & Kofoid, *Protogonyaulax catenella* (Whedon & Kofoid) Taylor

A medium sized species. Typically in field samples this species forms long chains of cells distinctly wider than long. In culture and some Japanese populations the cells may be isodiametric. The surface is lightly porulated. The epi- and hypotheca are equal in length. The first apical plate usually has strong contact with the APC. A ventral pore is consistently absent. The APC is broad and subtriangular. In chains both anterior and posterior attachment pores are present. The posterior sulcal plate is wide, almost five-sided, the attachment pore near the right margin. Length: 20-40 µm, transdiameter: 18-37 µm.

Distribution: Widely distributed in cold temperate waters, including the west coast of North America from California to Alaska, Japan, Chile, Argentina and Australia.

Comments: This was the first species to be linked to PSP. It seems to be consistently toxic. The cell shape is distinctive in chains and distinguishes it from other chain formers except *A. tamiyavanichi* Balech. The latter, a warm water species, has a distinct anterior projection to its anterior sulcal plate and a posterior sulcal which is not as wide. Chain length varies and the chains can break when sampled. In culture single cells can occur.

Alexandrium cohorticula (Balech) Balech Fig. 15.17

Syn.: *Gonyaulax cohorticula* Balech, *Protogonyaulax cohorticula* (Balech) Taylor.

A larger chain-forming species. The cells are rounded, slightly longer than wide. The surface is finely, densely porulated. The epitheca lacks angularity at the "shoulders" and is slightly longer than the hyoptheca. The first apical plate contacts the APC and its right upper margin is concave with a ventral pore present. The APC is broadly triangular with a larger anterrior connecting pore.The anterior sulcal plate has a distinct squarish to trapezoid anterior projection demarcated by a ridge on the plate. The margins of the posterior sulcal plate may be flange-like.

Distribution: A warm water species recorded from the Gulfs of Mexico and California (Balech) and Phuket, Thailand (Taylor, unpubl.).

Comments: The toxicity of this species is now in question since the culture from the Gulf of Thailand that was shown to be toxic is now referred to by Balech as a recently described species, *A. tamiyavanichi*..The latter is very similar (see its description later).

Alexandrium hiranoi Kita & Fukuyo Fig. 15.17

Cells are ovoid to round in ventral view, slightly longer than wide or subequal. The size range is considerable. The APC is narrowly rectangular with a hooklike apical pore. A small ventral pore is present on the upper right boundary of 1u. The girdle is median, displaced one girdle width. The sulcus is shallow, with the posterior sulcal homologue (Z of T-E) not depressed and thus resembling an antapical. Thecal pores sparse. An unusual, distinctive feature is its mode of division in a non-motile, cyst-like state. A smooth-walled resting cyst is also present. Length: 18-75 μm (most 40 μm), transdiameter: 18-75 μm (most 35-40 μm).

Distribution: Known from tide pools in Japan and British Columbia coastal waters.

Comments This species is closest to *A. pseudogonyaulax* but differs from it in that the latter is different in shape (flattened, wider than long). Other differences noted by the original authors are either the consequence of this broader shape (wider first apical homologue, 1u) or the result of more detailed examination. They may well prove to be conspecific but a difference in the resting cyst ornamentation (reticulate in *A. pseudogonyaulax*) may also serve to separate them.

Alexandrium minutum Halim 1960 Fig. 15.17

Syn.: *A. ibericum* Balech, *A.l usitanicum* Balech

A small, widely distributed species occurring singly, varying in shape from rounded (including the antapical profile) to elongate with some antapical flattening. The first apical plate usually does not appear to contact the APC but Balech reports the presence of a fine anterior projection from the first apical homologue reaching the APC. In some cells the main part of the first apical does have a narrow contact with the APC. The anterior right margin is somewhat concave. A ventral pore is present. The surface is delicately porulate and may have a very faint, irregular reticulation although some have been reported to have a strong reticulation confined to the hypotheca. The anterior sulcal plate is fairly conventional for the genus, slightly wider than long. The APC is narrowly triangular with no attachment pore. Length: 15-29 μm, transdiameter: 13-21 μm.

Distribution: Egypt, Turkey, Italy, Spain (both in the Mediterranean and on the Atlantic coast), Portugal, France, the east coast of the United States, Australia.

Comments: A few of the above records are for forms lacking a ventral pore and these may be confused with the closely similar species *A. angustitabulatum* (reports from New Zealand are probably the latter).

Alexandrium monilatum (Howell) Balech

Syn.: *Gonyaulax monilatum* Howell, *Gessnerium mochimaensis* (*sic*) Halim, *Gessnerium monilata* (Howell) Loeblich.

A large, distinctive chain-forming species. The cells in the chain are strongly flattened antero-posteriorly. Some angularity of the "shoulders" may be present. The plates have delicate porulation.The first apical homologue does not come close to the APC (metasert) and is five-sided, wider than long, lacking a ventral pore. The APC is large and triangular, slightly curving, usually with a large anterior attachment pore near the dorsal end of the apical pore. The posterior sulcal plate is large with relatively straight sides and a large notch where it contacts the other sulcals. A large posterior attachment pore is centrally located. Length: 28-52 µm, transdiameter: 33-60 µm.

Distribution: A warm water species known from several tropical Atlantic locations (east coast of Florida, Gulf of Mexico, Venezuela) and the tropical Pacific (Ecuador).

Comments: This species is a powerful fish killer. Halim, in a paper in which his drawings were optically reversed, reported a much larger size (length 57-70 µm). The species is somewhat similar to *A. pseudogonyaulax* but the latter has a ventral pore, is more rounded and has a narrower APC and a different posterior sulcal plate.

Alexandrium ostenfeldii (Paulsen) Balech & Tangen Fig. 15.17

Syn.: *Goniodoma ostenfeldi* Paulsen, *Gonyaulax ostenfeldi* (Paulsen) Paulsen, *Protogonyaulax ostenfeldi* (Paulsen) Fraga & Sanchez, *Heteraulacus ostenfeldi* (Paulsen) Loeblich, *Gessnerium ostenfeldi* (Paulsen) Loeblich & Loeblich, *Triadinium ostenfeldi* (Paulsen) Dodge, *Gonyaulax globosa* (Braarud) Balech, *Protogonyaulax globosa* (Braarud) Taylor, *Gonyaulax trygvei* Parke.

A distinctive, large, non-chained, globose species in which both the epithecal and hypothecal profiles are smoothly rounded. The first apical plate is distinctive in shape, the right upper margin abruptly concave with a large ventral pore excavated from the margin at the point of inflection. It usually contacts the APC. The cingulum is only slightly excavated. The anterior sulcal plate is wide. Length: 40-56 µm, transdiameter: 40-50 µm.

Comments: It is mildly toxic. It is very similar to *A. peruvianum* which is smaller and has a more smoothly curved right upper margin to its first apical plate.

Distribution: Known from Scandanavian, Icelandic, Spanish (Galicia), Washington State, British Columbian (unpubl.), Kamchatka, and Egyptian waters.

Alexandrium tamarense (Lebour) Balech Fig. 15.17

Syn.:*Gonyaulax tamarensis* Lebour, *Gonyaulax excavatum* Braarud, *Pyrodinium phoneus,* Woloszynska & Conrad, *Protogonyaulax tamarensis* (Lebour) Taylor, *Gessnerium tamarensis* (Lebour) Loeblich & Loeblich, *Alexandrium excavatum* (Lebour) Balech & Tangen, *Alexandrium fundyense* Balech.

A medium sized species, occurring singly or in pairs. It is somewhat pentagonal in shape, usually with a slightly indented posterior profile. The left posterior lobe is often slightly larger than the right. "Shoulders" may be apparent on the upper profile. The margins of the first apical plate are relatively straight although the upper right may be curved. The plate contacts the APC which variable in shape, from broadly triangular to almost narrowly rectangular. In pairs an anterior attachment pore and/or posterior attachment pore may be present. A small ventral pore is usually present (absent in the *fundyense* form). The anterior sulcal plate is narrow and relatively smoothly curving. Length: 22-51 µm, transdiameter: 17-44 µm.

Distribution: A widespread species in European waters, including Britain. Also on both coasts of North America, Japan, Korea, and Argentina. A tropical Atlantic record has now been referred to *A. tropicale*.

Comments: This was the first species of the genus to be described. Most strains are toxic but some produce only trace amounts. It is very similar to *A. tropicale* which differs in having a narrow, anteriorly pointed first apical plate and is somewhat more rounded.

Alexandrium tamiyavanichi Balech Fig. 15.17

A chain-forming species, the cells being isodiametric or slightly wider than long. The plates are strongly porulated. The epitheca is wider than long, conical. The first apical plate usually has substantial contact with the APC and has a small ventral pore. The sides of the first apical are relatively straight. The APC is broad with an anterior attachment pore. The anterior sulcal has a distinctive anterior flange demarcated by a ridge. This projects into a notch in the epitheca. The posterior sulcal is rather variable in shape, with a posterior attachment pore near the centre.

Distribution: Gulf of Thailand, Manila Bay.

Comments: A strain of this species from the Gulf of Thailand has been shown to be toxic. The species is very similar to *A. cohorticula* which also forms chains, has a similar size and has the anterior flange on the anterior sulcal plate. Balech considers the key differences to be the proportions of the epitheca, details of the APC with the apical pore more ventral in the plate in *A. cohorticula*, a narrower and more irregular first apical plate in the latter and other small differences.

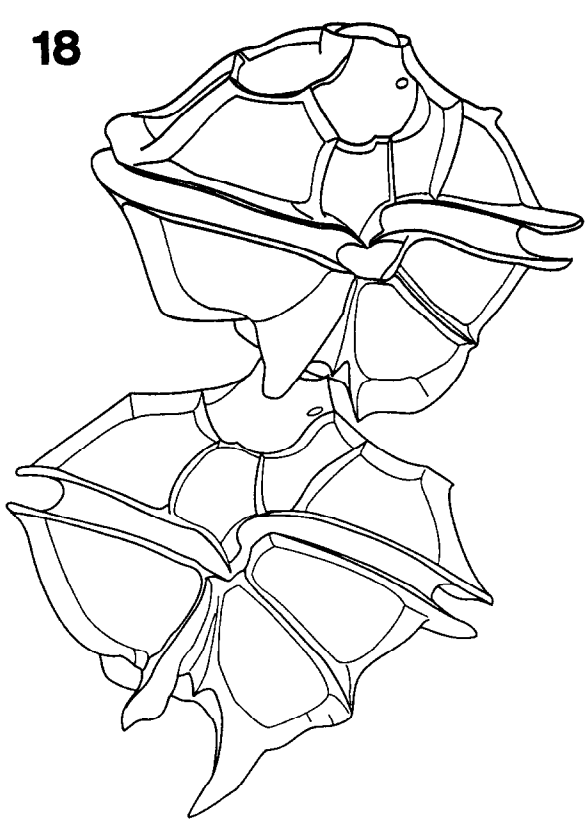
Pyrodinium Plate

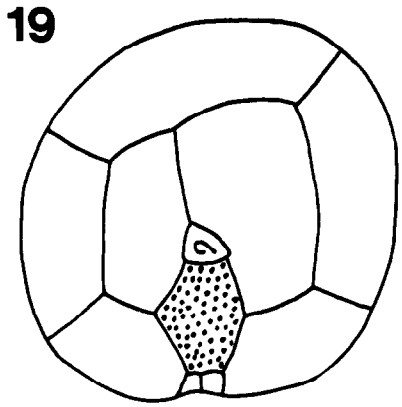
Cells with a very similar tabulation to *Alexandrium* but differing in having a much heavier theca with strong surface markings and flanged sutures. Left handed girdle displacement. The sulcal lists create a tunnel that includes the plate conventionally considered to be the first postcingular plate. Cysts spiny.

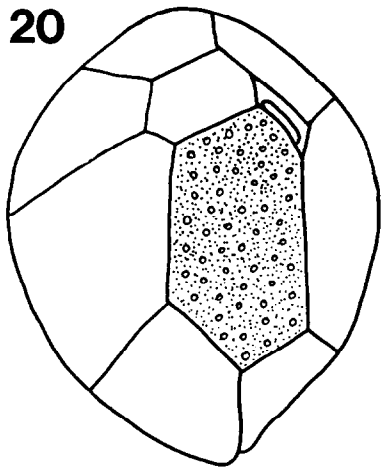
Pyrodinium bahamense **Plate *var. compressum*** (Böhm) Steidinger, Tester et Taylor Figs 15.18, 15.38 a

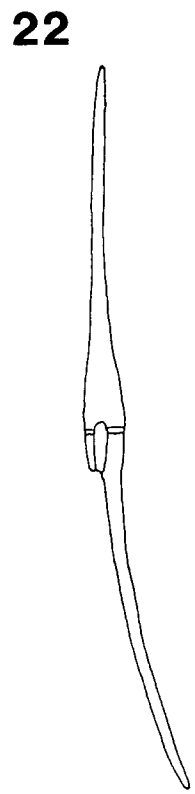
Syn.: *Pyrodinium bahamense* f. *compressa* Bohm, *Gonyaulax schilleri* Matzenauer, *Hemicystodinium zoharyi* Rossignol (cyst), *Polysphaeridium zoharyi* (Rossignol) Bujak *et al.*

Cells single or, more commonly, in chains. Single cells rounded, those in chains showing distinct apico-antapical flattening. Thecal surface covered with dense fine spinulae (only clearly visible with SEM) and large, prominent pores. Strong low flanges mark the edges of most sutures, some more developed than others. The first apical plate homologue does not reach the APC (metasert). A distinct ventral pore is present near the upper right junction with the fourth apical plate. The apex is a low horn. The APC is large, triangular, with a very distinct separation of the inner and outer pore plates (spinules only on the inner). An anterior attachment pore lies close to the right margin of the inner plate. The girdle has well developed lists. The sulcal lists are large and contact each other anteriorly. Single individuals have prominent anterior and posterior spines. In intact chains only the anterior individual has an apical spine and only the most posterior cell has an antapical spine. The posterior sulcal plate is narrow, median, with a slit-like posterior attachment pore. Length of the cell body 33-47 µm, transdiameter 37-52 µm. Cyst spiny (see Matsuoka & Fukuyo, chapter 20).









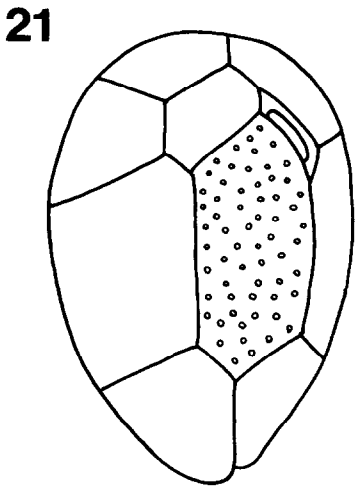


Figure 15.18. *Pyrodinium bahamense* var.*compressum*. Fig. 15.19 *Gambierdiscus toxicus*. Fig. 15.20. *Ostreopsis lenticularis*. Fig. 15.21. *Ostreopsis siamensis*. Fig. 15.22. *Ceratium fusus* [Fig. 18. by M. Yoshida; Fig. 15.19-21. by Y. Fukuyo; Fig. 22. by H. Inoue].

Distribution: Principally in S.E. Asian waters from the Philippines to New Guinea, including Palau, Solomon Islands, Halmahera, Sabah, Brunei and also northern Indian Ocean, Red Sea. Possibly the Pacific coast of central America. The var. *bahamense* occurs in the tropical west Atlantic.

Comments: The var. *bahamense* does not form chains and so all cells in a population are roughly isodiametric with large apical and antapical spines. The pores are often not as prominent. Toxic strains of it have not been found so far. The sketch of the sutural relations of the first apical homologue and the APC by Steidinger (1990) erroneously shows an exsert condition (the genus is metasert, as noted above).

Gonyaulax Diesing

From the literature it might appear that several species of this genus are toxic or harmful in other ways. Partly this is due to the original inclusion of several *Alexandrium* species in this genus. An early report of the production of PSP toxin by *Gonyaulax polyedra* has not been substantiated. Several species of *Gonyaulax*, such *G. polygramma* and *G. reticulatum,* have been implicated in marine fauna mortalities in Hong Kong, False Bay, South Africa and elsewhere, but these appear to be non-specific deaths due to oxygen depletion and any strongly blooming species of this genus could probably produce kills under the right circumstances.

Gambierdiscus Adachi et Fukuyo

This highly flattened genus has a tabulation which is fundamentally similar to *Pyrodinium* and *Alexandrium* even though it looks very different. The first apical homologue is small and exsert (referred to as a precingular by some authors). Only one species, which is benthic, has been described so far.

Gambierdiscus toxicus Adachi et Fukuyo Figs 15.19, 15.38 f

This large species is strongly flattened apico-antapically. In apical view it is round to broadly ovoid, sometimes somewhat squarish in outline with a distinct sulcal indentation. In ventral view it is lenticular and the girdle appears to be displaced in a curving right-handed way. The sulcus is deeply impressed and pocket-like. Lists not present. The plates are densely poroid. The APC triangular with a hook-like apical slit. The APC is inclined towards the cell's left. Although usually sessile on seaweeds the cells can swim when disturbed. Length: 24-60 µm, transdiameter: 42-140 µm.

Distribution: Circumtropical, on seaweeds in sheltered coral reef areas away from strong land run-off. Small, dry islands and offshore banks and reefs are preferred.

Comments: Believed to be the primary source of ciguatoxin and maitotoxin.

Ostreopsis Schmidt

Another large, strongly flattened benthic genus with a tabulation fundamentally similar to *Gambierdiscus* but with a high degree of distortion due to dorso-ventral elongation. The APC is narrow and displaced dorso-laterally to the left. The cells are elliptical and pointed toward the sulcus and there is no obvious sulcal notch when seen in broad view (unlike *Gambierdiscus* with which it might be confused under low magnification). The species are all epibenthic, often attached to the substrate by a fine thread, the flagella beating while tethered in this manner.

Generally less substrate specific than *Gambierdiscus*. There are two species in addition to those below, *O. ovata* Fukuyo (more slenderly ovoid) and *O. heptagona* Norris, Bomber et Balech (seven sided conventional first apical plate) which have been reported to produce mild, water soluble toxic compounds which are unlikely to contribute to ciguatera.

Ostreopsis lenticularis Fukuyo Fig. 15.20

Cells roundly lenticular, slightly pointed towards the sulcus. Plates covered with large pores as well as numerous fine pores. The girdle does not undulate and there is no girdle displacement. Dorso-ventral distance: 60-100 µm, transdiameter: 45-80 µm.

Distribution: Probably circumtropical, benthic. Presently known from French Polynesia (Gambier Islands), New Caledonia and several tropical Atlantic localities including Cayman Islands, Puerto Rico and the Virgin Islands.

Comments: A possible contributor to the ciguatera syndrome. The report of ostreopsis-toxin by this species appears to have been based on cultures of *O. siamensis*. Distinguished from *O. siamensis* chiefly by lack of undulation of the girdle and two types of pores.

Ostreopsis siamensis Schmidt Fig. 15.21

Cells ovoidal in apical view, some rounder than others, pointed towards the sulcus. In side view the cell undulates somewhat and the girdle also undulates. The plates are covered with large pores. Similar in size to *O. lenticularis*. Dorso-ventral distance: 60-100 µm, transdiameter: 45-90 µm.

Distribution: First described from the Gulf of Thailand. Subsequently found at numerous Pacific Island locations, the tropical Caribbean and the Mediterranean.

Comments: A producer of both fat and water soluble toxins, the former possibly contributing to ciguatera. Some of the Caribbean records of *O. lenticularis* may be this species. In the Marshall Islands a large, rounded (apical view) and very undulated form may be this species.

Ceratium Schrank

A gonyaulacoid genus characterised by the presence of two or three hollow horns (four in some fresh water species) and a broad, window-like depression on the ventral side. Although not shown to produce toxins, the common species below has been linked to oyster larval mortality. *Ceratium tripos* has been linked to several anoxic or hypoxic events.

Ceratium fusus (Ehrenberg) Dujardin Fig. 15.22

Syn.: *Peridinium fusus* Ehrenberg, several others from the 19th. Century.

Like other members of the Section Fusiformia the cells are very elongate with two long, slightly and smoothly curved horns, the apical and left antapical horns. The right antapical horn, well developed in many *Ceratium* species, is greatly reduced or completely absent. Length: 150-300 µm, transdiameter: 15-30 µm.

Distribution: Very widespread in coastal waters where it can tolerate a great range of salinities (5-70 parts per thousand).

Comments: Can cause harm to invertebrate larvae by an unknown mechanism.

DINOPHYSOIDS

Members of this group are the only thecate dinoflagellates fundamentally divisible into two lateral halves and possess a girdle (very anterior in position) and a short sulcus. They are laterally compressed. and their shapes in lateral view are most important for genus and species identification. Two families, Dinophysiaceae and Amphisoleniaceae, with about 15 genera are known. Most of their members except those belonging to the genus *Dinophysis* are rather rare species in tropical and temperate dinoflagellate communities, and never cause water discolorations. Toxins causing diarrhetic shellfish poisoning were detected in seven *Dinophysis* species, but toxicity of other dinophysioids has not been studied yet.

Dinophysis Ehrenberg

Syn:*Phalacroma* Stein, *Dinoceras* Schiller, *Prodinophysis* Balech

Dinophysis is one of the largest genera of dinoflagellates with more than 200 species described. The genus name *Phalacroma* had been used for members possessing a large convex epitheca. Abe (1967) and Balech(1967) independently observed micromorphology of the sulcal area and concluded to abandon the junior synonym *Phalacroma* due to lack of significant differences of taxonomic characteristics. For species identification of *Dinophysis* important features are size and shape of the cells, especially outline of the main body and sulcal lists, and presence or absence of chloroplasts. Thecal plate configuration and morphological characteristics of plates themselves may have importance for taxonomy, but it is premature to separate species on the basis of minor variations in some thecal plates.

Dinophysis reproduce asexually by a longitudinal binary fission. After the fission, two daughter cells having an incomplete sulcal list often attach at their dorsal margin. Sexual reproduction process is not well known yet. A small sized species simultaneously occurring with a larger size species may be a gamete of the latter. This phenomenon is found in *D. lapidostrigiliformis* with *D. fortii* (Fukuyo *et al.*, 1981), *D. dens* with *D. acuta* and *D. diegensis* with *D. caudata* or *D. tripos* (Moita and Sampayo, 1993). Close contact of the small species with the large one at the ventral side, often followed by engulfment of the small into the large cell, is suspected to be a process of sexual fusion. Productivity of toxins responsible for diarrhetic shellfish poisoning, okadaic acid or its derivative dinophysistoxin-1 or both, has been confirmed in seven species: *D. acuminata, D. acuta, D. fortii, D. mitra, D. norvegica, D. rotundata* and *D. tripos* (Lee *et al.*, 1989). Three other species, *D. caudata, D. hastata*, and *D. sacculus* are also suspected. The number of toxic species will most likely increase in the future, but the confirmation of their toxigenicity is not easy because of the difficulty in obtaining *Dinophysis* clonal cultures. Lee *et al.* (1989) confirmed the toxicity in 100-1000 cells of the above mentioned species using specimens collected from natural plankton samples under microscope by capillary manipulation. Toxin productivity varies considerably among species, and among regional and seasonal morphotypes in one species. For example, *D. fortii* in northern Japan during March and June contains high concentrations of toxins and is associated with significant accumulation of toxins in shellfish. But the same species in southern Japan during May and July does show slight toxicity and shellfish is free from toxins. In a strict taxonomic sense, a higher percentage of *D. fortii* cells in southern Japan have more convexed curvature in dorsal outline, but this morphological difference is not sufficient to distinguish northern and southern morphotypes (high and low toxin producers, respectively). Therefore clonal differences in toxin productivity, associated with minor morphological variations in a single species, are often found in *Dinophysis*.

Dinophysis acuminata Claparede et Lachmann Figs 15.23, 15.38 b

Syn: *D. lachmannii* Paulsen, *D. borealis* Paulsen, *D. boehmii* Paulsen

Cells oval or often narrow and elongated oval in lateral view. Dorso-ventral depth longest near the middle, about half of the cell length. Epitheca low, flat or weakly convex, invisible in lateral view. Dorso-ventral depth of epitheca is 1/3 to 1/2 of hypotheca. Antapex is rounded and smooth, or with two to four knob-shaped small protuberances. Left sulcal list rather narrow, often coarsely areolate, supported by three ribs, extending to 1/2 to 2/3 of cell length. Thecal plates thick, areolated. Length: 40-50 µm, dorso-ventral depth: 30-40 µm.

Distribution:This species is distributed widely in temperate waters and seems to appear abundantly in coastal waters, especially eutrophic areas. Blooms are often associated with toxification of shellfish (see Larsen and Moestrup, 1992).

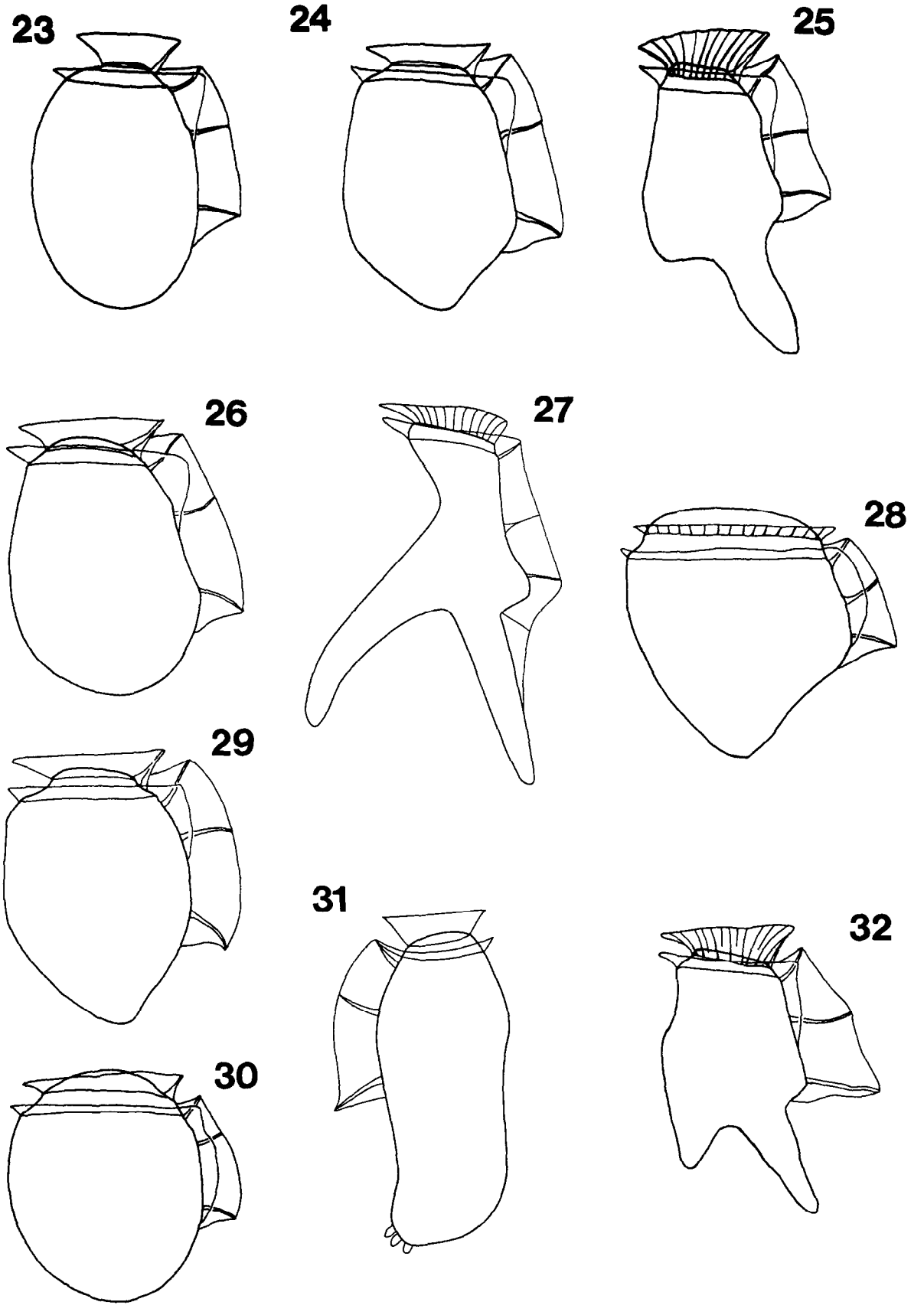
Comments: As size and shape varies considerably, differentiation of *D. acuminata* from morphologically similar species having "acuminated" features is rather difficult. Paulsen (1949) established three new species; *D. borealis*, *D. lachmannii* and *D.skagii*. Solum (1963) observed that morphological variation among the species was mostly caused by environmental salinity differences and contained no discontinuities. Furthermore Abe (1967) and Balech (1976) re-accommodated *D. borealis* and *D. lachmannii* in *D.acuminata*. *D.skagii* is also considered as an aberrant form (Dodge, 1982). Specimens collected from northern France (shown in Lassus and Bardouil 1991) and Norwegian waters (shown in Balech 1976) have more straight and flatter ventral outline than those in Japanese waters (shown in Abe 1967 and Fukuyo *et al.* 1990). The antapex moves ventrally and is more or less pointed in European cells, whereas Japanese ones have the antapex at the middle and a rounder posterior contour. Similar to in *D. fortii*, toxicity of *D. acuminata* varies considerably among seasons and areas where it blooms.

Dinophysis acuta Ehrenberg Fig. 15.24

Cells large, strongly flattened laterally. In lateral view, cells ovoid with pointed or somewhat rounded posterior end. The posterior 1/3 part of the hypotheca shows a triangular shape with the posterior end moving slightly ventrally. Epitheca low, flat or weakly covex, invisible in lateral view. Dorso-ventral depth largest below the middle, almost at 2/3 of total length, about 1/2 to 2/3 of cell length. Dorso-ventral depth of epitheca is 1/2 to 2/3 of hypotheca. Left sulcal list wider posteriorly, supported by three ribs, extending about 2/3 of cell length. Thecal plates thick, coarsely areolated. Length: 54-94 µm, dorso-ventral depth: 43-60 µm.

Distribution: This species is distributed widely in cold and temperate waters. Blooms are associated with toxification of shellfish (see Larsen and Moestrup, 1992).

Comments: This species looks similar to *D. norvegica*, but can be differentiated by the larger size and posteriorly located deepest position of the former species. Moita and Sampayo (1993) reported a gamete, which was identified as *D. dens*, and a resting cyst in natural plankton samples during blooming season of *D. acuta*. As the morphological character of the cyst was very different from those of other dinoflagellates, confirmation by culture experiments is necessary.



Figures 15.23-32. Dinophysoid dinoflagellates. Fig. 23. *Dinophysis acuminata*; Fig. 24.*D. acuta*; Fig. 25. *D. caudata*; Fig. 26. *D. fortii* Fig. 27. *D. miles*; Fig. 28. *D. mitra*; Fig. 29. *D. norvegica*; Fig. 30. *D. rotundata*; Fig. 31. *D. sacculus;* Fig. 32. *D. tripos.* [All figs by H. Inoue, except for Fig. 31, by M. Yoshida] .

Dinophysis caudata Saville-Kent Fig. 15.25

Syn: *D. homunculus* Stein

Cells large, irregularly subovate with fairly distinctive long ventral projection. Ventral side of hypotheca undulate or straight. Dorsal side straight or slightly concave in the anterior half, and straight or convex, running parallel with the ventral side in the posterior half. Anterior cingular list wide, supported by many ribs, forming a wide and deep funnel-like structure with very low epitheca on the bottom. Left sulcal list almost half of total length, supported by three ribs. Thecal plates thick, areolated. Length: 70-110 μm.

Distribution: This species is distributed widely in tropical and temperate waters and seems to appear abundantly in coastal waters. Red tides associated with mass mortality of fish were reported in the Gulf of Thailand and Seto Inland Sea in Japan (Okaichi 1967).

Comments: Cell shape, especially length of ventral projection and extent of dorsal expansion, is rather variable. Cells with a long projection usually have wide sulcal lists and well developed cingular lists, and differentiation from *D. tripos* becomes difficult. On the contrary, cells with short projection look similar to *D. diegensis*, which is suspected to be a gamete of the species (Moita and Sampayo 1993). Toxicity of the species was found using specimens collected in Philippines waters (Kodama, pers. comm.).

Dinophysis fortii Pavillard Fig. 15.26

Cells large, subovate with fairly straight ventral side in its anterior half, and broadly rounded posterior. Dorsal side smoothly convex with slight concavity near the cingulum. Dorso-ventral depth of epitheca is about a half of the hypotheca. Dorso-ventral depth largest below the middle at the base of the third rib. Anterior cingular list forms a wide and low cup-like structure with very low epitheca at the bottom. Sulcal list very long, supported by three ribs, extending to 4/5 of cell length. Reticulation on the list is obvious. Thecal plates thick, areolated. Length: 60-80 μm.

Distribution: This species is distributed widely in temperate waters. In northern Japan the species are brought by a warm current in spring and early summer, and propagate in coastal areas where shellfish aquaculture is intensive.

Comments: Many scientists have tried to establish cultures of *D. fortii* using several kinds of enriched sea water medium, but they can keep at most 30 cells/ml for several months. The inoculated cells did not die, but reproduced very slowly. As mean doubling time is reported as 1.4 days in natural conditions (Weiler and Chisholm, 1976), environmental factors for rapid growth must be overlooked in experimental incubations. The species is most noxious by its productivity of DTX1, PTX2 and OA, but clones in warmer waters often show very low toxicity.

Dinophysis miles Cleve Fig. 15.27

Cells very large, anterio-posteriorly elongated with two fairly distinctive long antapical and dorsal projections. Ventral side of hypotheca undulate. Dorsal side concave and smoothly continues to the dorsal projection which runs obliquely backwards. The distal end bends at a right angle, carrying a wing-like unabsorbed remnant of the megacytic zone. Six to eight daughter cells often attach at the remnant after asexual cell division. Posterior projections shorter or longer than, or as long as the dorsal process. Angle between the dorsal and posterior projections 50-90°. It starts at the base of the third rib. Anterior cingular list wide, supported by

many ribs, forming a narrow funnel-like structure with very low epitheca on the bottom. Thecal plates thick, round or angular areolated. Length: 125-150 μm.

Distribution: This species is distributed widely in tropical waters.

Comments: This species is easily identified by its large cell shape and especially the two projections. Kodama found toxicity of the species using specimens collected in Philippines waters. (Kodama, pers. comm.).

Dinophysis mitra (Schutt) Abe Fig. 15.28

Syn: *Phalacroma mitra* Schutt

Cells broad wedge-shaped. Dorsal side smoothly convex. Ventral side flat or somewhat undulate in anterior half, and slightly concave in posterior half. Epitheca flat or slightly convex, broad ellipsoidal in apical view. As the megacytic zone becomes wide due to cell growth, the epitheca becomes round in apical view and the posterio-ventral concavity becomes much less distinct. Dorso-ventral depth of epitheca and hypotheca almost the same and longest at the base of the second rib. Cingulum located most anteriorly. Both anterior and posterior cingular lists narrow, supported by many ribs. Sulcal list rather short, about half of the total length supported by three short ribs. Thecal plates thick, coarsely areolated. Length: 56-68 μm, width 48-58 μm.

Distribution: This species is distributed widely in temperate waters.

Comments: This species resembles *D. rapa*, but can be distinguished by the more stronglyprotuberant sulcal ridge at the base of the third rib and distinctive concavity at the posterio-ventral hypotheca of the latter species (Abe 1967). The production of DTX1 toxin was confirmed by Lee *et al.* (1989).

Dinophysis norvegica Claparede and Lachmann Fig. 15.29

Cells strongly flattened laterally, ovoid with pointed or somewhat rounded posterior end in lateral view. Dorsal side smoothly convex, often with a small protuberance. Posterior half of hypotheca tapers to make a triangular-shape. Posterio-ventral side straight or slightly concave. Epitheca low, flat or weakly covex, invisible in lateral view. Dorso-ventral depth longest at the middle, between the bases of the second and third rib. Dorso-ventral depth of epitheca is 1/2 to 2/3 of hypotheca. Left sulcal list often undulated, supported by three ribs, extending about 2/3 of cell length. Thecal plates thick, coarsely areolated. Length: 48-80 μm, dorso-ventral depth: 39-70 μm.

Distribution:This species is distributed widely in cold and temperate waters.

Comments: This species looks similar to *D. acuta*, but can be differentiated by its size and deepest position (see comment on *D. acuta*). DTX1 and OA production was reported by Lee *et al* (1989).

Dinophysis rotundata Claparede and Lachmann Fig. 15.30

Syn: *Phalacroma rotundatum* Kofoid and Michnener, *Dinophysis whittingae* Balech

Cells asymmetrically round-oval in lateral view, and ellipsoidal to round in apical view. Epitheca low and fairly evenly rounded, convex, and visible laterally. Both anterior and

posterior cingular lists narrow, lacking any ridges. Sulcal list also narrow, extending about 1/2 to 3/5 of total length. The list has three ribs, among which the second one is located nearer the first one. Dorso-ventral depth is largest between base of the second and third ribs. Thecal plates thick, finely areolated. Protoplasm does not contain chloroplasts, but often has many large food vacuoles. Length: 42-50 μm, dorso-ventral depth: 36-43 μm.

Distribution:This species is distributed widely in cold and temperate waters.

Comments: This species looks similar to *D. rudgei*, but is distinguishable by the low height of the epitheca of the former species. The epitheca of *D. rudgei* is quite easily visible in lateral view. *Dinophysis rotundata* is a phagocytic species, feeding on both loricated and non-loricated ciliates by piercing the prey cell with a feeding tube (Hansen, 1991, Inoue *et al.*, 1993). This is the first heterotrophic dinoflagellates in which toxin productivity has been confirmed (Lee *et al.*, 1989).

Dinophysis sacculus Stein Fig. 15.31

Cells irregular long oval. Dorsal side straight or undulating: convex below the cingulum, slightly concave in the middle, and convex again posteriorly. Ventral side also undulate: convex at the middle, concave below the middle. Anterior cingular list low without distinctive ribs. Dorso-ventral depth of epitheca is 1/3 to 1/2 of hypotheca. Deepest part of dorso-ventral width varies because of undulation of both dorsal and ventral side, but mostly deepest at between the second and the third rib. Sulcal list rather short, about 1/2 of cell length, reaching the middle of hypotheca. Thecal plate coarsely areolated. Length: 40-60 μm.

Distribution: This species is distributed widely in cold and temperate waters.

Comments: Although toxin production has not been confirmed, this species is considered to be responsible for DSP toxin occurrence in bivalves in Portuguese coastal waters (Alvito *et al.*, 1990, Sampayo *et al.*, 1990).

Dinophysis tripos Gourret Fig. 15.32

Cells large, anterio-posteriorly elongated with distinctive long antapical and short dorsal projections. Ventral side of hypotheca straight or slightly undulate. Dorsal side concave below the cingulum and then convex, smoothly continuing to the dorsal projection which runs backwards. The posterior end of both projections often bears several minute protuberances. Anterior cingular list wide, supported by many ribs, forming a narrow funnel-like structure with very low epitheca on the bottom. Sulcal list extends widely posteriorly with reticulation. Thecal plates thick, coarsely areolated. Length: 90-125 μm.

Distribution: This species is distributed widely in tropical and temperate waters, and occasionally appears in colder regions.

Comments: This species is easily identified by its two projections. Lee *et al.* (1989) found DTX1 toxin in this species.

PROROCENTROIDS

The species of this group are easily distinguishable from other dinoflagellates by two anteriorly inserted flagella and two large laterally flattened valves. One of the valves, the right valve, has a small indentation, which is occupied by 7 or 8 tiny periflagellar platelets forming a flagellar pore and an accessary pore. Two genera, *Mesoporos* and *Prorocentrum,* are known. The former has a large central pore at the centre of the valves.

Prorocentrum Ehrenberg

Syn.: *Exuviaella* Cienkowski

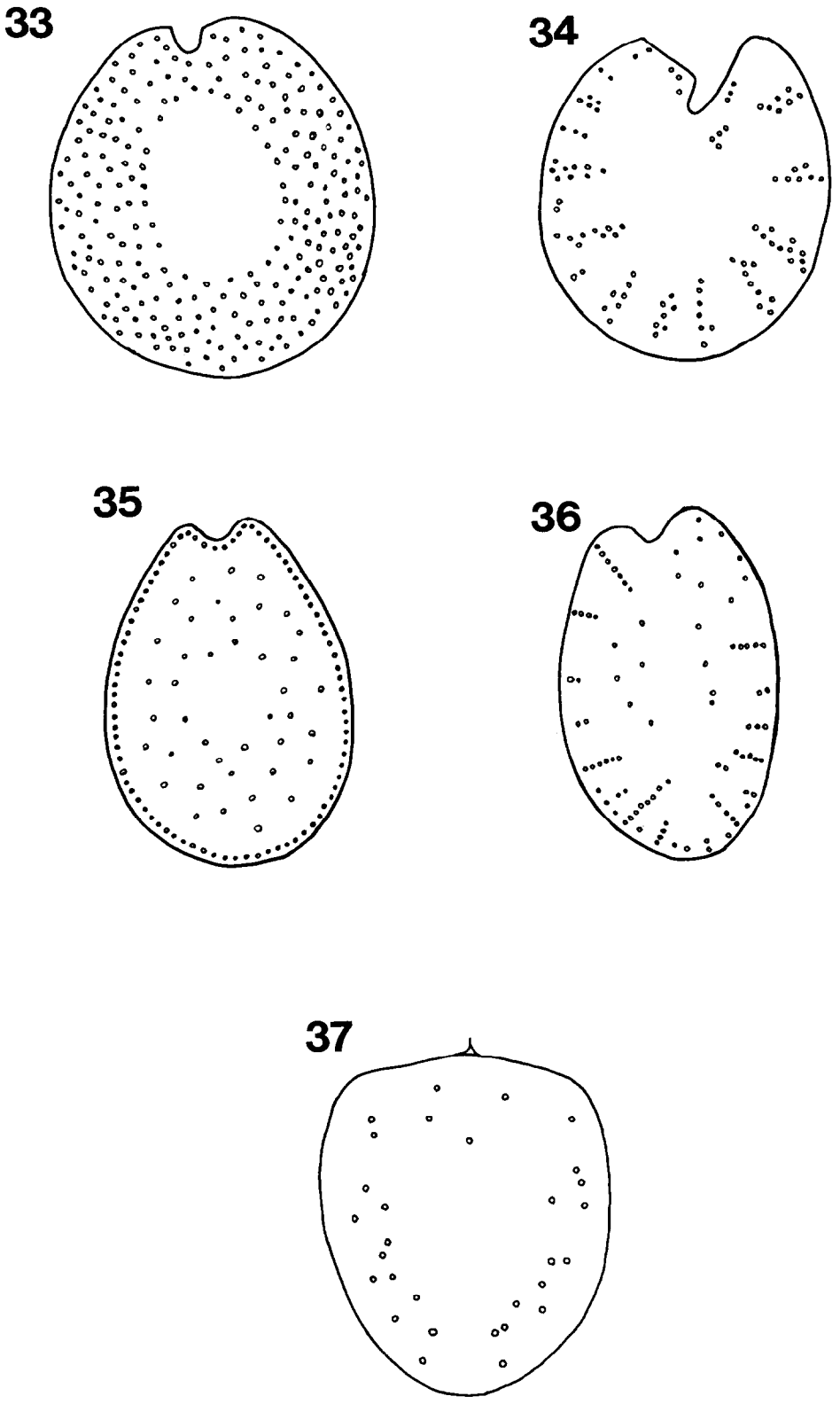
Classification of members of this genus is based on morphological characteristics such as presence of anterior spine, cell shape, ornamentation of valve surface (smooth, or covered with depressions or spinules), and distribution pattern of trichocyst pores. Two distinctive life forms, planktonic and epibenthic, are known. Planktonic species have been studied for a long time, and more than 60 taxa are reported under the genus name of either *Prorocentrum* or *Exuviaella*.. Dodge and Bibby (1973) combined the two genera after careful morphological observation using EM, which was followed by revision of taxonomy with reduction of species number from 64 to 21 by Dodge (1975). Although many planktonic *Prorocentrum* species are known to form red tide discolorations, most are harmless. In contrast to the planktonic forms, many epibenthic species found recently (see Faust *et al*, 1995) are suspected to have implications in poisonings endemic in tropical areas. For species identification, architecture of periflagellar area and intercalary bands are suggested to be important criteria in addition to those used for the planktonic species.

Prorocentrum concavum Fukuyo Fig. 15.33

Cells broadly ovate in valve view, widest behind the middle, lenticulate to ellipsoidal in lateral view with a flattened central area. The anterior end of left and right valves is straight and deeply concave, respectively. Both sides of the concavity rise roundly. Valve surface covered with many depressions. Trichocyst pores distributed all over the surface except the central area, but are denser near the margin. Pyrenoid at the centre and nucleus posteriorly. Length 45-55 μm, width 38-45 μm.

Distribution: Epibenthic species on macroalgae growing on dead corals in coral reefs (Fukuyo, 1981), and on floating detritus in mangrove (Faust, 1990) in tropical and subtropical areas.

Comments: Most epibenthic species share ovate or ovoidal valve shape without distinctive anterior spine, and it is difficult to distinguish *P. concavum* from similar species such as *P. lima, P. hoffmannianum,* and *P. sabulosum*. Important and reliable criteria are the surface ornamentation of the valve and the number and configuration of trichocyst pores. Valve surface of *P. lima* is smooth, whereas that of *P. concavum* and the two above mentioned species is areolate. The distinction among the three species can be made by number of areolae: ca.1000 for *P. concavum*, ca. 670 for *P. hoffmannianum* (Faust, 1990), and ca. 390 for P.*sabulosum* (Faust, 1994). Several toxins having different effects have been isolated (see Bomber and Aikman (1988/1989), but their implications in natural foodwebs in tropical areas are not known. This is mostly because of low population densities of the species.



Figures 15.33-37. Prorocentroid dinoflagellates. Fig. 33. *P.concavum*; Fig. 34. *P. emarginatum*; Fig. 35.*P.lima*; Fig. 36. *P.mexicanum*; Fig. 37. *P.minimum* [all figs. by Y.Fukuyo].

Prorocentrum emarginatum Fukuyo Fig. 15.34

Cells ovate in valve view, lenticulate to ellipsoidal in lateral view with a flattened central area. Anterior margin widely concaved and both sides of the concavity rise into a sharp point. A wide low list stands dorsally in the concavity. The right valve has long wide cuneiform indentation which is curved slightly dorsally. Length: 35-40 μm, width: 32 μm.

Distribution: Epibenthic species on macroalgae growing on dead corals in coral reefs (Fukuyo, 1981) in Ryukyu Islands.

Comments: The species is readily distinguishable from other benthic *Prorocentrum* except *P. sculptile* by its deep indentation at the anterior margin. Valve outline of *P. sculptile* is the same as *P. emarginatum*, but the former has round to oval depressions all over the valve surface. The former species is a little smaller (30-37 μm) than the latter (Faust, 1994). The toxicity of the species is unknown.

Prorocentrum lima (Ehrenberg) Dodge Fig. 15.35

Syn: *Exuviaella marina* Cienkowski, *Prorocentrum marinum* (Cienkowski) Dodge and Bibby

Cell ovate, widest behind the middle in valve view, lenticulate to ellipsoidal in lateral view with a flattended central area. The anterior end of left and right valves is straight and trianglarly concave, respectively. Valve surface smooth, having trichocyst pores sparsely all over the surface except the central area. Length: 30-40 μm, width: 26-30 μm.

Distribution: This species is distributed very widely in tropical waters such as French Polynesia and Carribean Sea to subantarctic waters such as the Swedish coast and northern Japan. Habitat is epibenthic on seaweeds and benthic embedded shallowly in sand.

Comments: Faust (1991) observed morphological characteristics of the species in detail, and suggested its wide variability of shape and size. But *P. lima* is distinguishable by its smooth valve surface from other *Prorocentrum* species, especially from *P. hoffmannianum*, which has an areolate surface. *Exuviaella marina (P. marinum)* is considered as junior synonym of *P. lima* by Dodge (1975), but some taxonomists still treat the former as an independent *Prorocentrum* species. Sexual and asexual reproductive cycles of *P. lima* were observed by Faust (1993a, b). The mature hypnozygote (resting cyst) was 70 μm in diameter, spherical, pale yellow, with a smooth organic \ triple layered wall (Faust, 1993a). The same author found a similar resting cysts of *P. marinum* from the same mangrove area (Faust 1990b). This species produce several kinds of toxins such as okadaic acid (see Bomber and Aikman 1988/1989), and then sometimes is called a ciguatera-causing organism (Faust, 1991) or DSP-causing organism (Jackson *et al.*, 1993), but the implication of the toxin in food webs are unclear.

Prorocentrum mexicanum Tafall Figs 15.36, 15.38 e

Syn: *Prorocentrum rhathymum* Loeblich, Sherley et Schmidt.

Cell oval, widest anterior or at the middle in valve view, lenticulate to ellipsoidal in lateral view. Anterior end slightly concave at the middle. Low list rising from periflagellar plate looks like a short spine. Valve surface smooth, having trichocyst pores lying in rows radially from the center perpendicular to the valve margin. Length: 30-40 μm, width: 22-25 μm.

Distribution: This species is distributed very widly in tropical to temperate waters. Habitat is epibenthic on seaweeds and planktonic.

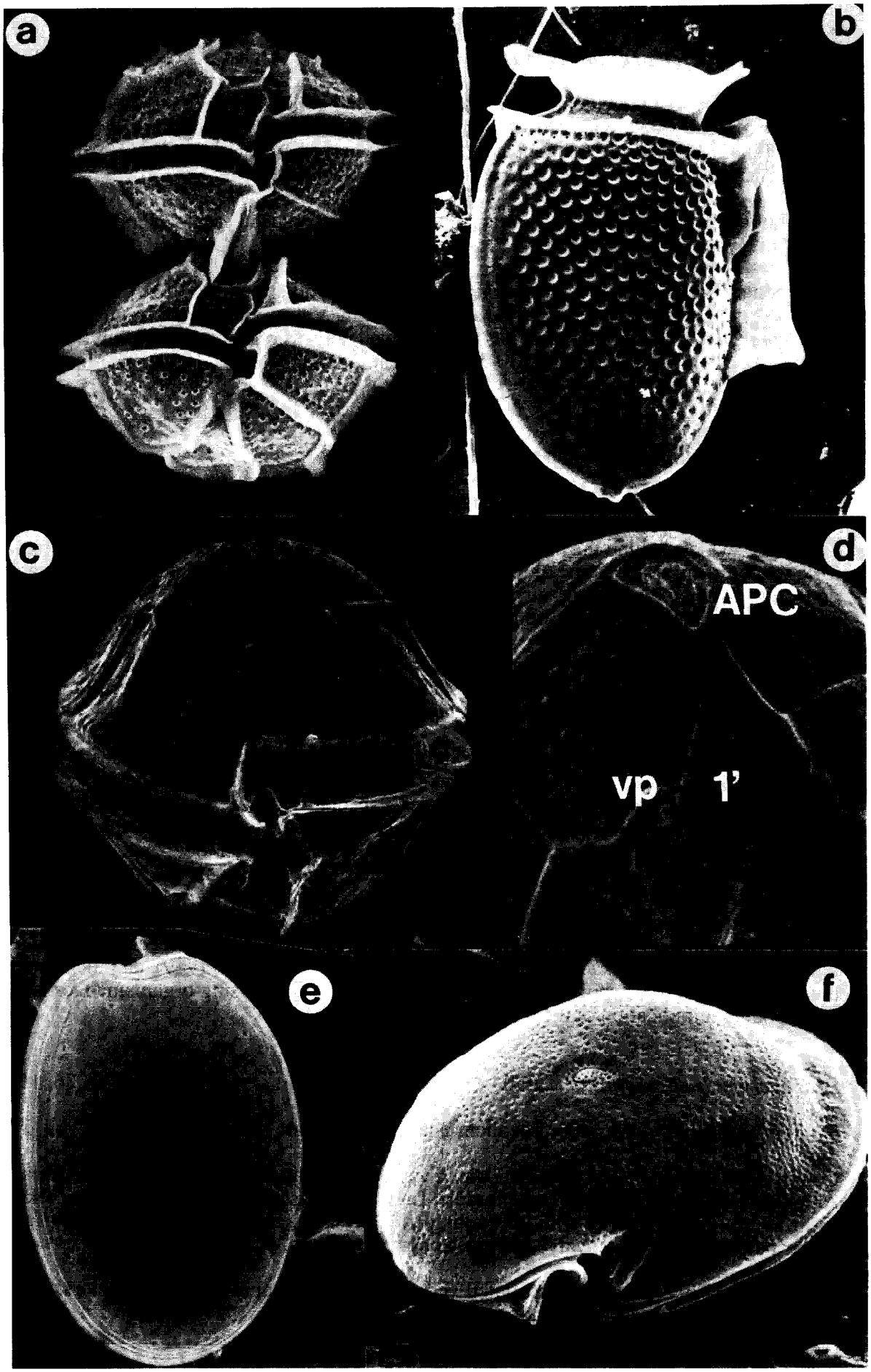


Figure15.38. SEM of selected armoured dinoflagellates: Fig. a. *Pyrodinium bahamense* var. *compressum* from Papua New Guinea; Fig. b. *Dinophysis acuminata* from Tasmania; Fig. c. *Alexandrium minutum* from South Australia; Fig. d. Detail of the same cell as in Fig. c, showing the apical pore complex (APC) and first apical plate (1') with ventral pore (vp); Fig. e. *Prorocentrum mexicanum* from Palau; Fig. f. *Gambierdiscus toxicus* from Australia. [all micrographs by G.Hallegraeff, except figs c,d by C.Bolch].

Comments: Hemolytic toxin and fast acting toxins were detected in culture of the species (Bomber and Aikman 1988/1989).

Prorocentrum minimum Fig. 15.37

Synonyms: *Exuviaella marina* Pavillard, *Prorocentrum triangulatum* Martin, *E. mariae-lebouriae* Park et Ballantine, *P. cordiformis* Bursa, *P. mariae-lebouriae* (Park and Ballantine) Loeblich III

Cell heart-shaped, triangular, or ovate in valve view, lenticular in lateral view. Anterior margin straight or slightly convex with a small depression at the middle. A short spine rises from a side of the depression. Valve surface covered with many spinules. Trichocyst pores present. Chloroplast yellowish brown, two in number, located peripherally. Nucleus spherical to ellipsoidal, located posteriorly.

Distribution: Planktonic species having a world-wide distribution, both in marine and brackish waters.

Comments: Shape of this species is so variable that several names either at a variety or specific rank have been given. The morphological variation, mostly due to differences in environmental parameters such as light intensity, salinity and temperature, is well observed at the curvature of lateral outline in ventral view. This species is often described as the causative organism of poisonings occurred by eating oysters and short necked clams. The toxic substance was named as venerupin, but the chemical structure and property has not been elucidated (Hashimoto, 1979). So far six poisoning cases have been reported in Japan, and in total 542 peoples became sick with 185 fatal cases (34% fatality rate) (Hashimoto, 1979). As *P. minimum* was observed abundantly in the digestive organs of toxic short necked clams, the species was thought to be responsible (Nakazima, 1968). But observations and laboratory experiments made by Nakazima (1965a,b,c) were considered insufficient to conclude *P. minimum* to be the causative organism (Hashimoto, 1979). Okaichi and Imatomi (1979) failed to detect venerupin in a culture of *P. minimum*, although they could extract two substances having mouse toxicity. It is recommended to pay attention to shellfish toxicity, if a bloom of *P. minimum* affects areas for shellfish aquaculture and collection. But it is not necessary and not practical to consider *P. minimum* as a persistent toxic organism.

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16. Taxonomy of Toxic Haptophytes (Prymnesiophytes)

Ø. Moestrup and H.A. Thomsen

The Haptophyceae was first recognized as a separate class of algae by Christensen (1962), following detailed studies by I.Manton and M.Parke in England. The 'third flagellum', one of the most characteristic features of many haptophyte cells, was shown by Manton and Parke to represent an entirely new organelle, which Parke *et al.* (1955) named the haptonema. Pigments of haptophytes are very similar to chrysophytes, and haptophytes were previously included in the Chrysophyceae, which differ markedly, however, in the heterokont flagellation (a hairy anterior and a smooth posterior flagellum) while haptophytes usually have two equal (isokont) or subequal (anisokont) flagella. More recently, gene sequencing studies have indicated that the Haptophyceae and the Chrysophyceae are only distantly related (Leipe *et al.* 1994). Much confusion arose as a result of the recommendation by the International Code of Botanical Nomenclature that names above the level of genera should be typified, i.e. based on a generic name. This led Hibberd (1976) to introduce the name Prymnesiophyceae as an alternative but typified class name (based on the genus *Prymnesium*) replacing Haptophyceae. The idea of typified names has not been generally accepted, however, and presently both names Haptophyceae and Prymnesiophyceae are equally valid. It is possible and likely, that the classification used in the future will comprise two rather than one class, united as the phylum Haptophyta (colloquial name haptophytes), and named Prymnesiophyceae (prymnesiophytes) and Pavlovophyceae (pavlovophytes), the latter differing so markedly in cell structure that separation at the class level is justified (Green & Leadbeater 1994).

All known toxic haptophytes belong to the Prymnesiophyceae. Presently only species of the genera *Prymnesium* and *Chrysochromulina* are generally recognized as potentially toxic, but recent findings from Norway indicate that *Phaeocystis,* a cosmopolitan genus of foam-producing species, is also a toxin producer (Eilertsen & Raa, in press). The future may demonstrate toxin production in more genera (the coccolithophorid *Ochrosphaera napolitana* was claimed by Jebram (1980) to be toxic) but in the absence of conclusive evidence, the present account will deal only with *Chrysochromulina, Prymnesium* and *Phaeocystis.*

DESCRIPTION OF THE CLASS

Haptophytes are generally unicellular or colony-forming flagellates which are particularly common in the marine plankton. Most species are small and belong in the nanoplankton, i.e. the cells are usually smaller than 20 µm. They may be recognized as haptophytes by the yellow colour of the (usually two parietal) chloroplasts, the green colour of chlorophylls *a* and *c* being masked by the carotenoid fucoxanthin which is present in large amounts and photosynthetically active (Jeffrey 1980). The cells contain a single nucleus which is difficult to see except under high-resolution light microscopes, using Nomarski interference contrast, while a relatively large vacuole in the posterior end is commonly visible. It contains chrysose (chrysolaminarin), a water soluble polysaccharide. The same colour and the same kind of vacuole occurs in the Chrysophyceae, from which haptophytes may be distinguished by their flagellation. The hairy front flagellum of chrysophytes beats in a sine wave as in other heterokonts, pulling the cell forward in the water or, in sessile forms, drawing water towards the cell. The smooth flagellum is often trailing. In prymnesiophytes both flagella are usually active in swimming, the proximal part being directed anteriorly, while the tip is bent backwards along and behind the cell. Two species of *Chrysochromulina* are quadriflagellate (*C. birgeri* and *C. quadrikonta*)

while other prymnesiophytes possess only two flagella. Cells lacking flagella often cannot be distinguished from chrysophytes by light microscopy. The marine plankton comprises only few nano-sized chrysophytes, however, and in the marine nanoplankton small flagellar-less unicellular algae with two parietal yellow chloroplasts are usually haptophytes which have lost flagella and haptonema. Some species lack a haptonema (e.g. *Dicrateria*, Green & Pienaar 1977) or this may be present as an internal remnant not visible by light microscopy (e.g. *Imantonia*, Green & Pienaar 1977). All species of *Chrysochromulina, Prymnesium* and *Phaeocystis* possess a haptonema. In *Phaeocystis* this is very short and often difficult to see under the light microscope while in *Prymnesium* and particularly in *Chrysochromulina* it is readily visible and in *C. camella* may attain a length of 160 μm (Leadbeater 1972).

Short haptonemata may be used as attachment organelles (*Prymnesium*) while longer haptonemata may serve in food uptake (Kawachi *et al.* 1991). An increasing number of haptophytes is presently being discovered to be mixotrophic. In *Chrysochromulina hirta*, small eukaryotic algae attach to the haptonema, are concentrated in a small packet at the haptonema tip and the haptonema then bends to deliver the packet at the posterior end of the cell (Kawachi *et al.* 1991). It is subsequently phagocytized in a food vacuole. In *Chrysochromulina ericina*, whose cells are covered with long spines, the prey is caught on the spines and transported to the haptonema, before being delivered at the posterior end of the cell for phagocytosis (Kawachi & Inouye 1995). A third food-uptake mechanism which does not involve the haptonema, has recently been found in *Chrysochromulina spinifera* (I. Inouye and M. Kawachi, pers. comm.). *Balaniger balticus* and several coccolithophorids are entirely apoplastidic (Marchant & Thomsen 1994) but whether these species are phagotrophic or osmotrophic is not known.When swimming slowly forward the haptonema is directed forwards while the flagella beat slowly backwards along the cell. Upon hitting an object, a long haptonema instantly rolls up and swimming ceases abruptly. Slow swimming recommences when the haptonema extends again. Short bursts of rapid swimming occur with all appendages directed backwards behind the cell.

Cells of many haptophytes are covered with several layers of scales whose function remains obscure. They are used extensively in taxonomy, but only the larger types are visible with the light microscope. Some scales of the coccolithophorids are calcified (coccoliths) while others are non-calcified (organic). Only organic scales occur in *Prymnesium, Chrysochromulina* and *Phaeocystis*. Identification of most haptophytes relies on scale structure, cell size, and length of the flagella and haptonema, the first feature being the most reliable. In many genera reliable identification to species level requires electron microscopical examination of scale morphology; this applies to nearly all species of *Prymnesium* and to most species of *Chrysochromulina*. Transmission electron microscopy of whole mounts is to be preferred (Moestrup & Thomsen 1980), as the resolution of the scanning electron microscope is often insufficient to resolve the minute details of the scales. It should also be borne in mind that many species of *Chrysochromulina* are as yet undescribed, thus few studies have been performed on the genus in tropical and oceanic areas.

Reproduction of haptophytes is usually by longitudinal fission, some species often dividing once per day (*Chrysochromulina polylepis, Prymnesium patelliferum* (Larsen *et al.* 1993; Moestrup & Arlstad, submitted)). Sexual reproduction is known in some cases (Billard 1994), but it is unknown in *Prymnesium, Chrysochromulina* and *Phaeocystis*. It is likely to occur, however, and the gametes may be morphologically identical to vegetative cells and therefore difficult to recognize unless fusion is observed.

Thick-walled resting stages occur, e.g. in *Prymnesium* (Pienaar 1980). The life cycle of many haptophytes remains poorly understood, but old cultures of e.g. *Chrysochromulina* often contain both coccoid and amoeboid stages and cells with odd numbers of flagella (Parke *et al.* 1955). Whether these are haploid or diploid is unknown.

Many haptophytes are distributed worldwide (e.g. *Emiliania huxleyi*, many species of *Chrysochromulina*, etc (Marchant & Thomsen 1994; Thomsen *et al.* 1994)). A major effort is required to study the non-coccolithophorid species in tropical areas, only few species have been reported (e.g. Manton 1982, 1983; Manton & Oates 1983) but preliminary studies have shown (Moestrup, unpubl.; Thomsen, unpubl.) that they are probably as common in the tropics as in

temperate and polar waters. Around 75 genera and 500 species of haptophytes have been described (Green *et al.* 1990).

Some species of prymnesiophytes have been known as fishkillers since the turn of the century and economic aspects of prymnesiophyte blooms have recently been reviewed (Moestrup 1994).

KEY TO GENERA

Description of genera

Chrysochromulina Lackey 1939.

(Type species *C. parva* Lackey 1939)

Since our first review (Moestrup & Larsen 1992), in which only a single species was described as toxic, studies in Norway have demonstrated that one more of the c. 50 described species is potentially toxic, and our attention was directed to an article by Jebram (1980), who found several species to be toxic in old cultures when served as food to the bryozoan *Electra pilosa*. Presently 5 species of *Chrysochromulina* are known to be potentially toxic (Figs 16.1a-e) while 7 have proved non-toxic when tested to the crustacean *Artemia* or the bryozoan *Electra* (see Table 16.1, Rhodes *et al.* 1994 and Moestrup 1995). Table 16.1 provides an overview of characters used to distinguish individual species of *Chrysochromulina* by light microscopy. The toxic species will be described in detail below. Most of the remaining species require further study, and so do the many undescribed species, *Chrysochromulina* probably comprises 75-100 species.

Chrysochromulina brevifilum Parke et Manton 1955

Description: Cells spheroidal-pyriform, the flagellar pole usually flattened, (3.5-) 4-7 (-11.5) µm in diameter (Fig. 16.1a). Two equal, homodynamic flagella, 2.5-3.5 times the cell diameter in length (Birkhead & Pienaar 1994: (11.3-) 13-17 (-19.5 µm), and the haptonema, (3-) 7.6-9.5 (-14.2) µm in length, arise at the flattened pole. The haptonema is nearly always extended during swimming, very rarely coiled up. It is always shorter than the flagella. Two (rarely one or four) golden brown parietal chloroplasts, each with a pyrenoid, which is usually difficult to see except in squashed cells. Oil droplets scattered throughout the cell, muciferous bodies line the plasmalemma. A vacuole of variable size is sometimes visible posteriorly (phagotrophy?; see below). Cells may become greenish with age.

Scale structure: In the original description only a single type of scale was seen, described as ellipsoid, c. 0.7 µm across, each scale with a central spine attached by four decurrent ridges (Parke *et al.* 1955). Later, Manton (in Leadbeater 1972, p. 76) mentioned the presence of plate scales and very recently Birkhead & Pienaar (1994) found a second type of plate scale in material from South Africa. The presence of three types of scales has been confirmed in a clone from Denmark (Moestrup, unpubl.) and Figs 16.2a,b show the two main types of scale. The large plate scales measure 0.76-1.02 µm in length and 0.68-0.92 µm in width. They carry a rim, which is 0.07-0.1 µm wide and shows a pattern of concentric fibres. Each scale is divided into four quadrants, each with c. 17 radiating lines visible on both sides of the scale. The spine scales, which occur in numbers similar to the large plate scales, are slightly longer than wide or almost isodiametric, 0.63-0.98 µm long and 0.55-0.84 µm wide. The central spine is 0.6-0.7 µm long with four decurrent ridges which do not extend to the rim. The scales carry an unstructured rim measuring c. 0.08 µm. Like the plate scale, the spine scale is divided into four quadrants. These are delineated by the decurrent ridges of the spine, each

Table 16.1. List of *Chrysochromulina* species described, including dimensions and shape of cell body, dimensions of flagella and haptonema, and an indication of habitat (hab), phagotrophy (ph) and toxicity.

SPECIES	HAB.	LENGTH			WIDTH			L/W	SHAPE	FLAGELLA			HAPTONEMA			HAPT./	HAPT./	SP.	SP.	COMMENTS	PH.	TOXIC	REFERENCES
		min.	avg.	max.	min.	avg.	max.			min.	avg.	max.	min.	avg.	max.	FLAG	C.L.	VIS.	POS.				
acantha	m	6	8	10	6	8	10	1,0	saddle		20			40		2,0	5,0	n				n	Leadbeater & Manton 1971
adriatica	m	5	7	8	6	8	9	0,9	subsphaerical	8	9	10	6	8	10	0,9	1,2	n					Leadbeater 1974
alifera	m	6	8	10	6	8	10	1,0	saddle	12	18	25	60	90	120	5,0	11,3	n			y		Parke, Manton & Clarke 1956
apheles	m	3	4	4	3	5	6	0,8		7	11	15	20	30	40	2,7	8,6	n				y	Moestrup & Thomsen 1986
bergenensis (type)	m		4			4		1,1	ovate		10			8		0,8	2,0	n			y		Leadbeater 1972
bergenensis	m		7		4	4	6	1,8			10			8		0,8	1,1	n			y		Manton & Leadbeater 1974
birgeri	m	13	18	31	8	10	11	1,9		30	33	35	10	15	18	0,5	0,8	n					Hällfors & Niemi 1974; Hällfors & Thomsen 1979
brachycylindra	m	4	5	7	4	5	7	1,0	subsphaerical	8	11	13	8	11	13	1,0	2,1	n					Hällfors & Thomsen 1985
brevifilum	m	4	6	12	4	6	12	1,0	sphaeroid	9	17	25	8	13	18	0,8	2,2	n			y	y	Parke, Manton & Clarke 1955
camella	m		14			16		0,9	saddle		25		160	160	160	6,4	11,4	n					Leadbeater & Manton 1969
campanulifera	m		10			10		1,0	saddle		25		40	50	60	2,0	5,0	n		hapton. substantially longer than fl.	y		Manton & Leadbeater 1974
chiton	m	4	7	10	4	7	10	1,0	sphaeroid	13	20	28	20	30	40	1,5	4,6	n			y		Parke, Manton & Clarke 1958
cyathophora	m		3			3		1,0	subsph.	10	11	12		7		0,6	2,3	n					Thomsen 1979; Manton, Oates & Sutherland 1981
cymbium	m		7			7		1,0	saddle		20			60		3,0	8,6	n					Leadbeater & Manton 1969
discophora	m		10			10		1,0	spherical		25		25	30		1,2	3,0	y		hapt. > 25 um			Manton 1983
elegans	m	3	4	4	3	4	4	1,0		14	16	17		25		1,6	7,1	y		hapt. longer than flagella			Estep et al. 1984
ephippium	m	6	8	10	6	8	10	1,0	saddle	18	29	40	72	106	140	3,7	13,3	n			y		Parke, Manton & Clarke 1956; Manton & Leadbeater 1974
ericina	m	5	8	12	4	7	10	1,2	ovoid	12	18	25	24	37	50	2,1	4,6	y	all		y	n	Parke, Manton & Clarke 1956; Leadbeater 1972
"eyelash"	m	10	11	12	10	11	12	1,0	saddle	20	22	23	6	7,5	9	0,3	0,7	n		hapt. non-coiling			Pienaar & Bandu 1984
fragilis	m		4			4		1,0	subsphaerical	10	13	15		7,5		0,6	2,1	n					Leadbeater 1972
fragilis aff.	m		6			5		1,2			13			7,5		0,6	1,4	n					Manton & Leadbeater 1974
herdlensis (type)	m		4			3		1,3	conical		35			15		0,4	3,8	n			y		Leadbeater 1972
herdlensis	m	6	7	7	5	6	7	1,1			35			15		0,4	2,3	n			y		Manton & Leadbeater 1974
hirta	m		6	12		6	12	1,0			20			30		1,5	5,0	y	all	hapt. somewhat longer when ext.	y	n	Manton 1978, Kawachi et. al. 1991
kappa	m	4	6	10	4	6	10	1,0	sphaeroidal	7	11	16		14		1,3	2,5	n		haptonema a little longer than fl.	y	y	Parke, Manton & Clarke 1955
latilepis	m		9			6		1,5	ovoid	10	15	20		25		1,7	2,8	y		hapt. long; > flagella			Manton 1982
leadbeateri	m	3	6	8	3	6	8	1,0		13	17	20	20	22	24	1,3	4,0	n				y	Throndsen & Eikrem 1991
leadbeateri (type)	m	2	3	4	2	3	4	1,0		11	12	13		15		1,3	5,0	n		hapt. longer than flagella			Estep et al. 1984
mactra	m	15	18	20	15	18	20	1,0		20	30	40	30	35	40	1,2	2,0	n		plate scales perhaps visible			Manton 1972
mantoniae	m		10		4	5	5	2,2			19			10		0,5	1,0	y		hapt. non-coiling			Manton & Leadbeater 1974
mantoniae (type)	m		6			3		2,0	subcylindrical	18	19	20		10		0,5	1,7	y	polar	hapt. non-coiling			Leadbeater 1972
megacylindra	m		5			4		1,3	conical		20			20		1,0	4,0	n			y		Leadbeater 1972; Manton 1972
microcylindra	m	4	6	7	4	6	7	1,0	subsphaerical	20	23	25	16	23	30	1,0	4,2	n					Leadbeater 1972
minor	m	3	4	8	3	4	8	1,0	sphaeroid	6	11	15		9		0,9	2,6	n		hapt. slightly shorter than flagella	y	n	Parke, Manton & Clarke 1955
novae-zelandiae	m		7			7		1,0			25			5		0,2	0,7	n					Moestrup 1979
orbiculata	m	4	6	7	4	6	7	1,0	sphaeroid	7	11	15	5	8,6	12	0,8	1,6	n					Rouchijainen 1972
pachycylindra	m		8			8		1,0	globose		20			15		0,8	1,9	y	all	hapt. shorter than flagella; fl.>20			Manton, Oates & Course 1981
parkeae	m	10	20	30	5	8	10	2,7	elongate	8	20	30	2,5	5	10	0,3	0,3	y	polar	hapt. non-coiling			Green & Leadbeater 1972
pelagica	m	6	8	10	6	8	10	1,0		22	24	25		35		1,5	4,4	y		hapt. longer than flagella			Estep et al. 1984
polylepis	m	6	9	12	5	7	9	1,3	ovoid	18	23	27	9	11	13	0,5	1,2	n			y	y	Manton & Parke 1962; Edvardsen & Paasche 1992
pontica	m	4	5	5	4	5	5	1,0		8	9	10		70		7,8	15,6	n					Rouchijainen 1966
pringsheimii	m	12	17	24	4	7	9	2,6	cylindrical	21	30	40	14	27	40	0,9	1,6	y	polar		y	n	Parke & Manton 1962
pyramidosa	m		3			4		0,8		10	12	13	5	5,5	6	0,5	1,8	n					Thomsen 1977
quadrikonta	m	10	18	25	10	14	18	1,3	subspherical	30	35	40	25	28	30	0,8	1,6	y	all	4 flagella; prox. part of hapt. covered by spine scales			Kawachi & Inouye 1993
simplex (type)	m	2	4	6	2	4	6	1,0	saddle	9	18	27		50		2,8	12,5	n		hapt. much longer than flagella			Estep et al. 1984
simplex	m		6			5		1,2		8	14	20		78	78	5,6	13,0	n				y	Birkhead & Pienaar 1990
spinifera	m	8	9	10	7	8	9	1,1		7	9	12	4	4,5	5	0,5	0,5	y	all	hapt. non-coiling	y		Pienaar & Norris 1979
spinifera (type)	m	6	7	8	3	4	5	1,8	bell	10	20	30	6	7	8	0,4	1,0	y	polar	hapt. non-coiling	y		Fournier 1971
strobilus	m	6	8	10	6	8	10	1,0	saddle	12	21	30	72	126	180	6,0	15,8	n			y	y	Parke, Manton & Clarke 1959; Leadbeater & Manton 1969
tenuispina	m	8	11	13	8	11	13	1,0	globose		25	30		20	25	0,8	1,8	y	all	hapt. not much shorter than fl.			Manton 1978
tenuisquama	m	2	4	5	2	4	5	1,0		7	13	19	39	45	52	3,5	12,9	n					Estep et al. 1984
vexillifera	m		8			6		1,3			20		20	25		1,3	3,1	y	polar	hapt. > 20 um			Manton & Oates 1983
breviturrita	f	6	10	16	6	10	16	1,0	sphaeroidal	15	22	27	6	12	16	0,5	1,2	n					Nicholls 1978
inornamenta	f	6	8	10	6	8	10	1,0	sperical-ovate	18	21	24	8	11	14	0,5	1,4	n					Wujek & Gardiner 1985
laurentiana	f	7	8	9	7	8	9	1,0	sphaeroid	18	19	20	10	13	15	0,7	1,6	n					Kling 1981
parva	f	3	5	7	3	5	7	1,0			15			75		5,0	15,0	n					Lackey 1939

quadrant with c. 15 radiating ridges. The small plate scales are much less numerous (Birkhead & Pienaar found four times as many of the large type of plate scale). They are 0.57-0.71 µm long and 0.52-0.66 µm wide and basically similar to the large plate scales. Each scale has a rim, c. 0.08 µm wide, with a concentric pattern. The scales are divided in four quadrants, each containing 12-13 radiating ridges which are visible from both sides of the scales. None of the three scale types are confined to any particular region of the cell.

Ecology and distribution: *C. brevifilum* was described from the sea near Plymouth (Parke *et al.* 1955) and has subsequently been found in Denmark (Manton & Leadbeater 1974), Norway (Leadbeater 1972) and South Africa (Birkhead & Pienaar 1994). It is probably very widely distributed but usually has not been identified to species level. The micrographs showing *C. brevifilum* in Australia (Hallegraeff 1983, fig. 7) and New Zealand (Moestrup 1979, as aff. *C. brevifilum*, figs 8,9) almost certainly belong to other species. The scales of the former are too small and with too few radiating ridges (c. 36) while the latter organism has two types of scales, smaller spineless plate scales and slightly larger spine-carrying scales.

Toxicology: Jebram (1980) found old cultures of *C. brevifilum* to be toxic to the bryozoan *Electra pilosa*. S. Simonsen (University of Copenhagen, pers. comm.) found no toxicity of *C. brevifilum* to the brine shrimp *Artemia* in complete or phosphorus-deplete medium.

Note: *C. brevifilum* is phagotrophic and ingests bacteria or graphite particles up to a size of 2 or 2.5 µm (Parke *et al.* 1955; Jones *et al.* 1993).

Chrysochromulina kappa Parke et Manton 1955

Description: Cells spheroidal (Figs 16. 1c), somewhat metabolic, (4-) 4.5-6.5 (-10.5) µm in diameter. The two flagella and the haptonema arise close together at one pole. The flagella are equal, homodynamic, the length 1.5-2.5 times the cell diameter. The haptonema is slightly longer than the flagella. The cell contains 2 or 4 golden brown chloroplasts (rarely 1 or none). This species has not been thin sectioned and there is no information on pyrenoids.

Scale structure: The original rather meagre description states that the cell is covered with 'very thin transparent sculptured scales, on any one cell ranging in shape from oval to polygonal and in size from 0.3 x 0.4 µm to 0.5 x 0.8 µm with, at the flagellar pole, a few scales bearing a soft central spine (Parke *et al.* 1955). Based on present knowledge of scale structure in *Chrysochromulina* and on the illustrations provided by Parke *et al.* (1955), it appears likely that this species has three types of scale (Figs 16.3a-c): plate scales of two sizes cover the cell throughout (Figs 16.3a,b), while the spine scales (Fig. 16.3c) are confined to the flagellar pole. Both types of plate scale are ovoid with a low rim, the largest measuring 0.65-0.73 x 0.33-0.35 µm, the smallest 0.4-0.43 x 0.23-0.31 µm. Both scale types show a pattern of radiating lines, in the small scales probably in four quadrants of each c. 9-10 lines, in the larger scales clearly in quadrants, each with c. 14 radiating lines. In the larger scales, the radiating lines appear to be visible only from one side of the scale. The spine scales are also ovoid and measure c. 0.6 x 0.5 µm. Fine radiating lines are visible on the spine-carrying side of the scale, indicating that such lines are present on the opposite side of the scale. A low rim is also present. The central spine is short, probably c. 0.25-0.3 µm but the length is difficult to measure in the published micrographs. Each spine is attached by four decurrent ridges, which extend to the rim of the scale or nearly so.

Ecology and distribution: *C. kappa* was described from the Isle of Man and the south coast of England (Parke *et al.* 1955). Leadbeater (1972) subsequently found it on the west coast of Norway. Additional finds, confirmed by examination of scale structure, have apparently not been made.

Toxicology: Old cultures of *C. kappa* were found by Jebram (1980) to be toxic to the bryozoan *Electra pilosa*. Edvardsen (1993) found it non-toxic to the brine shrimp *Artemia,* in both complete and phosphorus-deplete medium.

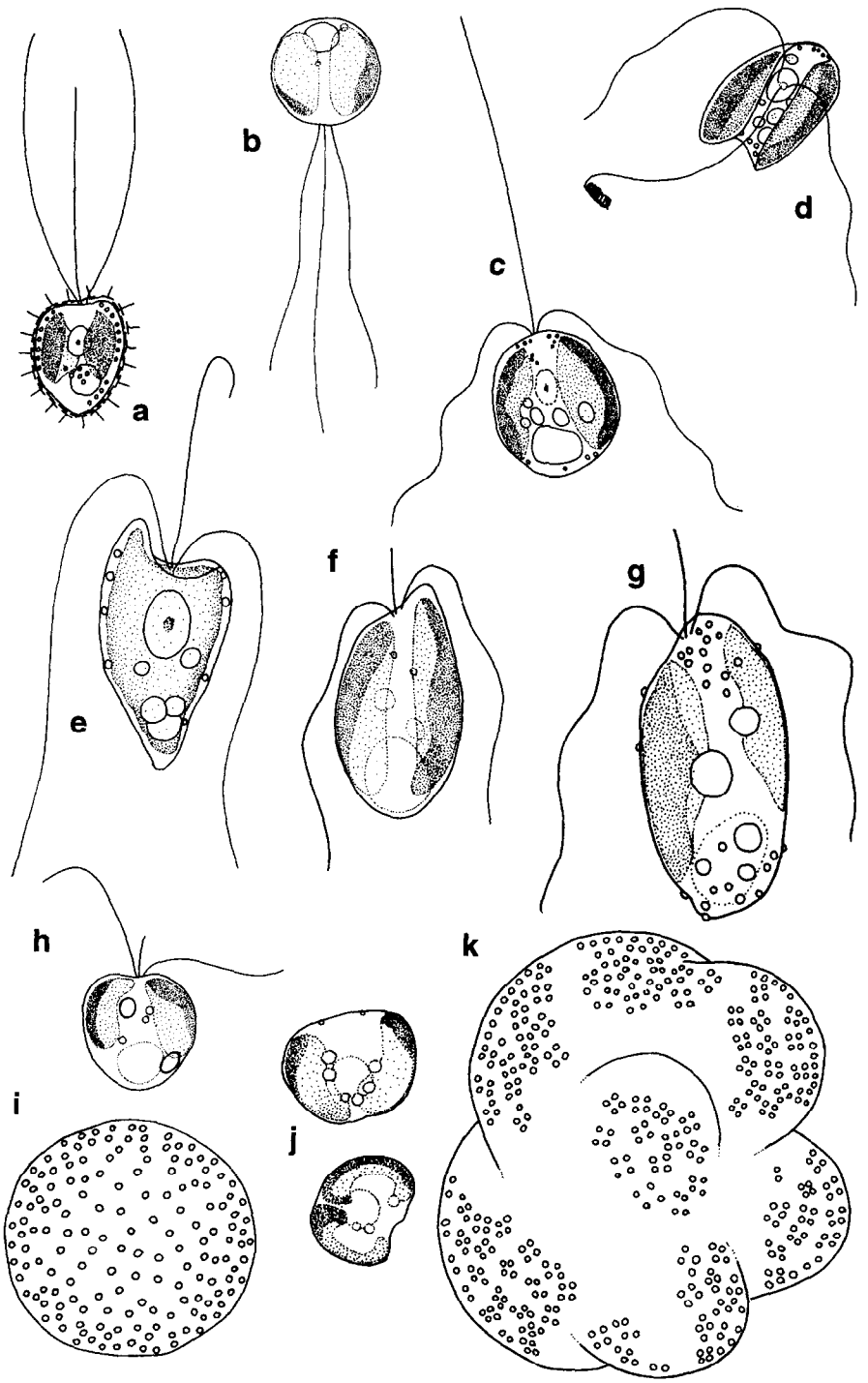

Figure 16.1a-k. Potentially harmful species of the Haptophyta. a, *Chrysochromulina brevifilum* (redrawn from Parke *et al.* 1955); b, *Chrysochromulina leadbeateri;* c, *Chrysochromulina kappa* (redrawn from Parke *et al.* 1955); d, *Chrysochromulina camella* (redrawn from Leadbeater & Manton 1969); e, *Chrysochromulina polylepis* (redrawn from Manton & Parke 1962); f, *Prymnesium patelliferum* (orig.); g, *Prymnesium annuliferum* (redrawn from Billard 1983); h, zooid of *Phaeocystis*; i, colony of *Phaeocystis globosa;* j, single cells of *Phaeocystis*; k, colony of *Phaeocystis pouchetii* (all orig.). Magnification x 3,000 Orig.

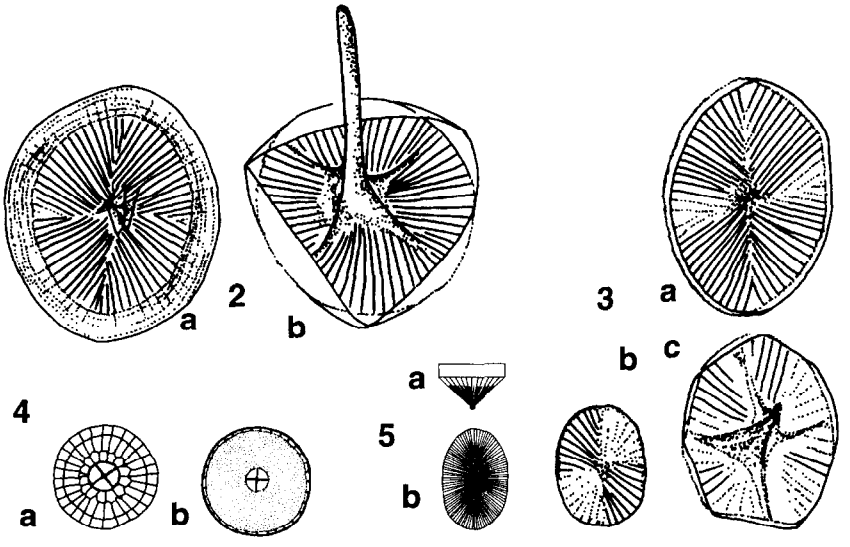

Figure 16.2a,b. Scales of *Chrysochromulina brevifilum* (drawn after Danish material, see text).
Figure 16.3a-c. The three scale types of *Chrysochromulina kappa.*
Figure 16.4a,b. Scales of *Chrysochromulina leadbeateri.*.
Figure 16.5a,b. Scales of *Chrysochromulina camella.* Magni-fication x 40,000. Orig.

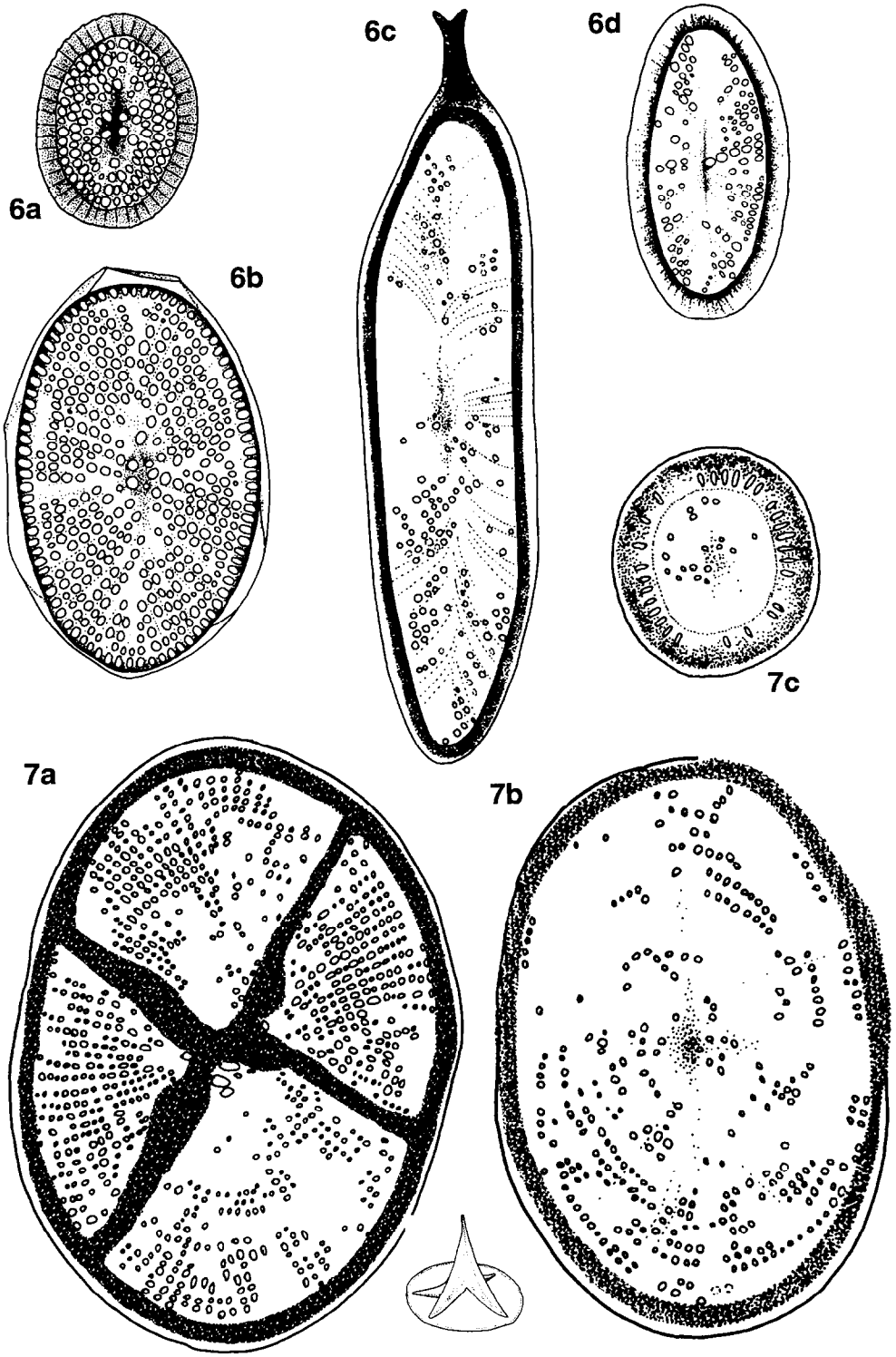

Figure 16.6. Scales of *Chrysochromulina polylepis*. a,b, the most numerous types, which cover the cell body throughout; c,d, the two scale types restricted to the anterior end of the cell. Figure 16.7a-c. Scales of the abberrant form of *Chrysochromulina polylepis*. Magnification x 40,000. Orig.

Note: *C. kappa* is phagotrophic, ingesting particles up to 2.5 µm in diameter, including bacteria (Parke *et al.* 1955).

Chrysochromulina leadbeateri Estep, Davis, Hargraves et Sieburth 1984

Description: Cells more or less spherical (Fig. 16.1b), 3-8 µm in diameter. The two flagella are 13-16 and 16-20 µm long, the haptonema 20-24 µm long. All appendages are attached anteriorly but the haptonema is usually coiled up. Cells contain two golden brown parietal chloroplasts, each with a pyrenoid. (This description is based mainly on Throndsen & Eikrem 1991).

Scale structure: The cell surface is covered with two types of scale (Figs 16.4a,b), each with a circular outline, and measuring c. 0.35 µm in diameter. One scale type is plate-like and consists of concentric and radiating ribs (Fig. 16.4a). It shows a central cross separating four perforations, surrounded by an elevated ring and a circle of 13-15 circular pores. C. 30 ribs radiate from the pores to the scale periphery, divided by a concentric rib into an outer ring of nearly square perforations and an inner ring of elongate ones. The other scale type (Fig. 16.4b) is less ornamented but surrounded by an upright rim. It possesses a central cross separating four openings and surrounded by a distinct elevated ring, but most of the scale is more or less structureless. Along the base of the scale rim there is a circle of very small perforations, in the order of 30-35. In some scales a few scattered pores may be present immediately within this ring of perforations or around the central cross.

Ecology and distribution: *C. leadbeateri* is known from Norway, the Southern North Atlantic, Florida, The North Pacific, and Australia (Estep *et al.* 1984; Hoepffner & Haas 1990).

Toxicology: *C. leadbeateri* was responsible for killing 600 tonnes of cultured fish in Northern Norway (Lofoten) in 1991 (Eikrem & Throndsen 1993). The toxic principle has not been isolated. Cultures of *C. leadbeateri*, however, proved non-toxic to the brine shrimp *Artemia salina* (Edvardsen 1993; Meldahl *et al.*; S. Simonsen, pers. comm.), and three other methods also failed to demonstrate any toxicity.

Note: *C. leadbeateri* has recently been established in pure culture in Norway, enabling studies on the autecology. It is mixotrophic (W. Eikrem, pers. comm.). In the cultures it forms stages with many flagella and non-motile cells (W. Eikrem, pers. comm.).

Chrysochromulina polylepis Manton et Parke 1962

Description: Cells of variable shape, usually ovoid or spherical (Fig. 16.1e), the flagellar pole obliquely truncate and depressed to form a groove. Cells 6-12 µm long and 5-9 µm wide. Flagella homodynamic, equal or subequal in length, 2-3 times cell length, haptonema 1.1-5 times cell length, coiling, inserted subapically. The haptonema is capable of attaching with its swollen tip. Two (rarely four) golden brown parietal chloroplasts.

Scale structure: Cells covered with four types of scale (typical form; aberrant form, see below). The entire cell is covered with two types of scale, arranged in two layers. Scales of the inner layer (Fig. 16.6a) are oval, measuring 0.7-0.8 x 0.5-0.6 µm. They possess an inflexed patternless rim on the dorsal surface, c. 0.08 µm in width. The number of perforations in the (usually) 6 concentric rings increases from c. 4 near the centre to c. 50 along the periphery. Perforations of the outermost ring are distinctly elongate, while other perforations are circular or slightly oval. A central thickening separates the four perforations near the centre. These scales are overlain by larger oval scales (Fig. 16. 6b), measuring 1.3-1.4 x c. 0.8 µm in Danish material. Somewhat wider or even almost isodiametrical scales were seen by Manton & Parke (1962) who gave the dimensions as 1.4 x 1.2 µm. These scales possess an upright patternless rim, 0.06-0.07 µm high. Perforations are more numerous than in the underlayer

scales. They are more distinctly arranged in four quadrants and in 9-10 rings. The scale in Fig. 16.6b has 85 perforations in the outermost circle, gradually decreasing in number towards the scale centre. A central cross-like thickening is present. The two remaining scale types occur only near the flagellar pole. One type carries a short spine (Fig. 16.6c), while the other, more numerous scale type, lacks a spine (Fig. 16.6d). Only very few spine scales are present on each cell, thus 4 were illustrated by Edvardsen & Paasche (1992). The few scales found in Danish material were c. 2.5 µm long (as in the type material from England), excluding the rim but including the 0.2-0.3 µm long spine, which bifurcated distally. The width was c. 0.5 µm, excluding the patternless rim, which was c. 0.07-0.09 µm wide (width of the scale given as c. 0.9 µm by Manton & Parke (1962)). In Norwegian material, Eikrem (pers. comm.) sometimes found scales with a spine extending from each end of the scale, and each spine was sometimes with more than one branching. This scale type also shows perforations, but most are filled in and not very conspicuous. The last scale type was seen repeatedly in thin sections of the Danish material (Throndsen *et al.* 1995), providing additional information not available to Manton & Parke (1962). The scales usually measure c. 1.0 x 0.5 µm in the Danish material (1.2 x 0.6 given as average in the English material) and surrounded by a c. 0.04 µm high upright rim a short distance from the scale periphery. Perforations are less numerous than in the two main types of scale, but are arranged in a radiating pattern.

Aberrant form: In cultures of Norwegian material Paasche *et al.* (1990) reported the existence of cells with a different scale complement. Only three types of scale were present, one carrying a distinct central spine with four decurrent ridges (Fig. 16.7a). The other scale types (Figs 16.7b,c) resemble the main scales of the type, but the largest type of scale (Fig. 16.7b) was found to be c. 30% longer and wider than in the type. The scales were described as thicker than in the type, and the pores, rather than being in the form of small holes, appeared as 'small irregular openings in a mesh created by radiating and concentric elements (fibrils)' (Paasche *et al.* 1990). This type of cell was interpreted as a stage in the life cycle of *C. polylepis*. It has now been found also in nature (Danish waters, Moestrup, unpublished observations).

Ecology and distribution: *C. polylepis* is presently known from the Irish Sea (Manton & Parke 1962), Norway, Sweden and Denmark (Barth & Nielsen 1989), and from Australia (P. Beech and D.R.A. Hill, pers. comm.). Arlstad (1991) found the species to grow equally well at salinities from 10 to 30‰, and slightly more slowly at 5 and 35‰ (in all cases c. one division per day under the light conditions used). During the massive occurrence of *Chrysochromulina* in Scandinavian waters in 1988 the cells in some areas were confined to the pycnocline, occurring as a band at several metres depth (Nielsen *et al.* 1990). Bacterivory was recently reported by Nygaard & Tobiesen (1993).

Toxicology: *C. polylepis* was responsible for the massive damage to the environment in 1988 in Scandinavia. Some 900 tonnes of fish died in Sweden and Norway (mainly cod, salmon and trout), and *Chrysochromulina* also affected invertebrates, macroalgae, zoo- and phytoplankton, and bacteria (the latter were absent in the water layer containing the toxic cells).The toxin(s) were examined by Yasumoto *et al.* (1990) who found two hemolytic and ichthyotoxic compounds. The major hemolytic compound was a galactolipid, 1-acyl-3-digalacto-glycerol. Small amounts of a polyunsaturated fatty acid, octadecapentaenoic acid, was also detected. Both the main type and the aberrant form are toxic.

Chrysochromulina strobilus Parke et Manton 1959

Description: Cells of variable shape: saddle-shaped, bell-shaped, obovoid or flattened globose (Fig. 16.1d), 5-12 µm in size. The flagella are subequal to equal, homodynamic when cells are moving rapidly, heterodynamic when cells move slowly or are stationary, 2-3 times cell length (average 20 µm). The haptonema is 12-18 times the cell body length (average 100 µm), coiling, the number of gyres in the coiled state 25-45. The appendages are inserted ventrally one third the cell length from the anterior end. Cells with two (rarely four) parietal

golden brown chloroplasts, each with an immersed pyrenoid (Parke *et al.* 1959; Leadbeater & Manton 1969a).

Scale structure: The cells carry an outer layer of mucilage which may obscure details of the scales. Cells are covered with scales of two types (Fig. 16.5a,b). Scales of the inner layer (Fig. 16.5b) are oval flat plates with a pattern of radiating ridges on both surfaces (44 are visible in Leadbeater & Manton 1969a, fig. 16), the ridges converging to a plain centre and showing some indication of being arranged in four quadrants. The scale margin is slightly thickened. The outer scales (Fig. 16.5a) are cup-shaped with a very short stalk, a cone-shaped mid-piece and an upper rim. The base of the cone and the rim are patternless, but the outer surface of the rest of the cone is delicately striated with a single row of perforations just below the junction with the rim.

Similar species: *C. strobilus* has given name to the strobilus-complex, a group of (presently) four species which are very closely related and unusual in containing 6 rather than 7 microtubules in the haptonema. They are difficult to distinguish without thin sections of the scales and it is likely, because of the very close relationship between the four species, that they behave similarly with regard to toxins. For a description of *C. camella* and *C. cymbium,* see Leadbeater & Manton (1969b), *C. campanulifera,* see Manton & Leadbeater (1974).

Ecology and distribution: *C. strobilus* was described from the open sea near Plymouth (England) and subsequently found in Denmark (Manton & Leadbeater 1974).

Toxicology: Old cultures of *C. strobilus* were reported by Jebram (1980) to be toxic to the bryozoan *Electra pilosa.* Rhodes *et al.* (1994) found a New Zealand isolate of the closely related species *C. camella* to be non-toxic to the brine shrimp *Artemia salina* under all conditions tested. Similar lack of toxicity was found by Edvardsen (1993) in *C.cf. campanulifera.*

Note: *C. strobilus* is phagotrophic, ingesting bacteria, diatoms, etc. (Parke *et al.* 1959).

The genus *Phaeocystis* Lagerheim 1893

(Type species *Phaeocystis pouchetii* (Hariot) Lagerheim 1893).

Phaeocystis is a genus of foam-producing species distributed world-wide and causing problems for fishing and tourism in areas as widely scattered as New Zealand (Tasman Bay slime) and the North Sea (review by Davidson & Marchant 1992). Recent evidence from Norway has shown that in addition to the problems caused by the foam, *Phaeocystis* may also form toxin(s) responsible for fish kills (Eilertsen & Raa, in press). *Phaeocystis* is a genus of colony-forming species (Fig. 16.1i,k) and the morphology of the individual colonies is highly variable. Several species of *Phaeocystis* were described mainly around the turn of the century but these 'old' species were all merged into one by Kornmann (1955) and Kashkin (1963), followed by Sournia (1988). This trend has now been reversed, and several authors have suggested that the genus comprises several species for which some of the old names are being applied (e.g. Baumann *et al.* 1994, see further below).

Morphology: At least two different stages occur in the life cycle, a colony-forming (Fig. 16.1i,k) and a single-celled stage (Fig. 16.1h). Colonies may attain a length of up to 2 cm and thus become visible to the naked eye. The cells are embedded in mucilage, forming a monolayer along the periphery of the colony. Each cell contains 2 (1-4) parietal yellow-green chloroplasts (Figs 16.1j) but lack flagella and haptonema. The unicellular stage (Fig. 16.1h) is biflagellate, 3-8 μm long, with a very short haptonema which is often very difficult to see with the light microscope. The cells usually contain two chloroplasts. The cell surface is covered with submicroscopic flat scales of two kinds. In some cases, trichocyst-like structures have been found, appearing as pentagonal or nonagonal stars after discharge from the cell (Parke *et al.* 1971). The life history of *Phaeocystis* is still unresolved, and both the colonies and the single cells may self-replicate, the colonies by fragmentation. It appears likely that some colonies originate from single cells which resorb the appendages and divide into colonies.

Single or few-celled stages attached to the setae of diatoms may represent flagellates that have settled. They may eventually give rise to the free-floating colonies, which can contain thousands of cells.

Taxonomy: The taxonomy of *Phaeocystis* is still unresolved, but Medlin *et al.* (1994), based on the morphology of the colonies, geographic distribution, growth in relation to temperature and 18S small subunit ribosomal RNA sequencing recognized three species, in addition to *P. scrobiculata,* which is known only from the flagellated stage and differs markedly from the others.

P. pouchetii (Hariot) Lagerheim (Fig. 16.1k) is a cold-water species which occurs in the Arctic and in temperate waters of the Northern Hemisphere. Colonies are rather small, up to 2 mm, small colonies are spherical, while colonies larger than 0.3 mm are lobed. Cells are generally in groups of four forming a square. Growth optimum is at 8°C, but the colonies will tolerate from less than -2 to 14°C.

P. antarctica Karsten is a cold-water species confined to the area around Antarctica. It grows to at least 9 mm (?), and colonies are spherical or derived from a spherical shape. Cells are uniformly distributed along the periphery of the colony. It has a growth optimim at 4.5°C and will tolerate from less than -2 to 14°C. It is closely related to *P. pouchetii* but the distribution of the two taxa does not overlap.

P. globosa Scherffel (Fig. 16.1i) occurs in temperate waters of both hemispheres. It grows to 8-9 mm and the colonies are - like *P. antarctica* - spherical or derived from a spherical shape. It also resembles *P. antarctica* in the uniform arrangement of the cells along the periphery. It has a growth optimum of 16°C but will tolerate -0.6 to 22°C.

These three species all form pentagonal trichocyst-like structures and the motile cells are scale-covered while cells of the colonial stage are not. No difference has been found between the motile cells, which are in need of a careful study, using transmission electron microscopy. Additional species seemingly occur in the Mediterranean (Medlin, pers. comm.) and in South Africa (Pienaar, pers. comm.)

P. scrobiculata Moestrup is known only as unicellular flagellate stage. It has considerably longer flagella (23-30 µm versus 6-15 µm), and the two scale types on the cell are markedly different. Both types show a pattern of radiating ridges on the ventral surface while the dorsal side is patternless. In *P. globosa* radiating ridges are visible on both surfaces. The scales in *P. scrobiculata* are oval and measure 0.6 x 0.45 µm, the small scales are circular-oval, measuring 0.19-0.21 µm. Scales in *P. globosa* are 0.18 x 0.19 µm and 0.10 x 0.13 µm. The trichocyst-like structures of *P. scrobiculata* are nine-rayed stars, as opposed to five-rayed in the other species. *P. scrobiculata* was originally described from New Zealand (Moestrup 1979) but has subsequently been found also in Australia (Hallegraeff 1983), the North Pacific (Hoepffner & Haas 1990), and in temperate and tropical waters of the Atlantic (Estep *et al.* 1984).

Toxicity: The main effects of *Phaeocystis* is clogging of fishing nets, destroying of fish and formation of masses of foam, which may reach several metres in thickness (Lancelot *et al.* 1987). The foam appears when blooms are washed ashore. Like many other marine plankton algae *Phaeocystis* blooms produce dimethylsulphide (DMS), which is believed to evaporate to the atmosphere and contribute to the acidity of rainwater (Davidson & Marchant 1992). A directly toxic effect is believed to have occurred in Norway in 1992, causing death of farmed salmon valued at 1 million NKr (Tangen, pers. comm.). The toxic compound(s) has not been identified.

The genus *Prymnesium* Massart ex Conrad 1926
(Type species: *P. saltans* Massart ex Conrad 1926)

Prymnesium (Fig. 16.1f,g) is one of the most serious fishkillers (reviewed by Moestrup 1994), and it is therefore unfortunate that the species cannot be identified by light microscopy. All described species appear very similar, if not identical, by light microscopy and identification to species level is not possible without examination of the submicroscopic scales. The exception is the most recently described species, *P. nemamethecum*, in which the scaly periplast covers also the haptonema, and this 'haptonema sheath' is visible in the light microscope (Pienaar & Birkhead 1994). The genus as such may be distinguished however. The shape of the cell varies from elongate pyriform to almost spherical, not compressed, 6-18.5 µm long by 3.5-11 µm wide. The front end is obliquely truncate and the appendages are inserted subapically in a slight depression of the cell (Fig. 16.1f,g). The flagella are subequal or unequal, 9.5-25 µm in length and heterodynamic. Green *et al.* (1982) describe the flagellar motion like this: the more posteriorly inserted flagellum is directed posteriorly often close to the cell body and beats with an undulating motion, while the other flagellum passes over the most anterior part of the cell, backwards along the side opposite to that on which the appendages are inserted and beats with a violent flickering movement. The haptonema is short, 2.2-6.3 µm long and non-coiling. It is kept stiffly in front of the cell. The cell possesses two parietal chloroplasts.

Behaviour: Perhaps the most typical feature of *Prymnesium* is the ability of the cells to attach to others objects. This was noted in some of the very first descriptions (Massart 1920) and is readily visible. The cells may attach to a coverslip by the tip of the haptonema, and the cell may rest in this position for some time. Conrad & Leloup (1938) noted that the cells attached to the gills of fish with the haptonema, which they suggest to function as a 'style inoculateur', injecting poison into the gills. Experimental evidence for this interesting claim is lacking, however. During attachment, the flagella beat homo- or heterodynamically along and behind the cell. In nature, the cells have been seen to attach to macroalgae or fishing nets (Johnsen & Lein 1989), or to sand grains (Pienaar & Birkhead 1994: *P. nemamethecum).* A thick layer of *Prymnesium* was noted by a fisherman after a salmon net had been in the water for a few hours (Johnsen & Lein 1989). It is possible that the cells graze bacteria in this condition, *P. parvum* was shown by Nygaard & Tobiesen (1993) to ingest bacteria. Under bloom conditions, cells of *Prymnesium* may be present also in the water column, but it is probably significant that in the only study where this has been examined carefully, Johnsen & Lein (1989) found cells in the water masses to be concentrated in the upper few metres of the water column. This suggests that the cells attach to the water surface like neustonic algae, using the haptonema.

We are not aware of any species of *Chrysochromulina* having been described as being capable of attachment and this feature may be a reliable distinguishing feature between *Prymnesium* and *Chrysochromulina* which are otherwise very similar.

Six species of *Prymnesium* have been studied ultrastructurally and these will be described briefly below. Two of additional species, *P. minutum* N. Carter and *P. czosnowskii* Starmach are poorly characterized and may be forms of other species. *P. papillatum* Tseng et J.F. Chen is very similar to and perhaps conspecific with *P. patelliferum*. The type species, *P. saltans,* has not been characterized ultrastructurally, but it is likely to be identical to *P. parvum.*

The three species tested for toxicity were all toxic and until proved otherwise, a bloom of *Prymnesium* should always be considered toxic.

Prymnesium annuliferum Billard 1983.

Scale structure: Cells covered with two types of scale (Fig. 16.8a-e). One type (Fig. 16. 8a,b), which occurs in one or more layers next to the cell, is oval, plate-like, with an inflexed dorsal rim. It measures c. 0.49 x 0.4 µm. The proximal side (Fig. 16.8a) shows a pattern of radiating ridges arranged in four quadrants, c. 14-15 ridges per quadrant. The rim is c. 0.08

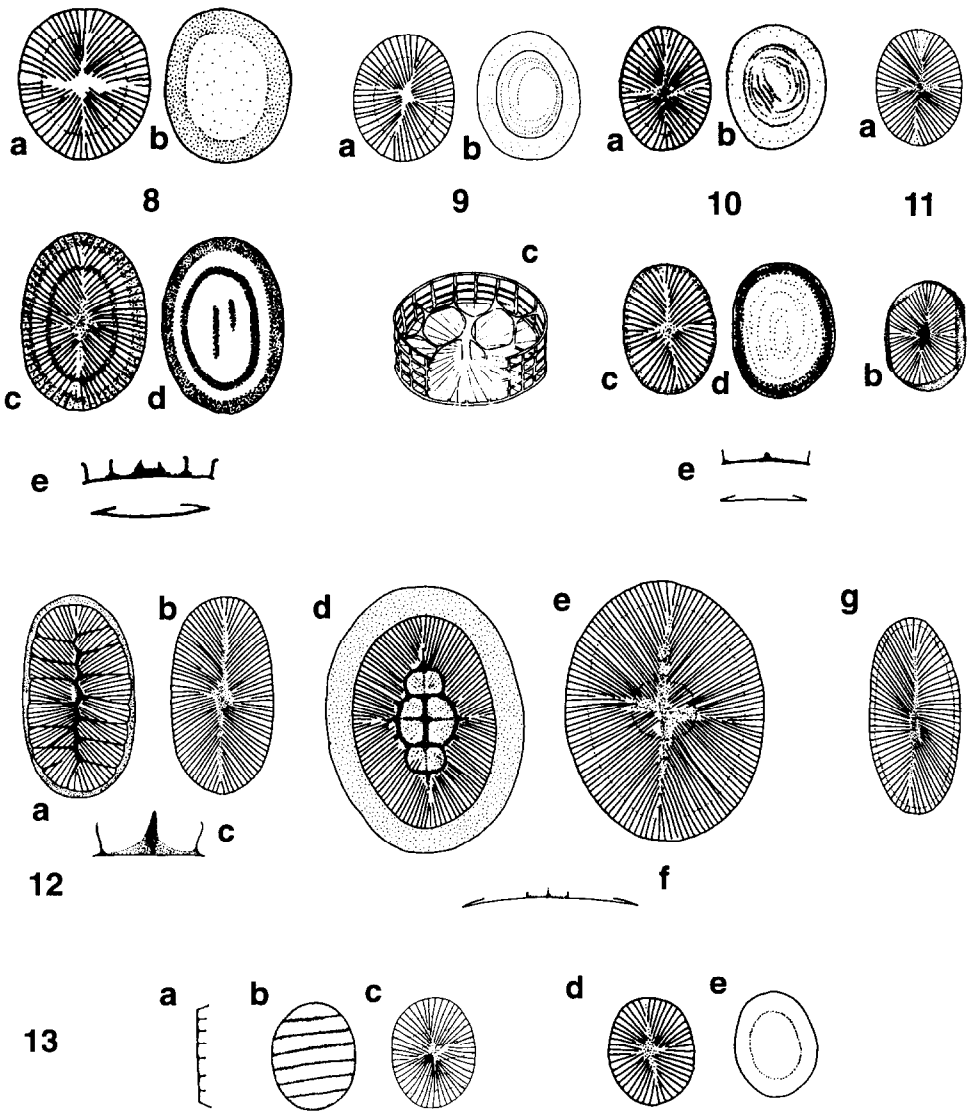

Figure 16.8a-e. Scales of *Prymnesium annuliferum.* a,b, underlayer scales seen from the ventral and dorsal side, respectively; c,d, scales from the outer layer seen from the ventral side and dorsal side, respectively; e, surface (top) and underlayer scales (below) in transverse section.
Figure 16.9a-c. Scales of *Prymnesium calathiferum.* a,b, underlayer scales in ventral and dorsal view, respectively; c, scale from the outer layer.
Figure 16.10a-e. Scales of *Prymnesium parvum.* a,b, underlayer scales in ventral and dorsal view, respectively; c,d, scales from the outer layer in central and dorsal view; e, outer (top) and inner layer scales (below) in transverse section.
Figure 16.11a,b. *Prymnesium patelliferum.* a, underlayer scale in ventral view; b, scale from the outer layer, dorsal view.
Figure 16.12a-f. *Prymnesium nemamethecum.* a-c, scales from the distal layer; a, seen from the dorsal side, b, seen from the ventral side, c, in transverse section; d-e, scales constituting the proximal layers; d, seen from the dorsal side, e, from the ventral side and f, in transverse section.
Figure 16.13a-e. *Prymnesium zebrinum.* a-c, scales from the outer layer, a, in transverse section, b, in dorsal and c, in ventral view; d-e, scales from the innermost layers, d, in ventral and e, in dorsal view.Magnification x 40,000. Orig.

µm wide. Scales of the outer layer oval, c. 0.53 x 0.39 µm (Fig. 16.8c,d). The proximal face (Fig. 16.8c) has ornamentation similar to that of the inner layers. The outer surface shows 2-3 concentric ridges (Fig. 16.8d,e), each c. 0.07 µm high, the outermost ridge forming the rim of the scale. The innermost ridge variable in appearance, sometimes U-shaped or reduced to a single or two parallel ridges of variable length.In addition to these two types of scale, fig. 17 of Billard (1983) shows two smaller plate scales, indicating the existence, perhaps, of a third type of scale. These scales are distinctly smaller than the plate scales mentioned in the diagnosis.

Geographic distribution: France only.

Toxicity: Unknown.

P. calathiferum Chang et Ryan 1985

Scale structure: Cells covered with two types of scale (Fig. 16.9a-c). One or two layers of oval, flat scales (Fig. 16.9a,b) are located immediately outside the plasmalemma. The proximal surface of these scales (Fig. 16.9a) bears radiating ridges in quadrants, c. 12 ridges per quadrant. The distal face (Fig. 16.9b) has a system of concentric fibrils and a wide inflexed rim (c. one third of the scale radius). The scales measure c. 0.45-0.55 x 0.30-0.35 µm. The outer layer of scale (Fig. 16.9c) has an oval base plate of similar size as the plate scales, but possesses an upright basket-like rim (c. 0.10-0.19 µm high), comprising both vertical and horizontal bars. A short pillar rises vertically from the centre of the scale and a network of fibrils extends from its top to the outer rim. The proximal face of the scale bears radiating ridges as in the plate scales.

Geographic distribution: Presently known only from New Zealand.

Toxicity: The toxin(s) are unknown, but *P.calathiferum* was tested toxic to fish (*Gambusia*) (Chang 1985). It is considered responsible for fish and shellfish mortalities in New Zealand in 1983 (Chang & Ryan 1985).

P. parvum N. Carter 1937

Scale structure: Cells covered with two types of scale (Fig. 16.10a-e). Scales of the inner layers oval (Fig. 16.10a,b), 0.29-0.36 x 0.26-0.32 µm, with radiating ridges on the proximal side (Fig. 16.10a), and a pattern of concentric fibrils on the distal face (Fig. 16.10b). The distal face with a wide (0.04-0.05 µm) inflexed rim. Scales of the outer layer very similar (Fig. 16.10c-e), 0.30-0.43 x 0.23-0.30 µm, but with a narrow rim only (Fig. 16.10c,d). The radiating ridges in both types of scale are arranged in quadrants, c. 11 ridges per quadrant.

Geographic distribution: *P. parvum* is very widely distributed and known from temperate and subtropical regions of both hemispheres (Green *et al.* 1982).

Toxicity: *P.parvum* is a noted fishkiller which is responsible for numerous fish mortalities, especially in low saline water (for a review, see Moestrup 1994). The toxins are rather poorly known. Several hemolytic compunds have been found, one of which was identified by Kozakai *et al.* 1982) as a mixture of two galactolipids. The major fatty acid component was a C18:4 and the minor a C18:5 polyunsaturated fatty acid. Igarashi *et al.* (1993) found two hemolytic compounds, known as prymnesin 1 and 2, which they identified as polyoxy-polyene-polyethers (MW = 2264 and 1970, respectively).

P. patelliferum Green, Hibberd et Pienaar 1982

Scale structure: Cells covered with two types of scale (Fig. 16.11a,b), both oval and measuring c. 0.36-0.37 x 0.25-0.27 µm. Innermost scales in up to 3 layers, each with a

narrow inflexed rim and a central thickening on the distal face and a system of radiating ridges on both surfaces (Fig. 16.11a). The ridges are in quadrants with 11-13 ridges per quadrant. Scales of the outer layer similar but with an upright rim (Fig. 16.11b), c. 0.06 µm high.

Geographic distribution: *P. patelliferum* (previously but uncorrectly known as *P. patellifera)* is known from the west coast of USA, England, Norway, Bulgaria, Australia (Green *et al.* 1982; Larsen & Moestrup 1989, Larsen *et al.* 1993). Identical to *P. papillatum* ?, see Chen & Zeng (1986).

Toxicity: The toxic potential is unknown. However, in a series of studies Arlstad (1991) showed that *P. patelliferum* was toxic to the dinoflagellate *Heterocapsa triquetra* and it is likely that the organism responsible for a sudden mass mortality of fish in the Varna lakes in Bulgaria in 1959 may have been this species (Green *et al.* 1982). It is also toxic to the brine shrimp *Artemia salina* (Larsen *et al.* 1993).

P. nemamethecum Pienaar & Birkhead 1994

Scale structure: Cells covered with three different types of scale (Figs 16.12a-g). One type is confined to the haptonema surface (Fig. 16.12g), and covers the haptonema as a sheath. These scales are elliptic, 0.6 x 0.3 µm, with radiating ridges visible on both surfaces, c. 20 per quadrant. The distal surface has a narrow inflexed rim c. 0.03 µm wide. In transverse thin section they frequently appear more or less v-shaped. The cell body is covered with up to six imbricate layers of flat oval scales, measuring c. 0.85 x 0.6 µm (Fig. 16.12d-f). The proximal face of each scale (Fig. 16.12e) bears a pattern of radiating ridges in quadrants, each quadrant with c. 30 ridges. The distal surface (Fig. 16.12d) possesses a patternless inflexed rim, c. 0.04 µm wide, and radiating ridges in addition to a central corona-like structure of somewhat variable construction. The outermost layer of scales consists of elongate, oval scales, measuring c. 0.65 x 0.35 µm (Fig. 16.12a-c). Both sides show a pattern of radiating ridges in quadrants, c. 20 per quadrant. A 0.11 µm high raised rim lines each scale (Fig. 16.12a,c), while the central part comprises a raised thickening (c. 0.14 µm high) that subtends branches alternately to each side (Fig 16.12c). The 17-22 branches extend to the peripheral rim and measure c. 0.045 µm in height (Fig. 16.12c).

Geographic distribution: Described from full salinity seawater near Cape Town and found also in Australia (Pienaar & Birkhead 1994).

Toxicity: Unknown.

P. zebrinum Billard 1983

Scale structure: Cells covered with two types of scale (Fig. 16.13a-e). Innermost scales flat, in one or two layers (Fig. 16.13d,e). Scales circular or slightly elongate, measuring c. 0.3 x 0.28 µm. The proximal face with a pattern of radiating ridges in quadrants, 10-11 ridges per quadrant (Fig. 16.12d). The distal face appears patternless (Fig. 16.13e), but has an inflexed rim which is c. 0.06-0.08 µm wide. Outer scales in a single layer, elliptic, c. 0.35 x 0.27 µm (Fig. 16.13a-c). The proximal face with radiating ridges in quadrants, 11-12 ridges per quadrant (Fig. 16.13c).The distal face with a raised rim 0.05-0.06 µm high, and 4-6 parallel cross bars, each 0.025-0.03 µm high (Fig. 16.13a,b). The cross bars are arranged perpendicularly to the long axis of the scale or nearly so. The distal face otherwise appears patternless.

Geographic distribution: Known from three locations in France only.

Toxicity: Unknown.

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17. Taxonomy of Diatoms

G.R. Hasle and G.A. Fryxell

The diatoms are one of the largest algal groups known. Although traditionally 10 000-12 000 taxa are accepted, a much higher number have been suggested. According to Round and Crawford (1984, p. 169 and 1989, p.574) "experienced diatom systematists estimate a probable final number of approximately 50,000 species" and "there are approximately 250 commonly recorded genera; the number of species described is in excess of 100,000". The diatoms are found in all types of aquatic habitats, in the marine plankton at all latitudes and through all seasons. Sournia *et al.* (1991) estimated between 1365 and 1783 diatoms (865-999 centric and 500-784 pennate species) to be from the marine planktonic habitat.

Although unicellular, many of the planktonic diatoms appear in various kinds of colonies. Cells embedded in mucilage extruded from the diatoms are especially relevant here. The diatoms are above all characterized by their siliceous cell covering that contains most of the features used for identification to family, genus, and species. Other distinctive features are cell shape, number and shape of chloroplasts, and structure of colony.

METHODOLOGY

Collection

Sampling with a tube or any kind of water-bottle (Venrick, 1978) provides ample material for identification of diatoms occurring in bloom concentrations. In most cases an additional sample collected by a fine-meshed net (Tangen, 1978) is required for preparation for critical light microscopy and electron microscopy. Crude cultures, serial-dilution cultures (Throndsen, 1978a) and unialgal cultures provide other means to obtain material for careful identification.

Preservation

Although information on the protoplast is best obtained by examination of non-preserved material, identification to species will usually require further procedures, including preservation. The preservatives in most common use in marine phytoplankton investigations (Throndsen, 1978b) are formaldehyde and potassium iodide plus iodine (Lugol's solution). Slightly acid preservative is preferred to hinder the dissolution of the silica wall. If stored for a longer time (months or years), the sample should not be too dense, and the sampling jar should be of glass with a non-metallic lid. Frequent openings of the sampling jar often seem to reduce the effect of the preservation. If feasible, two aliquots of the original sample, one to be archived being left unopened, would therefore be advantageous.

Preparation

Examination of raw (uncleaned) material in water mounts gives sufficient information to identify some genera and most of their species, e.g. *Chaetoceros, Corataulina*, and *Rhizosolenia*. These genera are recognized by specific structures such as setae, elevations, and special shapes of the valve and the bands, respectively. Species identification of other diatoms,

e.g. *Thalassiosira* spp. and *Pseudo-nitzschia* spp., requires examination of valve structure. Since the refractive indices of silica and water are about the same, the contrast has to be increased by embedding the diatom cell in a medium of a higher refractive index. The material to be embedded may just be rinsed to get rid of preservative and sea water or, in addition, may be cleaned to get rid of organic cell elements and to separate the various frustule components. A great number of methods for cleaning and preparing permanent diatom slides are in use (e.g. Hasle, 1978; Round *et al.*, 1990).

Microscopy

Most of the planktonic diatoms in question are weakly silicified. The use of phase or differential interference contrast optics is recommended, especially for light microscopy of water mounts, but also for examination of cleaned material embedded in a medium of a high refractive index. Electron microscopy can ensure correct identification of some of the diatoms mentioned as harmful, especially the *Pseudo-nitzschia* species. Cleaned material for light microscopy (LM) can be used also for electron microscopy and mounted on a formvar coated grid for transmission electron microscopy (TEM) and on a stub for scanning electron microscopy (SEM; Hasle, 1978; Round *et al.*, 1990). The transmission electron microscope is usually the best instrument for examination of the fine structure of the *Pseudo-nitzschia* valves, especially the striae.

Terminology and Gross Morphology

The shape of the external skeleton of a diatom, called a **frustule**, is usually compared with that of a box, with a larger half fitting over a separate, slightly smaller half. On the half of the frustule with the larger diameter, the lid of the box is an element called a **valve**, and the sides are made up of a series of bands that stay with the valve for the life of the cell. The smaller half of the frustule is made of similar parts with the exception that the sides of the box, or bands, are formed sequentially and slipped in place as the young cell matures and growth takes place before the next division cycle. All the bands together are called the **girdle**, and the cell may be seen in **girdle view** or in **valve view** (Fig. 17.1). The flattened part of the valve is called **valve face** and the rounded or steep peripheral part is called **valve mantle**. Valves as well as bands are usually not solid silica but have an open structure that lends strength without great weight. An individual opening, whether a pore or a chamber, is an **areola**. The areolae may be arranged in rows called **striae**. In centric diatoms the striae are oriented towards a **point**, and in pennate diatoms towards a **line**. (For further information see: Anonymous, 1975; Ross *et al.*, 1979; Round *et al.*, 1990, p.4).

HARMFUL EVENTS

Most harmful events have been noted in coastal waters where nutrients are high and observations frequent:

1. *Coscinodiscus concinnus* and *C. centralis* were found in May 1947 in the North Sea in concentrations discolouring the water and forming an oily film on the sea surface. This sticky oil adhered to birds' feathers, and bird mortality was observed (Tåning, 1951; Grøntved, 1952).

2. *Thalassiosira mala* forms mucilage colonies and caused discoloured water, clogging the gills of cultured bivalves in Tokyo Bay in 1951 (Takano, 1956; 1965). The reported loss amounted

to about 58 million yen (Takano, 1956, p. 65). The diatom is similar to a *Phaeocystis* cell in size and in shape of colonies, and the prymnesiophyte and the diatom may be confused.

3. In the late 1970's the fishermen in the Plymouth area complained that their nets became clogged with a heavy jelly-like material. Mucilage produced in cultures of *Coscinodiscus wailesii*, an important constituent of the phytoplankton in these waters from 1977, and mucilage from the nets were shown to have basically the same chemical structure (Boalch, 1984).

4. Death of benthic shellfish and bony fish was attributed to anoxia and clogging of the gills by a bloom of *Cerataulina pelagica* (Cleve) Hendey off the coast of northeastern New Zealand in 1983 (Taylor *et al.*, 1985).

5. A bloom of a diatom tentatively identified as *Rhizosolenia chunii* Karsten occurred in Port Phillip Bay from late August to mid October 1987 coincidently with the development of an unpleasant bitter taste of mussels, scallops and flat oysters, followed by high shellfish mortality 3-8 months after the bloom had ceased (Parry *et al.*, 1989).

6. Large concentrations of *Chaetoceros* spp. may clog the gills of farmed fish and also cause bleeding (Tangen, 1987; Horner *et al.*, 1990; Hallegraeff, 1993). This effect had been observed on lingcod by *C. convolutus* already in 1961, and the hypothesis was that the spiny *Chaetoceros* setae actually penetrated the gill tissue (Bell, 1961). Laboratory bioassays with Atlantic salmon and environmentally common (as well as greater) concentrations of *C. concavicornis* documented a physical action of the diatom on the fish gills. The gills started to produce mucus that induced hypoxia (or deficiency of oxygen to the tissues of the body) and hypercapnia (or excessive amount of carbon dioxide to the blood) (Rensel, 1993).

7. The first incident of amnesic shellfish poisoning (ASP) caused by the toxin domoic acid was documented in 1987 on Prince Edward Island (Bates *et al.*, 1989) coincidently with a bloom of *Pseudo-nitzschia multiseries* (syn. *Nitzschia pungens* f. *multiseries*). Three people died, and others lost their short-term memory permanently. In September 1991 deaths of pelicans and cormorants in Monterey Bay, California, were caused by *P. australis*. (syn. *Nitzschia pseudoseriata*).The toxin has been concentrated in the gut of filter feeders and the Dungeness crabs, but in the meat of anchovies and razor clams (Fritz *et al.*, 1992).

The connection between the occurrence of domoic acid in shellfish and other marine animals and the *Pseudo-nitzschia* species present in the plankton has been studied extensively, and a comprehensive literature on the subject exists (see: Bates, 1993).

8. A phenomenon in the Adriatic Sea, locally known as "mare sporco" (or dirty sea), reported from as far back as 1723 to the present, appeared in summer 1988 to such an extent that it detrimentally affected tourism and fisheries. Macroscopic mucilage aggregates of various shapes, present at various depths, were considered originally produced by diatoms, predominantly of pelagic origin (Stachowitsch *et al.*, 1990). *Cylindrotheca closterium* (Ehrenberg) Reimann *et* Lewin (syn. *Nitzschia closterium)* and *Pseudo-nitzschia* dominated among the diatoms in the aggregates (Stachowitsch *et al.*, 1990; Fanuko *et al.*, 1989). If any diatom could be considered predominant in 1988, it was, according to personal observation (GRH) of a net sample from Trieste, 16 August 1988, *Pseudo-nitzschia pseudodelicatissima* (syn. *Nitzschia delicatula).*

DESCRIPTION OF GENERA AND SPECIES

Any diatom frequently occurring in bloom proportion may be regarded as harmful. Reared fishes loose appetite and the power of resistance against infections when exposed to the

regularly appearing high abundances of *Skeletonema costatum* (Greville) Cleve along the Norwegian coast (Tangen, personal communication). A similar effect has been observed occasionally in South Chile with high concentrations of *Chaetoceros socialis* Lauder, which forms globular gelatinous masses. Fish kills were associated with high concentrations of *Leptocylindrus minimus* Gran off central Chile, although an eventual noxious effect of the diatom could not be established (Clement and Lembeye, 1993).

The genera and species dealt with here are those which are considered harmful due to (i) mucilage production (*Thalassiosira, Coscinodiscus, Chaetoceros* s), (ii) physical damage of the fish gills (*Chaetoceros*), and (iii) toxin production (*Pseudo-nitzschia).* Only the latter has been shown to have a toxic effect on humans.

Centric diatoms - Biddulphiales

Thalassiosira Cleve 1873 - Type: *T. nordenskioeldii* Cleve

Thalassiosira, a marine planktonic genus with more than 100 known species, has a world-wide distribution. For terminology see Fig. 17.2. The drum-shaped cells generally occur in chains, the cells being connected by one or more chitinous threads extruded from strutted processes on the disc-shaped valves. These processes are somewhat more heavily silicified tubes through the valve and can often be seen in phase contrast in the light microscope as tubes or even as dots. **Strutted processes (fultoportulae)** are typical for the family Thalassiosiraceae. In contrast, a **labiate process (rimoportula)**, also a tube through the valve and usually larger, is found in most centric diatoms (Fig. 17.3a). A third type of processes, **occluded processes**, long external tubes that do not penetrate the valve wall, are present in some species of the family Thalassiosiraceae.

About one-tenth of the known species of *Thalassiosira* are regularly found embedded as single cells or short chains in large gelatinous colonies (Figs 17.3c, e).

Generic characters:

- •Cells usually discoid to drum-shaped
- •Many strutted processes with organic threads
- •One or a few labiate processes
- •Areola cribrum ("sieve-plate") on inside wall

Characters showing differences between species:

- •Cell size
- •Valve shape
- •Areola pattern
- •Number of areolae in 10µm
- •Process pattern
- •Number of marginal processes in 10µm

Tables 17.1, 17.2, 17.3.

Comments: The process pattern (Figs 17.3b, d, as shown in Tables 17.1-17.3) is a useful tool for identification of the *Thalassiosira* species.

The shape of the gelatinous colonies is usually ill-defined and has been described as irregular gelatinous masses, cloud-like gelatinous masses, or just as cells embedded in mucilage. They have been found in high numbers even deep in the photic zone (>50 m) at a depth of sharply increasing density and nutrients (Gould and Wiesenburg, 1990). The chitinous threads (Fig. 17.3b), often with pennate diatoms on them, have been seen as especially abundant in colonies of *T. partheneia, T. diporocyclus, T. fragilis* and *T. minuscula*, and one or more cells of *T. minuscula* have been seen inside some kind of capsules inside the colonies (Hasle, 1972a, figs 46-48).

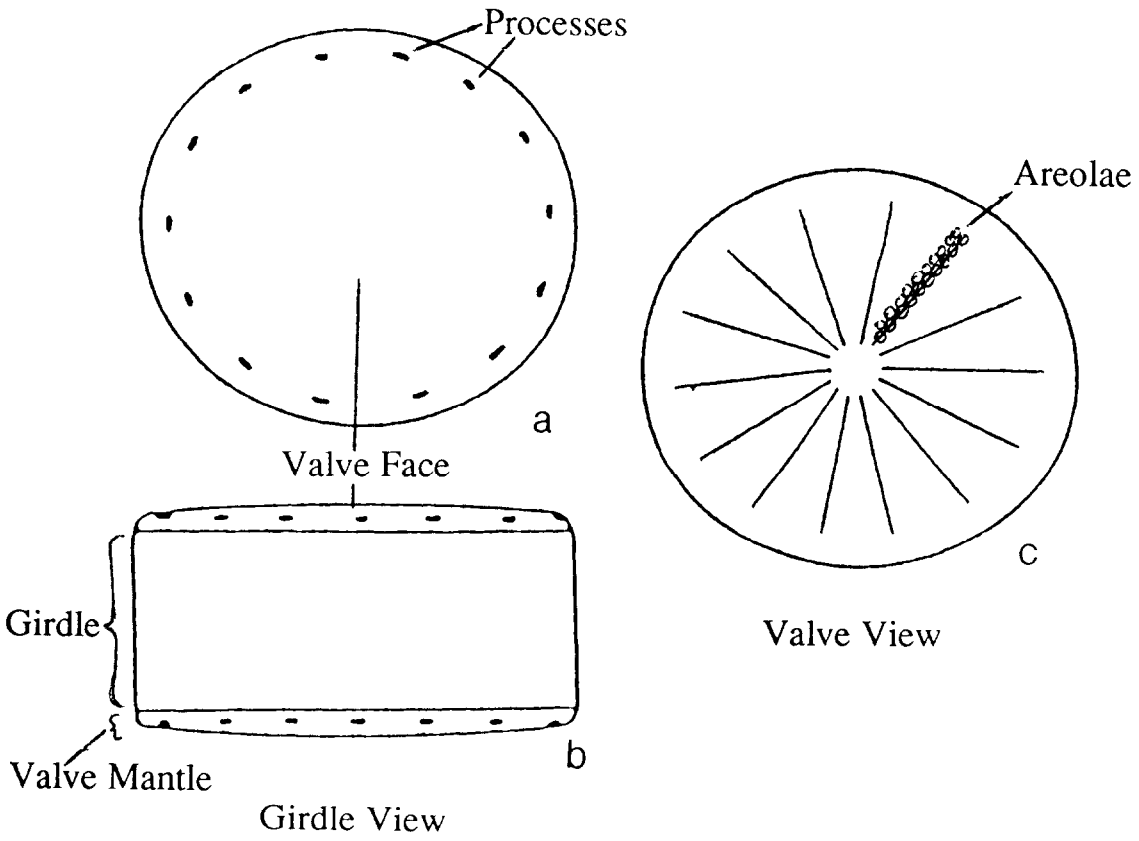

Figure 17.1. Centric diatom, schematic drawing, with terms. a) and c) Valve view, b) Girdle view. Processes, or tubes through the valve, and areolae in patterns related to a point.

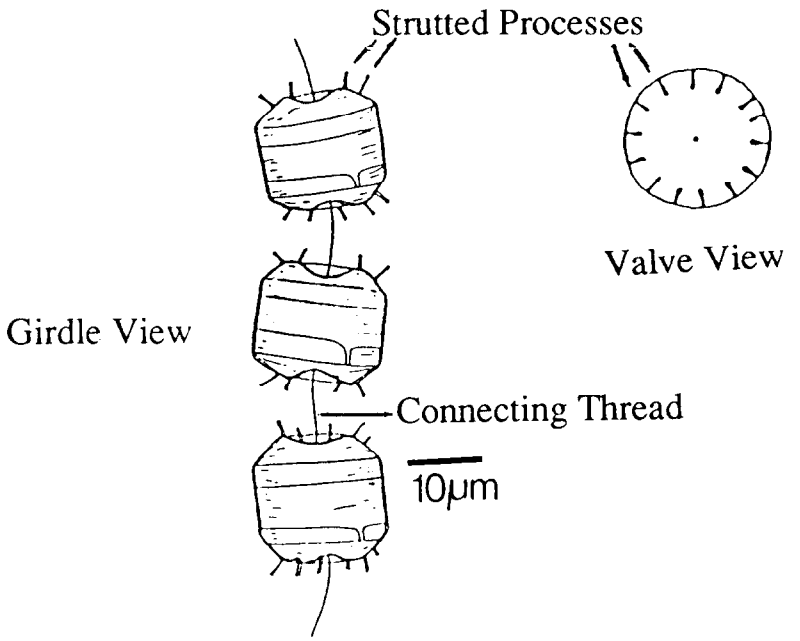

Figure 17.2. *Thalassiosira* with terms. Chain of three cells held together by thread(s). Valve view with one pattern of processes. Girdle view from Cupp (1943).

Table 17.1. Gelatinous colonies: *Thalassiosira* species with dome-shaped valves, radial lines of areolae arranged in sectors, with one central and at least one ring of strutted processes on the valve face in addition to a marginal ring.

Species	Diameter (μm)	Areolae in 10μm	Marginal Ring of Strutted Processes, Distance Apart	Process Patterns
T. diporocyclus	12 - 24	26 - 28	2.7 - 3.8μm, more crowded across from labiate process	1 labiate process, irregular ring of strutted processes on valve face
T. tubifera	15 - 33	ca. 20	3 - 4μm	1 labiate process, occluded processes present very near ring of strutted processes on valve face
T. subtilis	15 - 49	27 - 34	2.7 - 3.6μm	1 large labiate process in 2nd ring from margin, several rings of strutted processes
T. fragilis	19 - 30.5	40 - 50	3 - 5μm	2 marginal labiate processes, one irregular ring of strutted processes on valve face

Table 17.2. Gelatinous colonies: *Thalassiosira* species with one labiate process per valve and external extensions of strutted processes.

Species	Diameter (μm)	Areolae in 10μm	Marginal Ring of Strutted Processes, Distance Apart	Areola Patterns	Process Patterns
T. curviseriata	5 - 14.5	20 - 30	3.8 - 4.9μm	radial, but from offset centre	1-2 eccentric strutted processes, 2 wings on marginal tubes
T weissflogii	5 - 32	30 - 40	0.6 - 1.1μm, closely-packed	radial, areolae poorly developed	large labiate process, central ring of strutted processes
T. delicatula	9 - 30	22 - 26	2.0 - 2.5μm, three rings on mantle	sectors	labiate process away from margin; occluded present with larger external tubes, one or more rings of strutted processes on valve face
T. gravida	18 - 62	ca. 20	2 - 3μm?, several closely-packed rings on mantle with longer external tubes	radial, sectors	large labiate process, central cluster of strutted processes with many on valve face, resembles a pin-cushion.

Table 17.3. Gelatinous colonies: *Thalassiosira* species with a small, rounded appearance in girdle view, one ring of marginal strutted processes and one labiate process per valve, and without pronounced external tubes.

Species	Diameter (µm)	Areolae in 10µm	Marginal Ring of Strutted Processes, Distance Apart	Areola Patterns	Process Patterns
T. mala	2 - 10	25 - 30	1 - 1.5µm	center coarsely silicified	single strutted process on valve offset from center
T. proschkinae	2 - 10.5	25 - 30	1.5 - 1.9µm	radial	1 areola between labiate and single central strutted process on valve face
T. partheneia	4.4 - 13	38 - 50	2.4 - 3.8µm	radial, sectors	one central strutted process, longer internal tubes on strutted processes
T. mediterranea	6 - 20	ca. 30	2 - 4µm	areolae radial, usually poorly developed	stellate central ring of strutted processes
T. minuscula	10 - 24	32 - 37	3.0 - 3.8µm	sectors	labiate process slightly away from margin; one central strutted process and one adjacent to labiate process on valve face

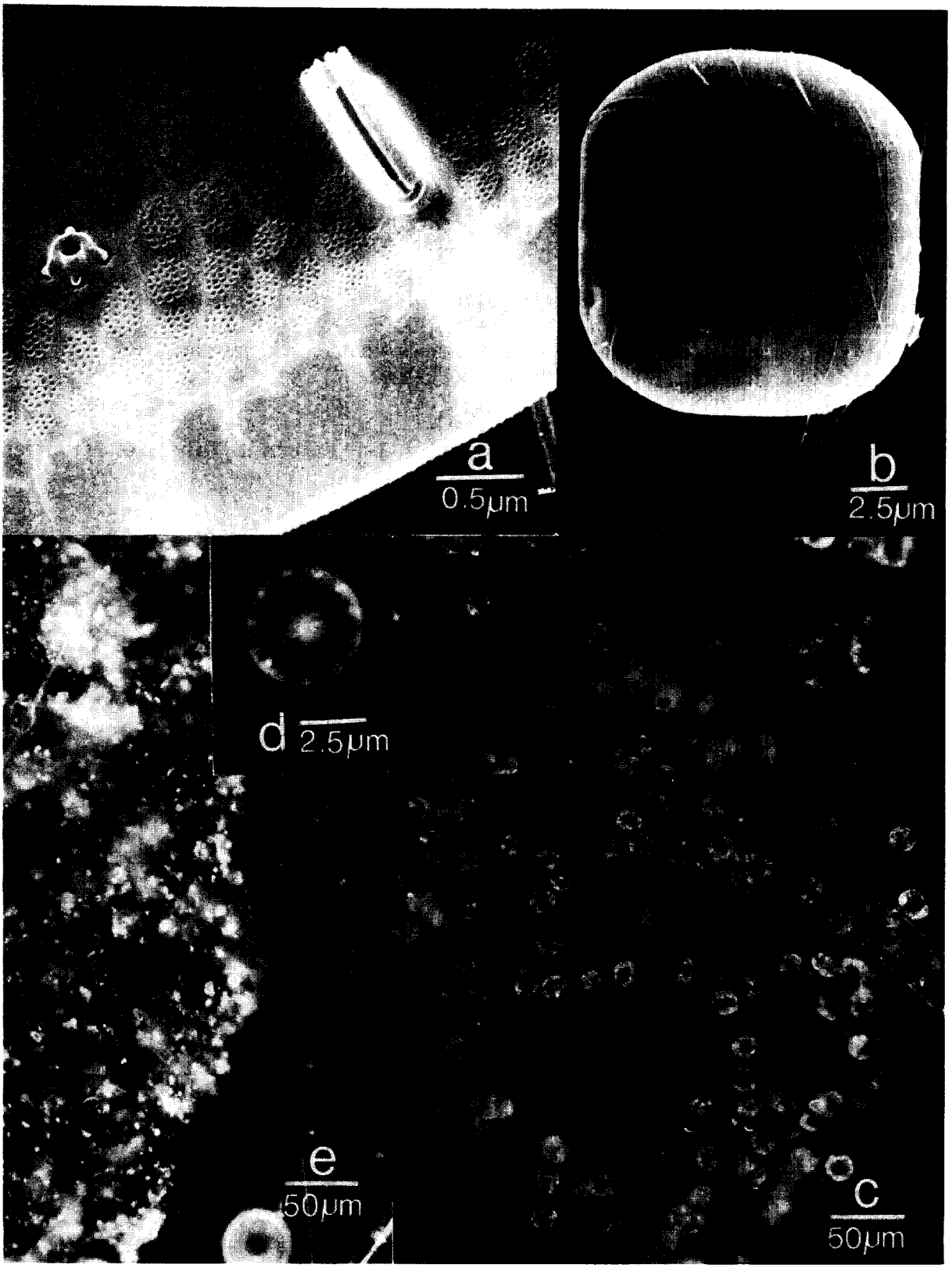

Figure 17.3. *Thalassiosira*
a) Valve inside, with strutted process to the left, labiate process to the right, cribra (SEM). b) and c) *T. diporocyclus*, b) Whole cell with threads from strutted processes (SEM), (ca.- 33°S, 18°E) South Atlantic Ocean. c) Colony (LM), NW Africa. d) and e) *T. mala* (LM), d) Valve with marginal processes, labiate process coarser (whiter than the strutted, central process to the left of coarser silicified central part), Indian Ocean. e) Part of colony and some free cells to the right, Trinidad, January 1968.

Under certain circumstances, most likely more species than those mentioned here may form gelatinous colonies, as suggested in 1926 by Hustedt, who referred to findings of *T. nordenskioeldii* in this type of colony.

Northernmost and Southernmost Records

Somewhat larger species with dome-shaped valves:
T. diporocyclus Hasle - ca. 40°N (Atlantic) - 40°S (Pacific)
T. tubifera G. Fryxell - ca. 40°N (Atlantic) - 05°S (Pacific)
T. subtilis (Ostenfeld) Gran - ca. 59-58°N (Atlantic) - 47°S (Chile)
T. fragilis G. Fryxell - Gulf Stream warm core rings

Species with flattened valves and external tubes of strutted processes:
T. curviseriata Takano - ca. 59-58°N (Atlantic) - ca. 35°S (Australia)
T. weissflogii (Grunow) G. Fryxell and Hasle - cosmopolitan (brackish water)
T. delicatula Ostenfeld - ca. 59-58°N (Atlantic) - 47°S (Chile)
T. gravida Cleve - cosmopolitan

Smaller species with rounded valves and no external tubes of strutted processes:
T. mala Takano - ca. 59-58°N (Atlantic) - ca. 35°S (Africa, Australia)
T. proschkinae Makarova - ca. 59-58°N (Atlantic), Japan, West Africa
T. partheneia Schrader - ca. 40°N (Atlantic) - 40°S (Pacific)
T. mediterranea (Schröder) Hasle - ca. 59-58°N (Atlantic) - 47°S (Chile)
T. minuscula Krasske - ca. 59-58°N (Atlantic) - 47°S (Chile)

Information on distribution is from Hasle (1972a; 1976; 1983), Rivera (1981), Takano (1981), Fryxell *et al.* (1984), Hallegraeff (1984), and Lange *et al.* (1992).

Coscinodiscus Ehrenberg 1839 - Lectotype: *C. argus* Ehrenberg

The *Coscinodiscus* species generally occur as single cells, and they are larger and more coarsely structured than *Thalassiosira* spp. They have no strutted processes or external tubes but at least one marginal ring of **labiate processes** (Figs 17.4b, c) The genus is marine and planktonic.
Generic characters:

- •Cells discoid to cylindrical
- •No central process
- •Many labiate processes
- •Two larger marginal labiate processes
- •Radial rows of areolae
- •Areola cribrum ("sieve-plate") on valve wall outside

Characters showing differences between species:

- •Cell size
- •Valve shape
- •Height of mantle, measured as number of areolae between marginal ring of processes and mantle edge
- •Number of areolae in 10µm
- •Areola pattern on valve face, e.g. fasciculation, spiralling rows
- •Interstriae (unperforated radial areas bordering fascicles, or bundles of areola rows)
- •Structure of valve centre
- •Process pattern

Table 17.4.

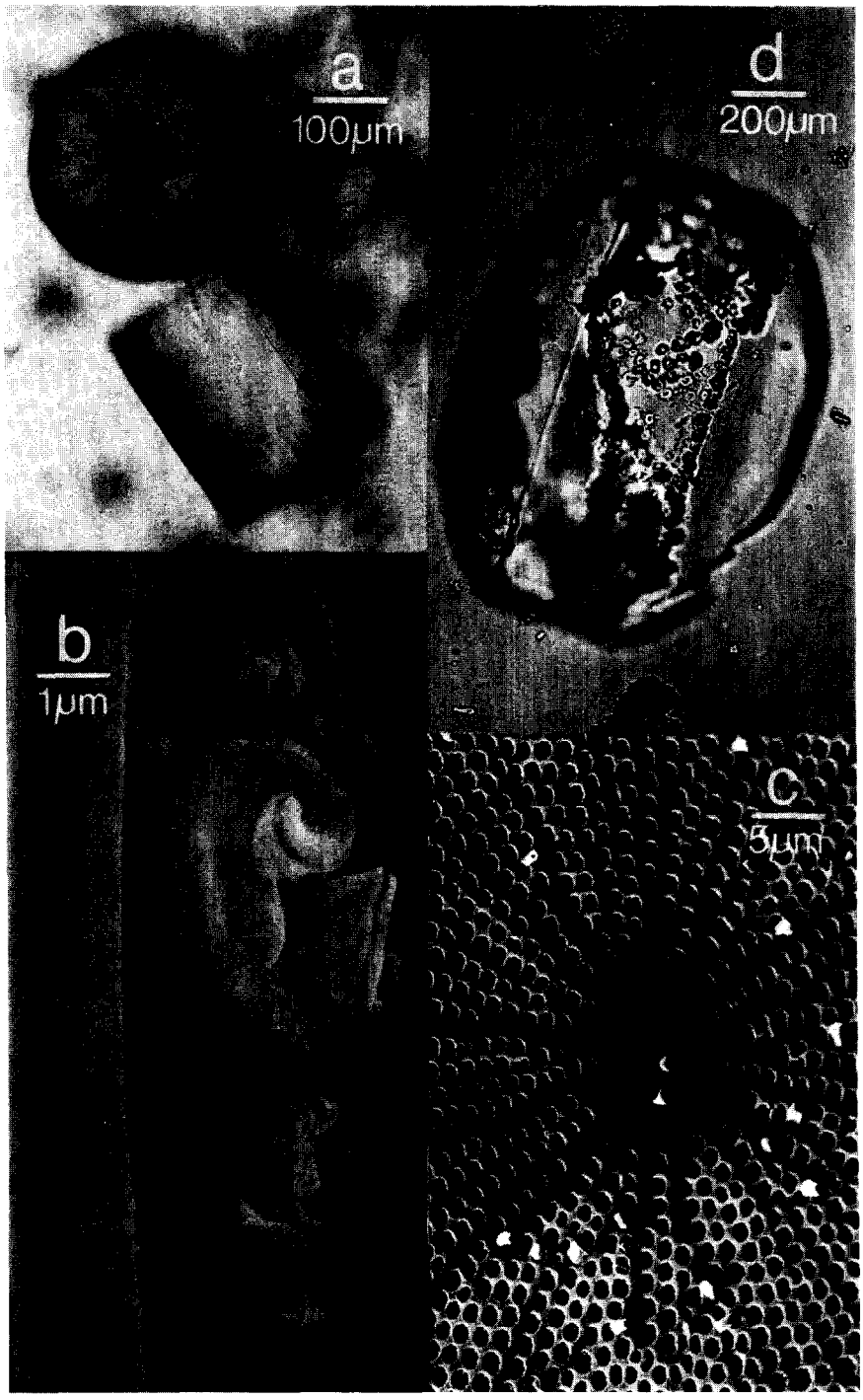

Figure 17.4. *Coscinodiscus*
a) - c) *C. wailesii*, a) Whole cells in water mount in valve view (size compared to the dinoflagellate *Ceratium furca*), and girdle view (LM), Skagerrak, NE Atlantic Ocean. b) Larger and smaller labiate processes (SEM), Departure Bay, British Columbia, NE Pacific Ocean, Aug. 1928. c) Valve centre, labiate processes on valve face (SEM), culture from NE Atlantic Ocean, harvested 18 Aug. 1982. d) *C. concinnus*, whole cell in water mount (LM), Skagerrak.

Table 17.4. *Coscinodiscus* species forming harmful masses.

Species	Diameter (µm)	Valve Shape	Areolae in 10µm	Areola Patterns	Central Area of Valve	Labiate Process Ring(s) near Margin	Labiate Processes on Valve Face
C. wailesii	280 - 500	flat valve face, mantle at right angle	5 - 6, smaller on valve face	radial	hyaline, irregular outline with extensions	2, one at juncture of valve face and mantle; one 2 - 3 areolae from margin	present
C. concinnus	110 - 500	convex, slightly flattened in centre	7 - 9, smaller on mantle	narrow fascicles, wavy appearance	star-shaped, hyaline centre; rosette of larger areolae	1, 3 - 9 areolae from margin	none
C. centralis	100 - 300	gently convex	4 - 6, smaller on mantle	radial, fasculated, or spiralling	rosette of larger areolae	1, 3 - 4 areolae from margin	none

Comments: *Coscinodiscus wailesii* has a high, steep mantle with the two larger processes in the ring close to the valve edge (Figs 17.4a, b). The species may thus be identified by fragments of the mantle, and with some experience, also by the ca. 50µm wide valvocopula having quincunx structure visible in the light microscope. *Coscinodiscus concinnus* (Fig. 17.4d) has more (but narrower) bands, and the valve has conspicuous interstriae running from the processes at a considerable distance from the valve edge towards the valve centre. *Coscinodiscus centralis* has a shorter pervalvar axis but the same number of bands as *C. wailesii*, since each of the bands is narrower. The main distinctive character of *C. centralis* is the central rosette of larger areolae.

Distribution

C. wailesii Gran and Angst - cosmopolitan, exclusive of Arctic and Antarctic, increase in abundance in North Atlantic waters since 1970's
C. concinnus W. Smith - cosmopolitan, exclusive of Antarctic
C. centralis Ehrenberg - cosmopolitan

Information on distribution is from Boalch (1984), Rincé and Paulmier (1986), Hasle and Lange (1990, table 3), Lange *et al.* (1992).

Chaetoceros Ehrenberg 1844 - Lectotype: *C. tetrachaeta* Ehrenberg

Chaetoceros is one of the larger planktonic marine genera (approximately 175 species, Rines and Hargraves, 1988) with a world-wide distribution. The genus is recognized by its **setae** (or hollow outgrowths of valve projecting outside the valve margin, with structure different from that of the valve). For terminology see Figs 17.5, 17.6.

Generic characters:
- •Chain formation by setae
- •Two setae per valve
- •Cells more or less rectangular in girdle view and elliptical to (rarely) circular in valve view.

Characters showing differences between species:
- •Number of chloroplasts
- •Terminal setae (setae of end valve of a chain) different or similar to the others in direction and structure
- •One seta of a cell longer than the three others
- •Shape and size of the aperture (or "window", the opening between adjacent cells)
- •Height of girdle compared to cell height (pervalvar axis)
- •Direction of chain (e.g. straight, curved, spiralled)

The genus is divided into two sub-genera *Phaeoceros* and *Hyalochaete*. The species most commonly reported as harmful to fish belong to *Phaeoceros*.

Phaeoceros Gran
- •Numerous small chloroplasts throughout the whole cell, the setae included
- •Large robust species
- •Setae strong, thick, striated, armed with conspicuous spines
- •Mostly oceanic species

Comments: Since the coarse, spiny setae of *C. convolutus* and *C. concavicornis* are regarded as the injurious parts of the cell, the whole sub-genus may be harmful to fish in net-pens. In waters of Washington State, Pacific coast of Canada, and South Chile, the harmful events

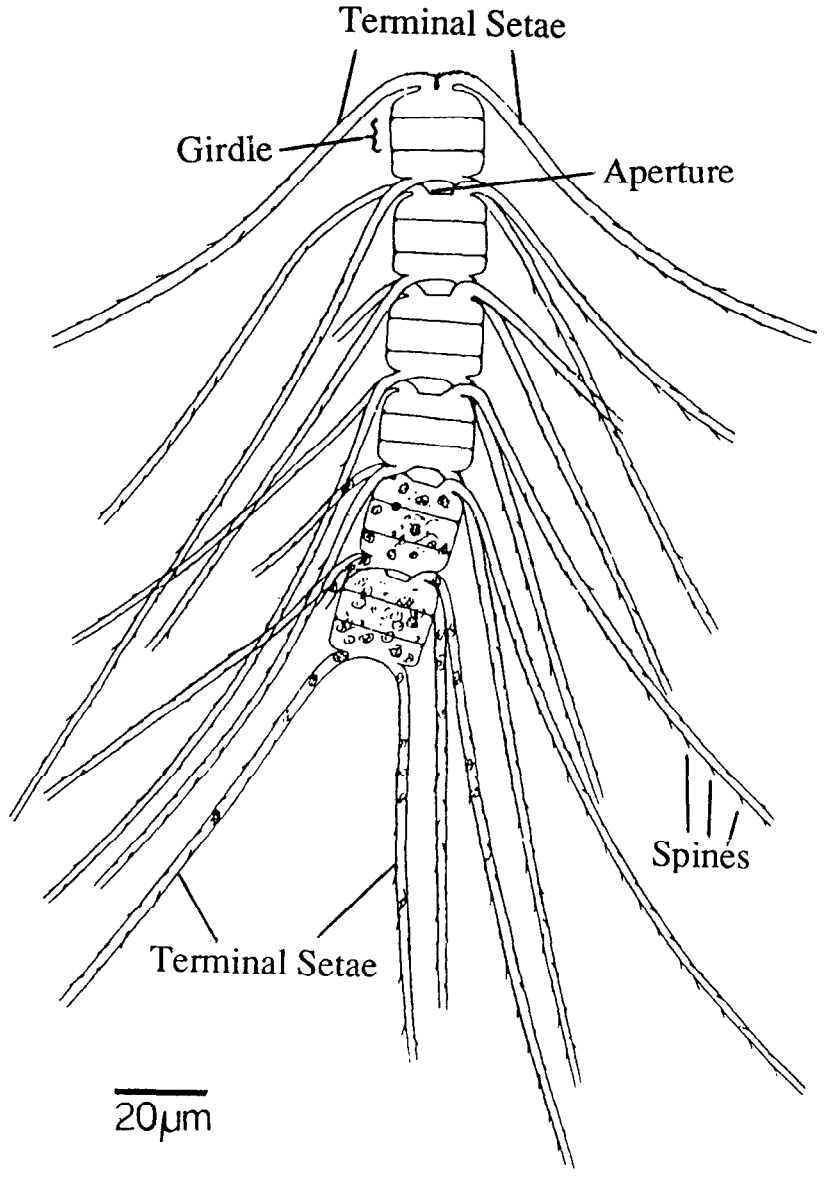

Figure 17.5. *Chaetoceros convolutus* with terms (from Hustedt, 1930).

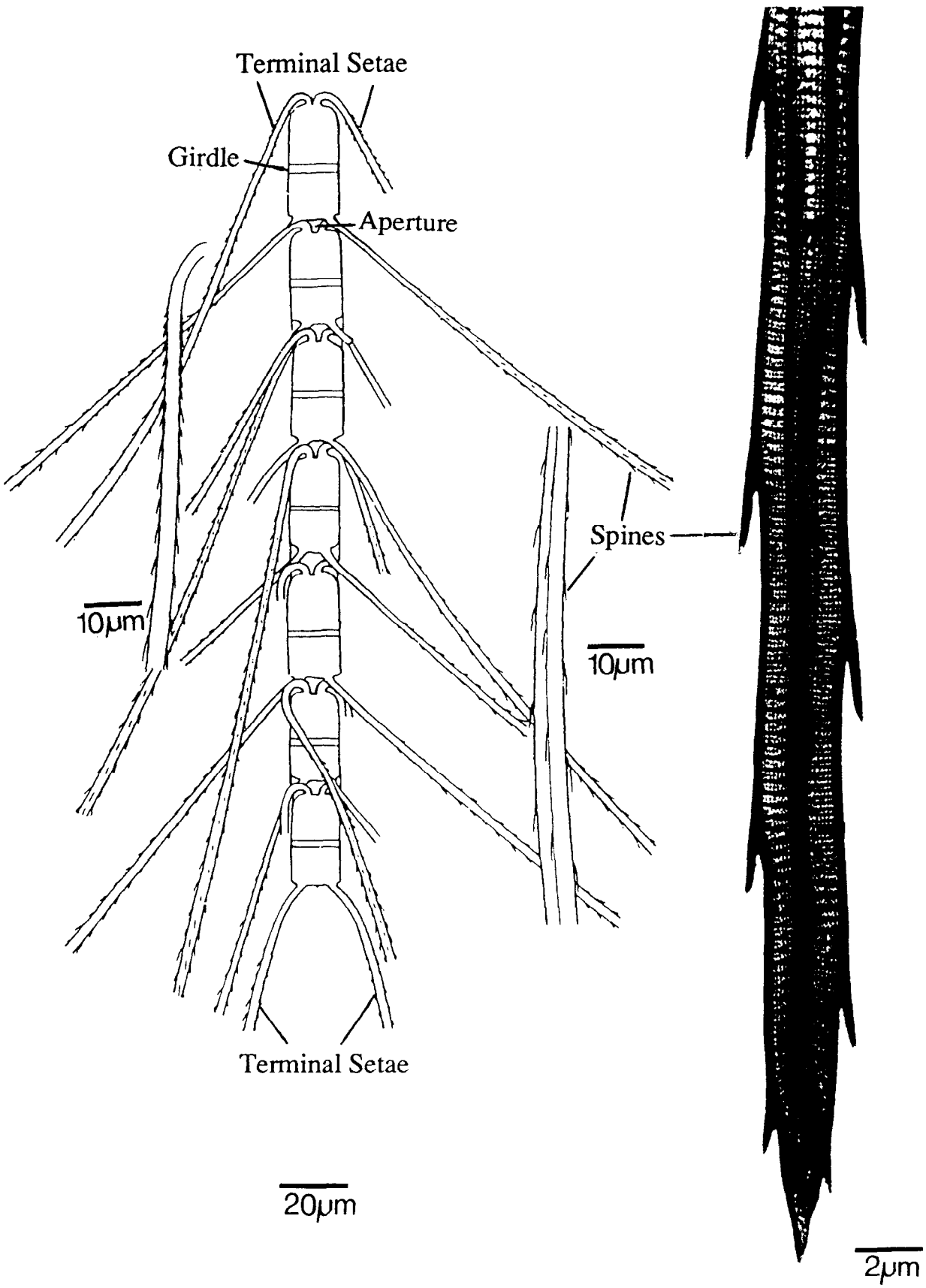

Figure 17.6. *Chaetoceros concavicornis* with terms (from Hustedt, 1930). Seta (SEM), D. Swift culture #189, from Gulf of Maine, NW Atlantic Ocean.

caused by just these two species (and not by other species of the sub-genus) may be due to the coincidence of geographical distribution of *Phaeoceros* species and fish farming.

Chaetoceros concavicornis and *C. convolutus* are differentiated by (i) height of the entire girdle and (ii) the thickness of the setae: the girdle of *C. concavicornis* being less than one-third of cell height and the girdle of *C. convolutus* being about the same height as each valve; and the setae of *C. concavicornis* increasing in width and in *C. convolutus* having the same width from the base and outwards. (Compare Figs 17.5, 17.6).

Distribution

Cells solitary or in short chains:
C. danicus Cleve - cosmopolitan
C. peruvianus Brightwell - warm water region to temperate
C. aequatorialis Cleve - warm water region
C. rostratus Lauder - warm water region
C. criophilus Castracane - southern cold water region

Terminal setae differentiated from the others by length and direction:
C. atlanticus Cleve - cosmopolitan
C. dichaeta Ehrenberg - southern cold water region

Terminal setae not distinctly differentiated from the others, setae often diverging in all directions:
C. borealis Bailey - cosmopolitan
C. concavicornis Mangin - cosmopolitan
C. convolutus Castracane - cosmopolitan
C. densus (Cleve) Cleve - cosmopolitan
C. eibenii Grunow - warm water region to temperate
C. coarctatus Lauder - warm water region
C. dadayi Pavillard - warm water region
C. tetrastichon Cleve - warm water region
C. castracanei Karsten - southern cold water region

The species here listed as cosmopolitan have probably their greatest abundances in temperate and/or cold waters.

Hyalochaete Gran

- •One or a few plate-like chloroplasts
- •Setae thin, often hair-like, without chloroplasts
- •Spines mostly too small to be seen with LM
- •Greatest distribution in coastal waters

Comments: This sub-genus has the greater number of species of the two. *Chaetoceros debilis*, the extract of which caused bradycardia (or slow heartbeat rate) on smolts of *Salmo salar* L. (Wildish *et al.*, 1991), and *C. socialis* belong to this sub-genus. The harmful effect of *C. socialis* could just as well be caused by its presence in mucilage colonies as by the setae. *Chaetoceros debilis* is recognized by its curved and spirally twisted chains and almost rectangular apertures (as distinguished from *C. curvisetus* Cleve with elliptical to almost circular apertures). Intact spherical colonies of *C. socialis* may not be found in preserved material. The species is then recognized by three short setae of two adjacent valves and a fourth straight, elongated one. The latter entwines with the elongated setae of other chains to form a spherical colony.

Distribution

C. debilis Cleve - cosmopolitan, abundant in cooler waters
C. socialis Lauder - cosmopolitan

Information on distribution of *Chaetoceros* is summarized from Hustedt (1930), Hendey (1937; 1964), Cupp (1943), and Rines and Hargraves (1988).

Pennate diatoms - Bacillariales

Pseudo-nitzschia H. Peragallo in H. & M. Peragallo 1900 - Lectotype: *P. seriata* (Cleve) H. Peragallo in H. & M. Peragallo
Pseudo-nitzschia was established for *Nitzschia seriata* Cleve, *N. fraudulenta* Cleve and *N. sicula* Castracane, but was reduced to a section of *Nitzschia* by Hustedt (1958). Approximately 20 species share the most prominent ecological and morphological features of the type, *P. seriata*, and form a well-defined group demarcated from other genera of the family Bacillariaceae. The genus is marine and planktonic. For terminology see Fig. 17.7.

Generic characters:
- •Stepped chains formed by overlap of valve ends
- •Cells strongly elongate, spindle-shaped or rectangular in girdle view, narrowly lanceolate to spindle-shaped or linear with rounded or pointed ends in valve view
- •Valves shallow, flattened, weakly silicified
- •Raphe extremely eccentric, along one margin, not elevated above the general valve level
- •Chloroplasts two plates along the girdle

Characters showing differences between species:
- •Valve outline (margins curved/straight, one curved and the other straight)
- •Shape of valve ends in valve and girdle views
- •Size of central interspace or presence/absence of central nodule (or pseudonodulus in older literature)
- •Number of fibulae (or keel punctae in older literature) compared to number of interstriae (formerly transapical costae)
- •Cell length (apical axis)
- •Cell width (valve width, or transapical axis)
- •Cell height (or pervalvar axis)

Table 17.5

Distinctive characters

P. delicatissima: - Cell ends rounded in valve view, cut-off in girdle view, short overlap (ca. one-ninth of cell length), with LM (acid-cleaned, mounted valves) fibulae visible, but not interstriae (Figs 17.8e, f).

P. pseudodelicatissima: - Cell ends pointed in valve and girdle views, fibulae and central larger interspace distinct, interstriae occasionally, with acid-cleaned, mounted valves, visible with LM (Figs 17.8d-c).

P. seriata: Overlap of cell ends *ca.* one-fourth to one-third of cell length, valve interstriae visible with LM (uncleaned cells in water mounts), raphe along either straight or curved margin, fibulae visible with LM as continuations of interstriae (on cleaned, mounted valves), difference in curvature of the two margins most expressed in middle valve (Fig. 17.9a).

P. pungens - Overlap of cell ends *ca.* one-third of cell length, coarsely silicified, interstriae visible in water mounts, fibulae visible as continuation of interstriae (acid-cleaned, mounted valves), perforation of striae mostly revealed under optimal optical conditions,

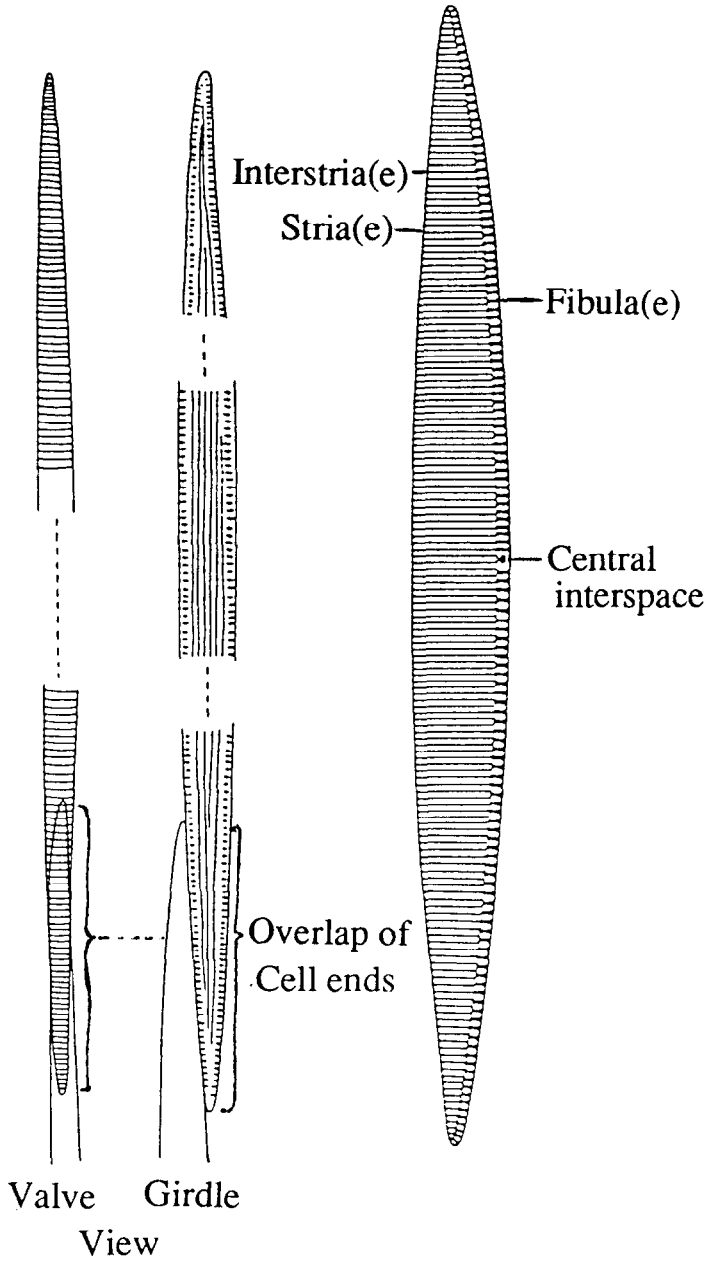

Figure 17.7. *Pseudo-nitzschia*, with terms.

Table 17.5. *Pseudo-nitzschia* species that have been involved in domoic acid studies.

Species	Apical Axis	Transapical Axis	Striae in 10μm	Fibulae in 10μm	Central Larger Interspace	Valve Margins (Plan View)
P. delicatissima	40 - 76	1.0 - 2.0	36 - 40	20 - 23	present	gently curved until some distance from poles
P. pseudodelicatissima	59 - 140	1.3 - 2.5	30 - 46	16 - 26	present	straight (linear) until some distance from poles
P. seriata f. *obtusa*	61 - 100	4.5 - 5.5	15 - 20	15 - 20	lacking	one almost straight, one slightly curved;. rounded poles
P. seriata	91 - 160	5.5 - 8.0	15 - 20	15 - 20	lacking	one curved, one almost straight
P. pungens	74 - 142	2.9 - 4.5	9 - 15	9 - 15	lacking	linear or gently curved until pointed poles
P. multiseries	68 - 140	3.4 - 5.0	10 - 15	10 - 15	lacking	curved or almost straight; poles pointed
P. australis	75 - 144	6.5 - 8.0	12 - 18	12 - 18	lacking	curved to straight; poles slightly rostrate

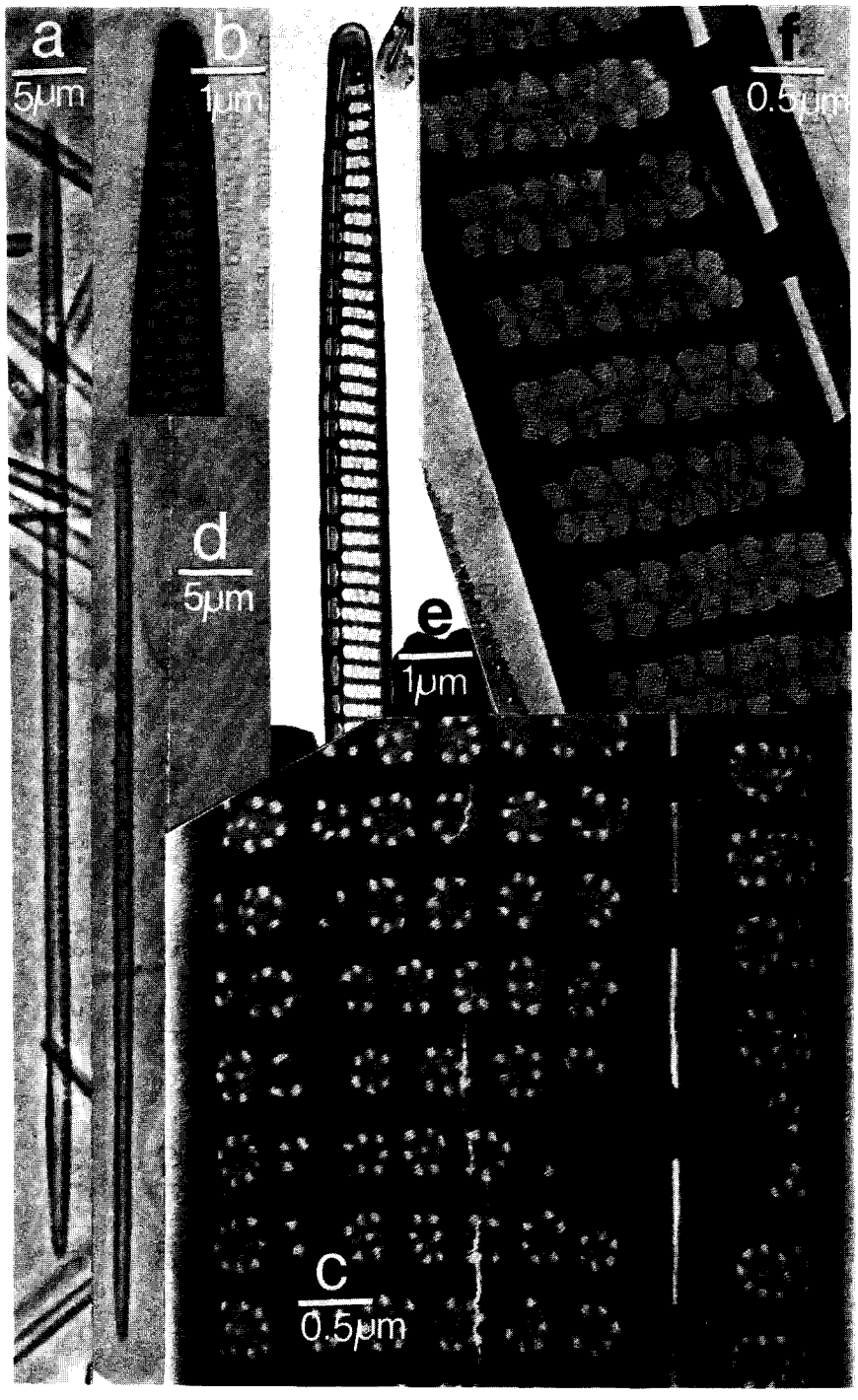

Figure 17.8. *Pseudo-nitzschia delicatissima* and *P. pseudodelicatissima*
a) - c) *P. pseudodelicatissima* (can be toxic). a) Single valve, fibulae and central larger interspace visible (LM; Naphrax mount), Outer Oslofjord, Norway, Sept. 1991. b) Pointed valve and striae with one row of areolae (TEM), Skagerrak, Oct. 1991. c) Part of valve face, raphe with central larger interspace, central nodule, and valve mantle areolae structure (TEM), Skagerrak, Oct. 1991.
d) - f) *P. delicatissima*. (can be toxic). d) Single valve, fibulae just discernible (LM, Naphrax mount), Skagerrak, May 1953. e) Rounded cell end, striae with two rows of areolae (TEM), Skagerrak, May 1953 . f) Part of valve face and raphe, areolae structure (TEM), Skagerrak, May 1953.

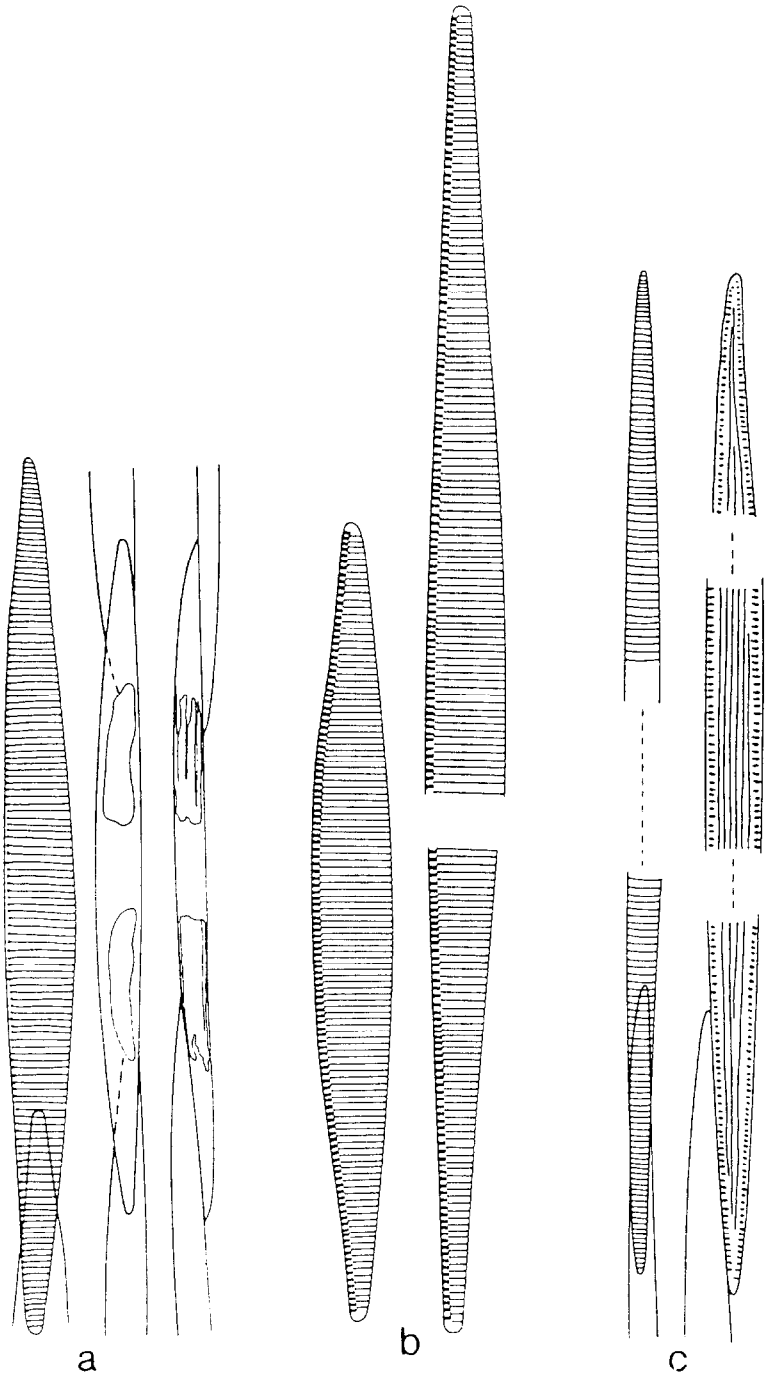

Figure 17.9 a) *Pseudo-nitzschia seriata* (can be toxic). b) *P. australis* (can be toxic). c) *P. pungens* (not known to be toxic) or *P. multiseries* (can be toxic), since they would be indistinguishable at this level of detail (from Hasle, 1972b).

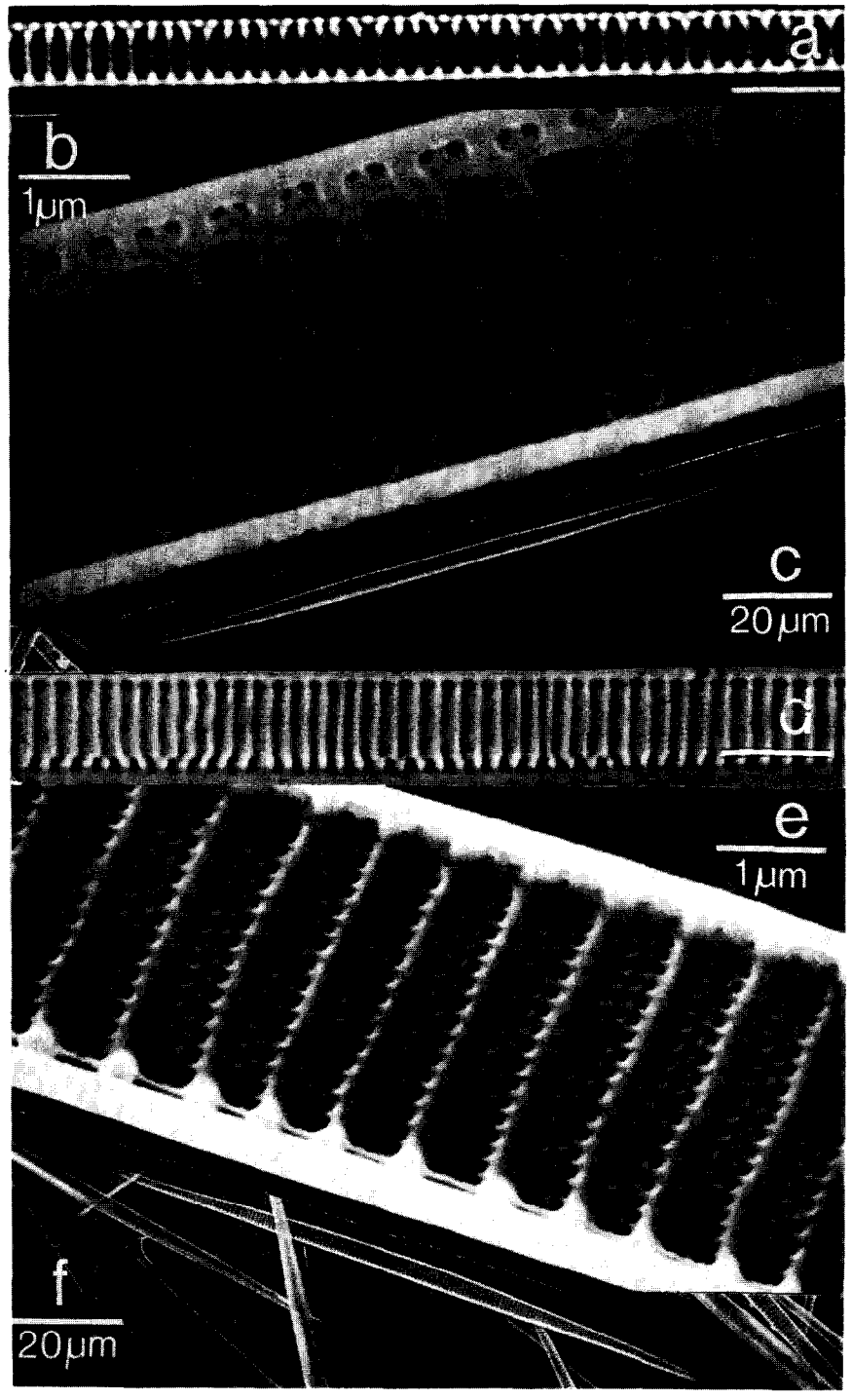

Figure 17.10. Two similar species of *Pseudo-nitzschia.*
a) - c) *P. pungens* (not known to be toxic). a) Areolate striae visible (LM), Skagerrak, North Atlantic, 12 Sept. 1990. b) Two rows of striae between interstriae (SEM), Gulf of Mexico culture F242. c) Orientation view of valve in Figure b (SEM).
d) - f). *P. multiseries* (can be toxic). d) Areolate striae not visible (LM), Skagerrak, North Atlantic, 7 Dec. 1989. e) Three (or more) rows of areolae between interstriae (SEM), Gulf of Mexico culture MD-2. f) Orientation view of valve detail in Fig. e. Scales in Figs a, d = 5 µm.

such as acid-cleaned valves in a medium of refractive index 1.6-1.7, phase contrast, objective oiled, x 100, n.a. 1.32 (Figs 17.9c, 17.10a-c).

P. multiseries: - Interstriae visible in water mounts (uncleaned cells), fibulae more distinct than interstriae, perforation of striae unresolvable with LM, irregularities often noted (Figs 17.9c, 17.10d-f).

P. australis - Valve middle part (ca. one-third of cell length) with straight to slightly curved margins, valve ends slightly rostrate with rounded poles (Fig. 17.9b).

Comments: *Pseudo-nitzschia multiseries* and *P. australis* as the sources of ASP have been documented by observations in the field as well as by studies of cultures (Garrison *et al.*, 1992; Villac *et al.*, 1993). *Pseudo-nitzschia pseudodelicatissima* was determined to be the source of domoic acid in shellfish in the southwestern Bay of Fundy (Martin *et al.*, 1993); cultures established from the area did produce domoic acid, although at very low concentrations (Martin *et al.*, 1990). Smith *et al.* (1991) found that *P. delicatissima* (syn. *Nitzschia actydrophila)* appeared to produce domoic acid under culture conditions, and Lundholm *et al.* (1994) found the same for *P. seriata. Pseudo-nitzschia pungens* and *P. multiseries* (syn. *P. pungens* f. *multiseries)* are now considered separate species because of morphological (Hasle 1965, 1995) and genetic differences (Manhart *et al.*, 1995).

Distribution

P. delicatissima (Cleve) Heiden in Heiden and Kolbe - Atlantic: Norwegian coastal waters, Danish waters, Skagerrak, Northwest Africa, Rhode Island; Pacific: California.

P. pseudodelicatissima (Hasle) Hasle- Atlantic waters of Europe and Africa (Denmark Strait to northwest Africa, including Norwegian and Danish coastal waters, Skagerrak, Kiel Bay), Canada and USA (Arctic to Gulf of Mexico); Pacific: California and British Columbia waters.

P. seriata Cleve H. Peragallo f. *seriata* - Barents Sea (ca. 80°N), Norwegian Sea, North Sea, Norwegian coastal waters, Skagerrak, Kiel Bay, English Channel, Greenland to New Foundland (45°N), Alaska, British Columbia.

P. multiseries (Hasle) Hasle - Atlantic: waters of North America, Europe, and South America; Pacific: waters of North America and Northeast Asia

P. australis Frenguelli - Atlantic: coastal waters of Spain, Portugal, Southwest Africa and Argebtina; Pacific: coastal waters of Chile, Peru, New Zealand, west coast of USA from San Diego, California to Puget Sound, Washington, British Columbia.

The diatoms not found associated with DA and ASP but included here for comparison, have known distributions as follows:

P. seriata f. *obtusa* (Hasle) Hasle - Norwegian coastal waters from 63°N and northwards, Hudson Strait, the Sound between Sweden and Denmark - all in the cold season.

P. pungens (Grunow *ex* Cleve) Hasle - Cosmopolitan.

Information on distribution is from Hasle (1965; 1972b; 1976); Martin *et al.* (1990); Lange *et al.* (1994); Lundholm *et al.* (1993); Taylor (1993); Villac *et al.* (1993), Lange *et al.* (1994), and Villereal *et al.* (1994), Fraga and Koeman (personal communication) and personal observations (GRH).

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18. Taxonomy of Harmful Marine Raphidophytes

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Small golden-brown (fucoxanthin-containing) flagellates within the class Raphidophyceae Chadefaud ex Silva (= class Chloromonadophyceae Papenfuss) of the Division Chrysophyta can pose a serious threat to finfish aquaculture. Throndsen (1993) created the order Chattonellales for the four marine genera *Chattonella* Biecheler, *Heterosigma* Hada, *Fibrocapsa* Toriumi et Takano and *Olisthodiscus* N.Carter and tentatively also included *Oltmannsia* Schiller. Freshwater species (not discussed here) are coloured green with pigment affinities to the Xanthophyceae (without fucoxanthin; Fiksdahl *et al.*, 1984) and were classified in the order Raphidomonadales Chadefaud, family Vacuolariaceae Luther. In the 1970s and 1980s much attention focused on species of the genus *Chattonella* as the cause of mortality of cultured yellowtail and red sea bream in Japanese inland seas (Okaichi, 1983) but in the 1990s attention has shifted to *Heterosigma* as the cause of fish kills in New Zealand, Chile and British Columbia (Chang *et al.*, 1990). Considerable confusion exists regarding the taxonomy of the group and various members have often been mistakenly assigned to other algal classes such as cryptomonads, chrysophytes, dinoflagellates and euglenoids.

Basic features of the cell structure include two subequal, heterodynamic flagella arising from a more or less pronounced flagellar groove (*Olisthodiscus, Heterosigma*): the forward flagellum bears two rows of fine tripartite hairs, while the trailing flagellum is smooth and lies close to the surface of the cell. The flagellar bases have a unique system of cross-banded, fibrous roots (Vesk and Moestrup, 1987; Inouye *et al.*, 1992). The cells are naked, dorsiventrally flattened and contain numerous ejectosomes (*Heterosigma)*, trichocysts and mucocysts (*Chattonella, Fibrocapsa)* that readily discharge, thereby rendering these species difficult subjects for microscopic studies (Vesk and Dwarte, 1980; Vesk and Puttock, 1980). The cells contain numerous golden-brown chloroplasts in the peripheral cytoplasm (termed ectoplasm), which upon cell disintegration gives a characteristic "raspberry-like" appearance to the cell remains. Ultrastructural studies are now available for *Heterosigma carterae* (Leadbeater,1969 [as *Olisthodiscus luteus*], Hara and Chihara, 1985 [as *Heterosigma akashiwo*]), *Olisthodiscus luteus* (Hara *et al.*, 1985), *Fibrocapsa japonica* (Hara and Chihara, 1985), *Chattonella subsalsa* (Mignot, 1976), *C. marina* and *C.antiqua* (Hara and Chihara, 1982), and four newly described species *Chattonella globosa*, *C.minima, C.ovata* and *C.verruculosa* (Hara *et al.*, 1994).

The killing mechanism of raphidophyte blooms is still poorly understood. Both physical clogging of gills by mucus excretion as well as gill damage by haemolytic substances such as polyunsaturated fatty acids, may be involved (Shimada *et al.*, 1983, Chang *et al.*, 1990). Increasing evidence is now pointing towards the production of superoxide radicals as the major mechanism of fish mortality by both *Chattonella* (Tanaka *et al.*, 1994) and *Heterosigma* (Yang *et al.*, 1995). Onoue *et al.* (1989) also reported HPLC evidence for brevetoxin production by *Chattonella*, but further mass spectroscopic confirmation is still required.

Heterosigma carterae (Hulburt) Taylor 1992 Fig. 18.1

Basionym: *Olisthodiscus carterae* Hulburt 1965
Synonyms: *Entomosigma akashiwo* Hada 1967, *Heterosigma akashiwo* (Hada) Hada 1968, ? *Heterosigma inlandica* Hada 1968, "*Olisthodiscus luteus* " Plymouth cultures 12A and 239, *Chattonella akashiwo* (Hada) Loeblich III.
Illustrations: Hara and Chihara 1982 (LM, TEM); Hara in Fukuyo *et al.*, 1990, p.346-347 (LM, SEM, TEM).

Cell shape and size: The potato-shaped cells (8-25 x 6-15 µm) are slightly compressed dorso-ventrally and vary in shape from spheroidal to ovoid or oblong according to culture conditions and cell age.
Flagella: Two subequal, heterodynamic flagella are present, an anterior pulling flagellum and a posterior rigid flagellum, both arise from an oblique groove in the ventral side of the cell which starts subapically and terminates half-way the length of the cell.The cells exhibit a spiralling swimming pattern.
Chloroplasts: Many (10-30) yellow-brown to brown discoid chloroplasts are located in the periphery of the cell. A pyrenoid protrudes from the inner chloroplast surface towards the centre of the cell and is invaded by thylakoids.
Others: The centrally located nucleus is tear-drop shaped. Eyespots or contractile vacuoles are absent. Mucocysts are present, similar to those found in *Chattonella* and *Fibrocapsa.*
Life cycle: As part of its life cycle, this species produces benthic resting cells consisting of agglutinated masses of non-motile brown cells of variable size and shape (Tomas 1978). For a description of the cyst stage, see Chapter 20.Sexual reproduction is unknown.
Distribution: Coastal and brackish waters in the Pacific and Atlantic. A common red tide species in Japan ('akashiwo'=red tide), where it never has caused major fish kills. A problem organism for finfish aquaculture in British Columbia, Chile, New Zealand and possibly Singapore.
Note: This species has often been confused with *Olisthodicus luteus* (see Gibbs *et al.*, 1980, Leadbeater 1969, Tomas 1978), but can be distinguished by its cell shape, colour and swimming pattern. *H. carterae* is probably also conspecific with *H. inlandica* Hada 1968 (Throndsen 1993).

Olisthodiscus luteus N.Carter 1937 Fig. 18.2

Illustrations: Hara *et al.*, 1985 (LM, SEM, TEM); Hara in Fukuyo *et al.*, 1990, p. 348-349 (LM, SEM, TEM).

Cell size and shape: Strongly dorsiventrally compressed, 15-25 µm long, 10-16 µm wide, 5-7 µm thick.
Flagella: Anterior flagellum 1-1.2 times the cell length, posterior flagellum 0.8-1 times cell length, both arising from a longitudinal furrow on the mid-ventral side of the cell which is extended into a deeper pit. The cells don't rotate but exhibit a smooth forward swimming pattern.
Chloroplasts: Many (5-13) flattened, disc-shaped, pale green-yellow chloroplasts ('luteus'=yellow) are located in the cell periphery. The ventral side of the cell is devoid of chloroplasts.The pyrenoid is free from thylakoids, but is invaginated by tubular structures.
Others: Eyespots, contractile vacuoles, mucocysts and lipid bodies are absent.
Life cycle: Siliceous statospores with a simple circular opening are formed asexually. Sexual reproduction is unknown.
Distribution: A benthic species, gliding along bottom sediments in salt marshes of Europe, North America, South Africa and Japan.
Note: *Olisthodiscus carterae* belongs in *Heterosigma* (see Taylor 1992), while *O. magnus* Hulburt probably belongs in *Chattonella.* The toxic potential of this species is unknown.

Fibrocapsa japonica Toriumi et Takano 1973 Fig. 18.3

Synonym: *Chattonella japonica* (Toriumi et Takano) Loeblich III et Fine 1977, "*Exuviella* sp." (isolate FCRG 51)
Illustrations: Hara and Chihara 1985 (LM, TEM); Hara in Fukuyo *et al.*, 1990, p.344-345 (LM, TEM).

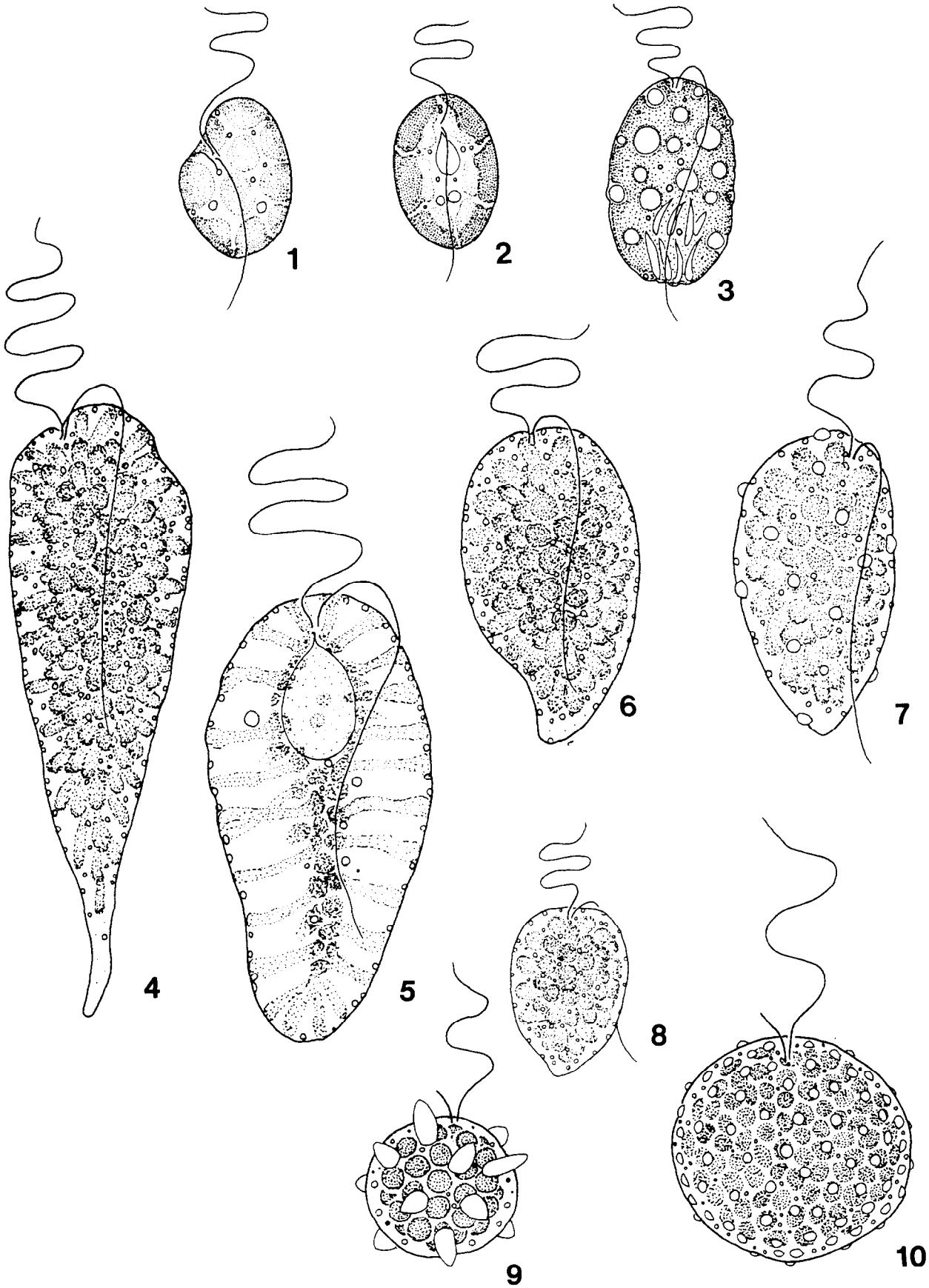

Figure 18.1. *Heterosigma carterae*; Figure 18.2. *Olisthodiscus luteus*; Figure 18.3. *Fibrocapsa japonica*; Figure 18.4. *Chattonella antiqua*; Figure 18.5. "*Chattonella ovata*" form; Figure 18.6. *Chattonella marina*; Figure 18.7. *Chattonella subsalsa*; Figure 18.8. "*Chattonella minima*" form; Figure 18.9. *Chattonella verruculosa*; Figure 18.10. *Chattonella globosa* form.

Cell size and shape: Slightly flattened, ovate to obovate in ventral view, 20-30 x 15-17 µm.
Flagella: The anterior flagellum is as long as the cell, and the posterior trailing flagellum is 1.2 times the cell length. Both emerge from an anterior gullet.
Chloroplasts: Many discoid, yellow-brown to golden-brown chloroplasts are densely packed in the cell, giving the appearance of a single reticulate chloroplast. A pyrenoid is present in each chloroplast.
Others: Rod-shaped mucocysts in the posterior end of the cell eject long threads (up to 300 µm). The nucleus is located in the centre of the cell. Eyespots and contractile vacuoles are absent. Spherical cyst stages can be found adhering to diatom frustules (see Chapter 20). Multinucleate benthic stages are also known, but sexual reproduction has not been reported.
Distribution: Coastal waters of Japan, Australia, New Zealand, California, North America and France. In the Seto Inland Sea harmful effects have been reported to mariculture.

Chattonella antiqua (Hada) Ono 1980 Fig. 18.4

Basionym: *Hemieutreptia antiqua* Hada 1974
Illustrations: Hara and Chihara 1982 (LM, TEM); Hara, in Fukuyo *et al.*, 1990, p.332-333 (LM, TEM).

Cell size: Large cells, 70-130 µm long, 30-50 µm wide, with a posterior tail.
Flagella: Two subequal, heterodynamic flagella emerge from the bottom of an anterior gullet.
Chloroplasts: Many ellipsoid chloroplasts are radially arranged. A naked pyrenoid is located at the inner pole of the chloroplast. Several less stacked thylakoids pass through the pyrenoid matrix.
Others: Numerous electron-dense particles are located in the cytoplasm, immediately below the cell surface. A tear-drop shaped nucleus is situated in the centre of the cell. Contractile vacuoles, eyespots or mucocysts are absent.
Life cycle: Asexual reproduction is by binary division. A diplontic life cycle has been described by Yamaguchi and Imai (1994), with cyst formation occurring after meiosis in vegetative cells. The hemispherical cysts have a simple pore on top and a circular wing on the bottom edge (see Chapter 20).
Distribution: A causative organism of massive fish kills, particularly well-studied in South-East Asia and Japan.
Note: The form newly described as *C.ovata* Hara et Chihara (*nomen nudum*, in Fukuyo *et al.*, 1990, p.340-341; validated by Hara *et al.*, 1994, Figs 19-22, 24-25; present Fig. 18.5) may be an ecotype of *C. antiqua*.

Chattonella marina (Subrahmanyan) Y.Hara et Chihara 1982 Fig. 18.6

Basionym: *Hornellia marina* Subrahmanyan 1954
Illustrations: Hara and Chihara 1982 (LM, TEM); Hara, in Fukuyo *et al.*, 1990, p.336-337 (LM, TEM).

Cell size and shape: The cell, 30-70 µm long x 20-30 µm wide, is asymmetrical in lateral view, slightly flattened, oblong to obovoid in shape, with a posterior tail.
Flagella: The two subequal flagella are approximately equal to the length of the cell and emerge from the bottom of an anterior depression in the cell.
Chloroplasts: Many, green to yellowish-brown, ellipsoid chloroplasts, are arranged radially. A naked pyrenoid is located on the inner pole of the chloroplast.
Others: The tear-shaped nucleus is situated in the centre of the cell. Contractile vacuoles, eyespots and mucocysts are absent. Asexual reproduction is by binary division. A diplontic life cycle has been described by Yamaguchi and Imai (1994), with cyst formation occurring after

meiosis in vegetative cells. The hemispherical cysts have a simple pore on top and a circular wing on the bottom edge (see Chapter 20).
Distribution: Brackish coastal areas rich in organic material from India, Australia and Japan.
Note: This species is often regarded as synonymous with *Chattonella subsalsa* Biecheler 1936 (Fig. 18.7), but Hara and Chihara 1982 discuss reasons to keep them separate, *i.e.* no thylakoids penetrating the pyrenoids, and oboe-shaped mucocysts present in *C. subsalsa* but not *C. marina*.. The form described as *C. minima* Hara et Chihara (*nomen nudum*, in Fukuyo *et al.*, 1990, p.338-339, validated by Hara *et al.*, 1994, Figs 13-15, 17-18; present Fig. 18.8) may be an ecotype of *C. marina*. The species referred to as *Olisthodiscus magnus* Hulburt 1965 most likely is conspecific with *C. marina*, but its description is incomplete and a reexamination of material from the type locality in Woods Hole ponds is required before a conclusion can be drawn on its precise identity.

Chattonella subsalsa Biecheler 1936 Fig. 18.7

Illustrations: Mignot 1976 (TEM)

Cell size and shape: The cell, 30-50 µm long x 15-25 µm wide, is slightly flattened, and lanceolate in lateral view.
Flagella: The two subequal, heterodynamic flagella emerge from the bottom of an anterior depression in the cell.
Chloroplasts: Many, green to brown, ellipsoid chloroplasts, are arranged radially within the vacuolated ectoplasm. A pyrenoid is located on the inner pole of the chloroplast, and is difficult to recognise by light microscopy.No thylakoids enter into the pyrenoid matrix.
Others: The tear-shaped nucleus is situated in the centre of the cell within the cytoplasmic endoplasm. Contractile vacuoles and eyespots are absent. Many mucocysts with "oboe" shaped inclusions are distributed around the cell periphery. Cyst formation and sexual reproduction are unknown.
Distribution: Eutrophic coastal areas of the Mediterranean from France to Algeria.
Note: This species appears closely related to *C. marina*, but can be distinguished by its colour and protrusion of heads of mucocysts on the cell surface. Considering that this is the type species of *Chattonella*, a reexamination of Mediterranean material of *C. subsalsa* is needed in order to reach a conclusion on the distinction between the species *C. subsalsa* and *C. marina*, but also to reexamine the validity of the delineation of the closely related genera *Heterosigma* and *Chattonella*..

Chattonella globosa Y.Hara et Chihara, in Hara *et al.*, 1994 Fig. 18.8

Nomen nudum, in Fukuyo *et al.*, 1990, p.334-335; validated by Hara *et al.*, 1994, Figs 1-12 (LM, TEM); Hosaka *et al.*, 1991 (LM); also known as "flagellate X" (Scotland, Ireland).

Cell size: nearly globose, 40-55 µm diameter.
Flagella: Two unequal flagella emerge from the shallow depression at the cell anterior.
Chloroplasts: Numerous pale-brown to golden-brown, small elliptical chloroplasts without pyrenoid are located throughout the cytoplasm.
Others: A spherical nucleus is located in the centre of the cell. Several large mucocysts with nail-shaped inclusions are distributed along the cell periphery. No contractile vacuoles nor eyespot are present. Asexual reproduction takes place by binary fission while swimming. Cyst formation and sexual reproduction are unknown.
Distribution: Eutrophic coastal areas of Japan, southeast Asia and Canada.This species is often confused with *C. antiqua*, but can be distinguished by characteristics of chloroplasts, flagellation and mucocysts. This species is known to cause respiratory damage to fish, similar to that caused by other *Chattonella* species.

Chattonella verruculosa Y.Hara et Chihara, in Hara *et al.*, 1994 Fig. 18.9

Nomen nudum, in Fukuyo *et al.*, 1990, p.342-343 (LM, TEM); validated by Hara *et al.*, 1994, Figs 26-33.

Cell size: globose, 12-45 µm long.
Flagella: Two unequal flagella emerge from the cell anterior.
Chloroplasts: Numerous pale-yellow to yellow-brown, small discoid chloroplasts with a single embedded pyrenoid.
Others: No electron-dense (osmiophilic) particles are present in the peripheral cytoplasm (as found in *C.antiqua* and *C.marina*). A spherical nucleus is located in the centre of the cell. Several large mucocysts with bullet-shaped inclusions are distributed along the cell periphery. Verrucose protrusions of their heads are visible by light microscopy.
Distribution: Only known thusfar from the Seto Inland Sea in Japan.The form described as *C. globosa* Hara et Chihara also has mucocysts with nail-shaped inclusions but verrucose appendages have not been reported.

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19. Taxonomy of Cyanobacteria

E.J. Carpenter and W.W. Carmichael

Marine and brackish water cyanobacteria can produce toxins which result in neuromuscular and organ distress as well as external contact irritation. These toxins can affect humans as well as terrestrial animals and marine life. At least nineteen species, belonging to nine genera, have been shown to be toxin producers among the brackish water and marine cyanobacteria. A review of the structure and characteristics of cyanotoxins has recently been published (Carmichael, 1992). Here we describe methods for collecting and identifying these cyanobacteria as well as a brief description of symptoms. For most of the species herein we have used the classification scheme and illustrations as presented by Geitler (1932). Note that this report is not intended as an absolute authority on the taxonomy or identification of toxic marine cyanobacteria. For precise identification we refer the reader to specialized publications. A key to toxic genera has recently been published (Skulberg *et al.*, 1993).

COLLECTION

Cyanobacteria can exhibit a patchy distribution in nature, and if a quantitative estimate of their abundance is desired, care should be taken to sample them adequately. Many, but not all, cyanobacteria have gas vesicles and are buoyant, thus causing them to accumulate in blooms on the water surface or in the long narrow surface streaks associated wth Langmuir circulation cells. Others, can exhibit depth-keeping and have dense accumulations at specific depths. For example, *Trichodesmium* spp. typically has a depth maximum at about 15 m in tropical and subtropical waters. Thus care should be taken to sample surface waters as well as accumulations in Langmuir circulation cells and specific depths in the water column. Generally, if water bottles are used for collection, samples taken at 5 meter intervals from surface to the bottom of the euphotic zone, will reveal any vertical accumulations, and one can visually determine sample sites in Langmuir circulation cells.

One additional caution is to draw your sample from the water bottle as soon as possible after collection since gas vacuolate cyanobacteria will rise to the top of the bottle, and the sampling port is typically at the bottom. If water cannot be drawn immediately, then the contents of the bottle should be mixed prior to taking the sample.

PRESERVATION

Identification is preferably done on live material as solutions used to preserve can distort cells. However, if the cyanobacteria cannot be examined when fresh, preservation with fixatives is an acceptable alternative. It is important that the preservative used for cyanobacteria be acidified. This discharges gas vesicles and allows the organism to be concentrated either by settling chambers or by centrifugation. Without discharge of these vesicles, the cyanobacteria would not fall to the bottom of either the settling chamber or centrifuge tube, and population density would be underestimated. To prepare a stock acidified Lugol's solution, add: 200 g KI, 100 g I_2, 2000 ml H_2O, and 190 ml glacial Acetic Acid. About 10 drops of this solution should be added to 200 ml of sample water to give the color of strong tea. Sample bottles should be

tightly capped, placed in the dark, and preferably in a cool place. If the color of the sample water begins to fade, add more Lugol's solution and check that the caps are tight. As long as the color remains in the sample, these will be preserved indefinitely.

An acidified formalin (ca 40% formaldehyde) solution can be made by mixing equal parts of formalin and concentrated acetic acid. For preservation, add 2 ml of this acidified solution to a 100 ml sample (giving a 0.4% formaldehyde solution). If tightly-capped, these samples will be stable.

IDENTIFICATION AND QUANTIFICATION

Noxious species can be benthic or planktonic and can also exist as cells, free trichomes or in the colonial state. For the larger filamentous cyanobacteria, identification can be made using a microscope at 400x magnification with the sample placed on a standard glass slide with a # 1 cover slip. However, for quantitative counts it is important that the method of counting be adapted to the size and density of the organism, since the range in size from the smallest toxic cyanobacterium to the largest is about three orders of magnitude. There are many acceptable methods for identifying and quantifying cyanobacterial populations. These have been summarized in a UNESCO manual (Sournia, 1978), and we refer the reader to it for details on counting chambers and concentrating techniques. Generally, a Sedgwick Rafter chamber (20 mm x 50 mm x 1mm deep) can be used for quantitative counts. This chamber holds 1 ml of sample, and the investigator can either put a concentrated (ca 10 ml or 15 ml concentrated with a centrifuge) or unconcentrated sample in the chamber. Transects are then made, and so long as the width of the field of view is known, one can calculate the density of different species present. One disadvantage of the Sedgwick Rafter cell is that magnifications of 400x cannot be used with a standard 40x objective. Long working distance 40x objectives can be purchased. Palmer Maloney counting chamber is shallower, with a depth of 0.4 mm, and a standard 40x objective can be used. This chamber is of 17.9 mm diameter and it holds 250 mm^2. All but the smallest cyanobacteria can be identified in this chamber, but because of its limited volume, it is generally necessary to concentrate the sample prior to filling the chamber. Methods for isolating and culturing cyanobacteria are given by Stein (1973).

Counting chambers such as the Sedgwick Rafter or the Palmer Maloney cells can be purchased from some major scientific suppliers. A relatively inexpensive plastic Sedgwick Rafter cell is available from Electron Microscope Sciences, Box 251, Fort Washington, PA 19034, USA.

The toxic strains of the coccoid cyanobacterium, *Synechococcus* cannot be identified to the species level using light microscopy. Identification of these picoplankters to genus is typically done using epifluorescence microscopy, and plankton samples are mounted on membrane filters. Formalin-killed samples should be used for epifluorescence counts and stored in a cool (preferably refrigerated) dark place. Since *Synechococcus* spp. are very abundant and widespread, and since toxic and nontoxic strains cannot at this moment be distinguished, it would be difficult to associate this organism with a toxic event. In this case it would be prudent to attempt to culture these coccoid cyanobacteria and carry out toxicity assays on isolates.

TOXIC GENERA

Anabaena is a freshwater genus that is sometimes present in brackish and marine waters. Within this genus there are six species associated with toxin production. These toxins are the potent neuromuscular blocking alkaloid anatoxin - a, several of the paralytic shellfish poisons

(PSP), the anticholinesterase organic phosphate anatoxin - a(s) and the hepatotoxic cyclic peptide microcystins. The species associated with one or more of these toxins include: *A. circinalis* Rabenh., *A. flos-aquae* (Lyngb.) Breb., *A. hassallii* (Kutz.) Wittr., *A. lemmermanni* P. Richt., *A. spiroides* var *contracta* Kleb., *A. variabilis* Kutz. In addition, in the related genus *Anabaenopsis,* the species *A. milleri* Woron. has also been shown to be toxic.

Anabaena flos-aquae (Lyngb.) De Brebisson (Fig. 19.1), is planktonic in brackish water, and colonies may be either solitary or in a twisted mass. Cells are spherical to subcylindrical, 4-8 µm wide and 6-8 µm long. Heterocysts are elliptical, 4-9 µm wide and 6-10 µm long, and are numerous. Cell contents have conspicuous pseudovacuoles, and cells link up in bead-like fashion to form trichomes. Akinetes are cylindrical or sausage shaped. *Anabaena lemmermannii* is similar to *A.flos-aquae,* but differs in that the akinetes are adjacent to, rather than remote, from the heterocysts. *Anabaena circinalis* (Fig. 19.1) have cells which are 8 to 14 µm in diameter with heterocysts from 14 - 18 µm wide and from 22 - 32 µm long. Trichomes are planktonic and can be straight, twisted or contorted, solitary or in colonies. *Anabaena hassallii* is similar to *A. circinalis*, and may not be a separate species.

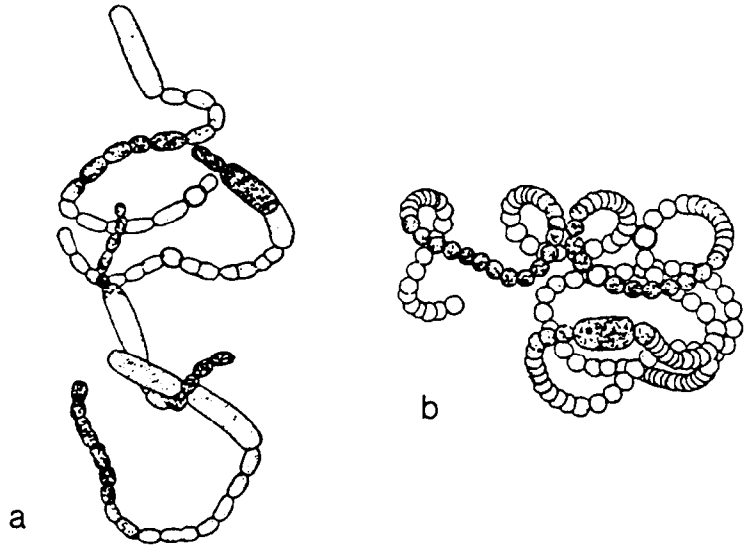

Figure 19.1.a. *Anabaena flos-aquae,* b. *Anabaena circinalis (A. hassallii).* From Geitler (1932).

Anabaena spiroides (Fig. 19.2) has trichomes which are spiral and enclosed in a thin mucilagenous sheath. Cells are spherical or compressed-spherical, 6.5-8 µm in diameter, and heterocysts are spherical.

Anabaena variabilis trichomes have cells which are compressed-globose, 3.7 to 6.5 µm in diameter and heterocysts are globular or ovate 5.5 - 8.0 µm in diameter and 5.8 - 6.5 µm long. Akinetes are ovate, from 6.8 - 9 µm wide and 7.5 - 14 µm long.

Species in the genus *Anabaenopsis* have paired intercalary (mid-trichome) heterocysts. *Anabaenopsis milleri* (Fig. 19.2) have spiral trichomes which have from 2.5, or more, complete spirals and with cells from 6 - 7 µm wide.

Aphanizomenon flos-aquae L. (Ralfs.) produces the paralytic shellfish poisons saxitoxin and neosaxitoxin. The species is present in fresh and brackish water environments. It has been associated with toxic events in Canada, USA and Europe. Colonies are usually very

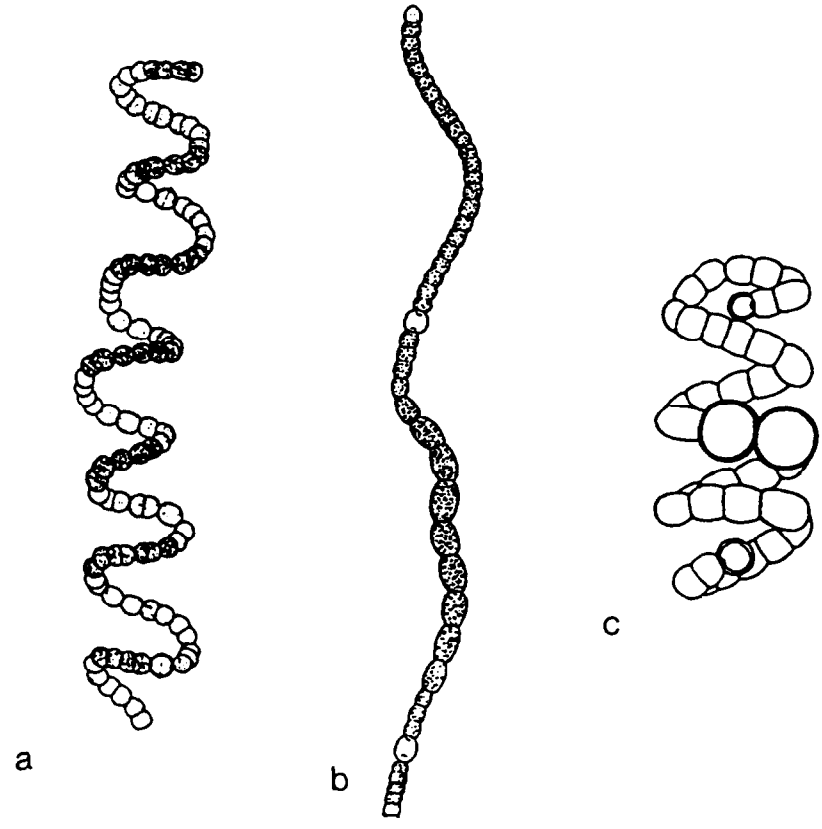

Figure 19.2. a. *Anabaena spiroides,* b. *Anabaena variabilis*, c. *Anabaenopsis milleri.* From Geitler (1932).

buoyant and present near the water's surface. Its trichomes are typically arranged in parallel as a colony, or as individual trichomes. When preservative is added, trichomes in colonies often disperse. Cells are 5 to 6 μm in diameter, and longer than wide, becoming progressively longer near the ends of the trichomes (Fig. 19.3). Cells are cylindrical, giving a straight, almost flat-sided appearance to trichomes. Heterocysts are 5-7 μm wide, 5-15 μm long.

Lyngbya majuscula Harvey, contains lyngbyatoxin A and debromoaplysiatoxin. Direct external contact causes "Swimmers Itch", which results in irritation of human skin. Generally *L. majuscula* grows attached to rocks or on sediments, but it can tear loose and drift for long distances. The toxins can also be concentrated by some marine invertebrates which graze on the cyanobacterium. It has been reported from the Pacific and Atlantic Oceans, and ranges from tropical to temperate locations.

Trichomes of *L. majuscula* are straight and long and enclosed in a distinct sheath (Fig. 19.3). Sheaths are unbranched. Cells are about 12 μm in diameter and only 1 - 1.5 μm long. The sheath is 4 μm thick, thus giving a total thickness for the filament of about 20 μm.

Microcystis is a freshwater genus which occasionally occurs in brackish water. Species in this genus are the most studied and most widely distributed geographically among the toxigenic cyanobacteria. The most common toxic species within this genus is *M. aeruginosa* Kutz., with *M. viridis* (A. Br.) Lemm. and *M. wesenbergii* (Komarek) Starmach also reported to be toxic. Species in this genus appear to produce several peptide toxins, known as microcystins, which usually produce liver damage as well as having tumor promoting activity.

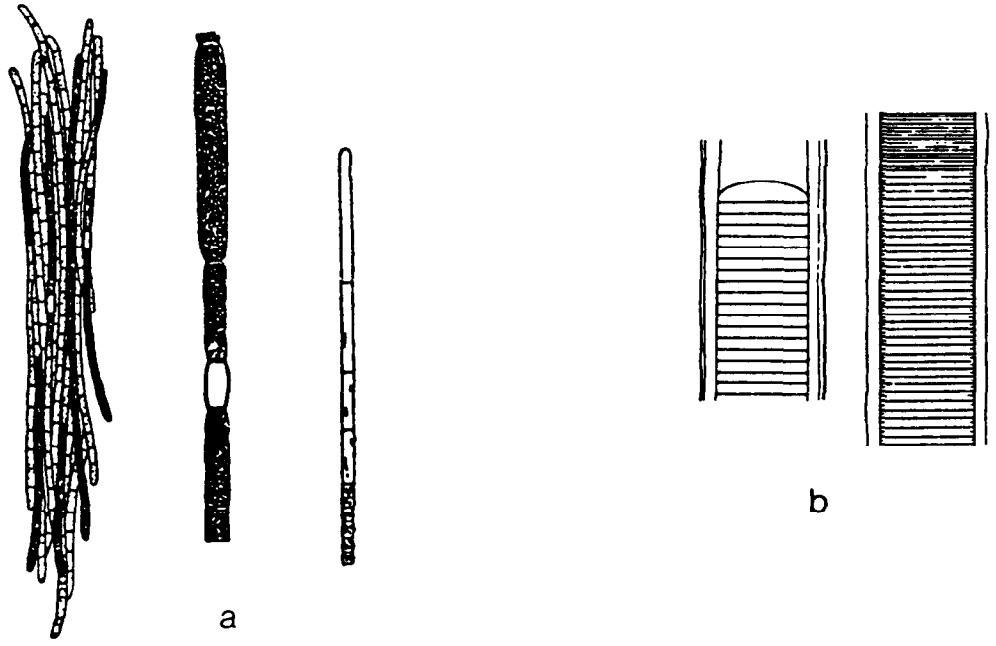

Figure 19.3. a. *Aphanizomenon flos-aquae,* b. *Lyngbya majuscula.* From Geitler (1932).

The colonies of *M. aeruginosa* can be spherical, ellipsoidal, or irregularly lobed (Fig. 19.4). Colonies can be up to several cm in diameter. Within the colony cells are spherical, about 4.5 μm (range ca 3 - 7 μm) in diameter. The cells have many gas vesicles which results in a granular appearance.

Nodularia spumigena Mertens is well known for its toxic blooms, along with other cyanobacteria, which occur in the Baltic Sea and brackish water lakes and estuaries of Australia and New Zealand. It can live either in the plankton or attached to macroalgae or sediments. The toxin produced is the cyclic pentapeptide nodularin. Nodularin is related to the microcystins and acts in a similar manner causing animal death through its effect on liver function. The filamentous colonies of *N. spumigena* have trichomes enclosed with more or less firm sheaths (Fig. 19.4). The unbranched filaments have disc-shaped vegetative cells as well as disc-shaped heterocysts. Filaments are often twisted, and cells are from 8 - 14 μm in diameter and only about 3 - 4 μm long. The sheath is thin and colorless. Heterocysts are compressed and elliptical in shape, 14 μm in diameter and 7 μm long.

Oscillatoria is a prominent genus of filamentous unbranched cyanobacteria with broad geographical distribution. Several marine species are known for their production of cytotoxins, but the most prominent is *O. nigro-viridis* Thwaites which produces the contact irritant debromaplysiatoxin and oscillatoxin - a. Other toxic species are better known from fresh and brackish water, and they include *O. agardhii/rubescens, O. agardhii, O. acutissima,* and *O. formosa.* The toxins they produce are the neurotoxins anatoxin - a and homoanatoxin - a plus the cyclic peptide microcystins. *Oscillatoria* species are filamentous, without a sheath and are unbranched. The mature filaments often show an apical region with the entire filament showing an oscillating or gliding movement. The trichomes vary in size but are generally 2 -10 μm in diameter and are without heterocysts or akinetes.

Oscillatoria nigro-viridis Thwaites produces oscillatoxin a. This is a cosmopolitan species which is found along the coast attached to rocks, macroalgae and sediments. The

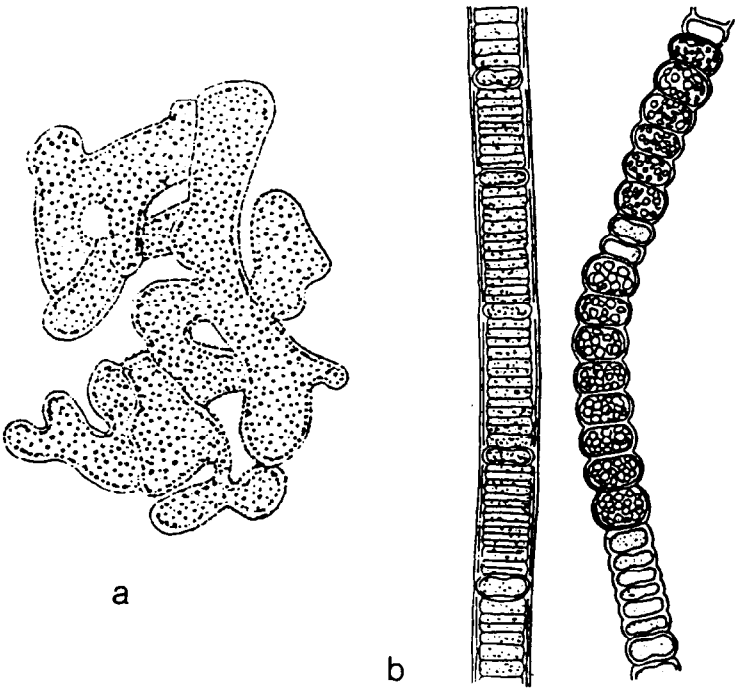

Figure 19.4. a. *Microcystis aeruginosa*, b. *Nodularia spumigena*. From Geitler (1932).

trichomes are olivegreen in color, and cells are 7 - 11 µm wide, and 3- 5 µm long (Fig. 19.5). The trichomes taper at the tips and terminate in a cone-shaped apical cell. In *O. agardhii* the trichomes are straight or slightly bent. Ends of trichomes can be polymorphic, and shape can range from a pointed calyptra to a blunt end. Cells usually not as long as they are wide (ca 4 - 6 µm wide). For *O. formosa* trichomes are straight, the ends are lightly bent, and cells 4 - 6 µm wide (Fig. 19.5). Cells can range from quadratic in shape to about half as long as wide. The end cell has no calyptra. Cells of *O. acutissima* are 1.5 - 2 µm in diameter, 1.5 - 3 times longer than wide and also without a calyptra.

Schizothrix calcicola (Ag.) Gom. is a filamentous species which characteristically has numerous trichomes enclosed in a mucilagenous sheath (Fig. 19.6). The sheath can be rather wide and laminated. Cells are 1-2 µm wide and 2-6 µm long. Trichomes within the sheath typically twist around each other. It is often found growing attached to a substrate in freshwater and marine locations, and has been implicated in toxic events near certain Pacific Ocean islands. The toxin produced is debromoaplysiatoxin, and exposure to it causes a "Swimmer's itch". Furthermore, the toxin can be accumulated by the sea hare (*Stylocheilus longicauda*).

Trichodesmium thiebautii Gomont ex Gomont, has recently been shown to possess a neurotoxin which is similar in action to anatoxin-a. This planktonic tropical marine species has been shown to be toxic to some, but not all, marine invertebrates which graze on it. Furthermore, there are reports of breathing difficulties from people who have been near "red tide" blooms of *Trichodesmium*. Other species may also be toxic.

Colonies of *T. thiebautii* consist of many trichomes which are bundled together parallel or twisted in a rope-like fashon (Fig. 19.6). A large fraction of the population may have trichomes in a radiate or spherical form (Fig. 19.6). Colonies are usually buoyant and are about 1 x 3 mm in size. They usually appear golden brown in color, but color can vary from gray to

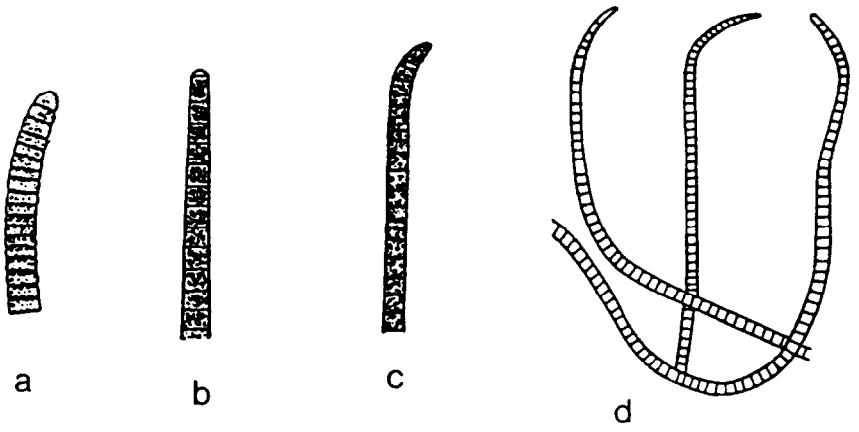

Figure 19.5. a. *Oscillatoria nigro-viridis*, b. *O. agardhii*, c. *O. formosa* (Geitler, 1932), d. *O. acutissima*. (Prescott, 1962).

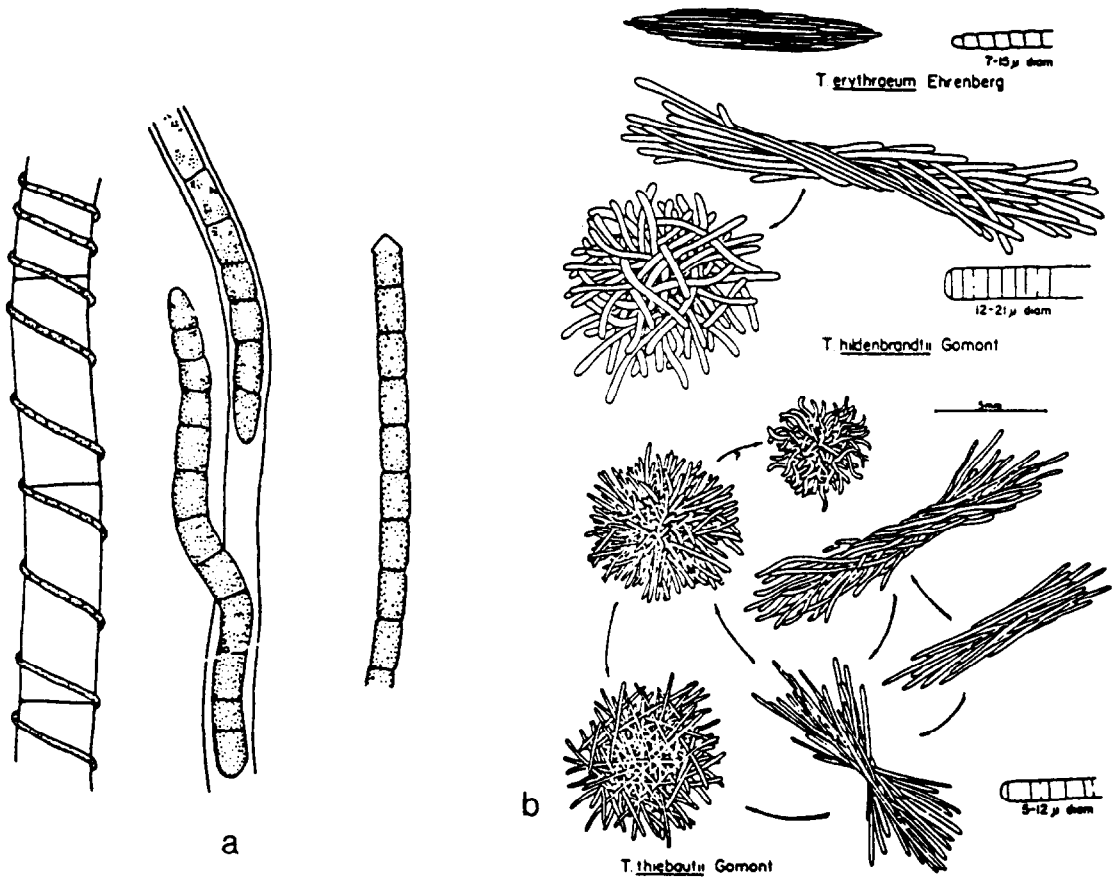

Figure 19.6. a. *Schizothrix calcicola*, from Humm (1979). On the left, a filament spirals around a green alga; b. *Trichodesmium erythraeum, hildebrandtii,* and *thiebautii*. Shown are radiate and parallel forms (from Borstad, 1978).

brown to red. Cells diameter ranges from 7 to 16 μm, and cells are usually as long as they are wide, or can be up to twice as long as they are wide.

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20. Taxonomy of Cysts

K. Matsuoka and Y. Fukuyo

DESCRIPTIVE TERMINOLOGY OF DINOFLAGELLATE CYSTS

The important morphological characters for identification of dinoflagellate cysts are the shape of the cyst body and its ornamentation, wall structure and color, and the type of aperture or archeopyle through which germinating cells leave the cyst. Archeopyle type can be a very useful character in determining the higher classification rank (genus and family) of cyst species. However, since cysts possess no archeopyle before excystment, it is impossible to use this feature for routine identification. In comparison to the morphology of motile forms, cysts usually have a relatively simple, mostly spherical to peridinioid shape. As a result, identification of cysts based on a single morphological character is not always reliable, and other characters such as the type of archeopyle, morphology of ornaments, wall structure, wall color, and paratabulation are indispensable for classification.

Morphology of the cyst body

Dinoflagellate cysts are classified into three major groups based on the position of its formation within the planozygote (Fig. 20.1).

The proximate cyst is formed directly beneath the theca of the planozygote and therefore its volume occupies approximately one-half to one-third of the original cell. Some cysts of this type have a characteristic ornamentation that possibly reflects the original plate, cingulum, sulcus and other structures such as apical groove of the motile forms. Proximate cyst bodies vary from spherical to peridinioid and sometimes have several projections on the surface.

The chorate cyst is characterized by various kinds of ornaments rising from the cyst surface. These ornaments functionally support the cell wall of the planozygote from the cyst surface and are morphologically very variable. The process formation during maturation from the planozygote toward the hypnozygote (resting cyst) has been observed in *Lingulodinium polyedra* (Stein) Dodge by Kokinos and Anderson (1992). Generally the cyst body of this type is spherical, subspherical or ovoidal. The volume of the cyst cavity is reduced compared to the planozygote, mostly less than one-third of the original.

The cavate cyst consists of more than two walls clearly separated, and usually possesses a cavity partly or entirely around the cyst body. Therefore, the outline of the cyst is variable. The volume of the cyst containing the protoplasm is much reduced during the maturation from planozygote to hypnozygote (resting cyst), and the inner cyst body is probably less than one-fifth of the original in volume.

In a strict sense, some fossil cyst terminologies are not directly relevant to modern cysts, but these terms are still useful not only for paleontologists but also modern planktologists. All types of cavate, chorate and proximate form cysts can be observed in modern sediments. For example, cysts of *Protoperidinium americanum*, although not a causative organism for red tides, consist of two layers which are partly detached, and probably cavate in form. Recently Fensom *et al.* (1993) tried to unify two different classification systems proposed independently by paleontologists for fossil cysts and by planktologists for modern motile forms.

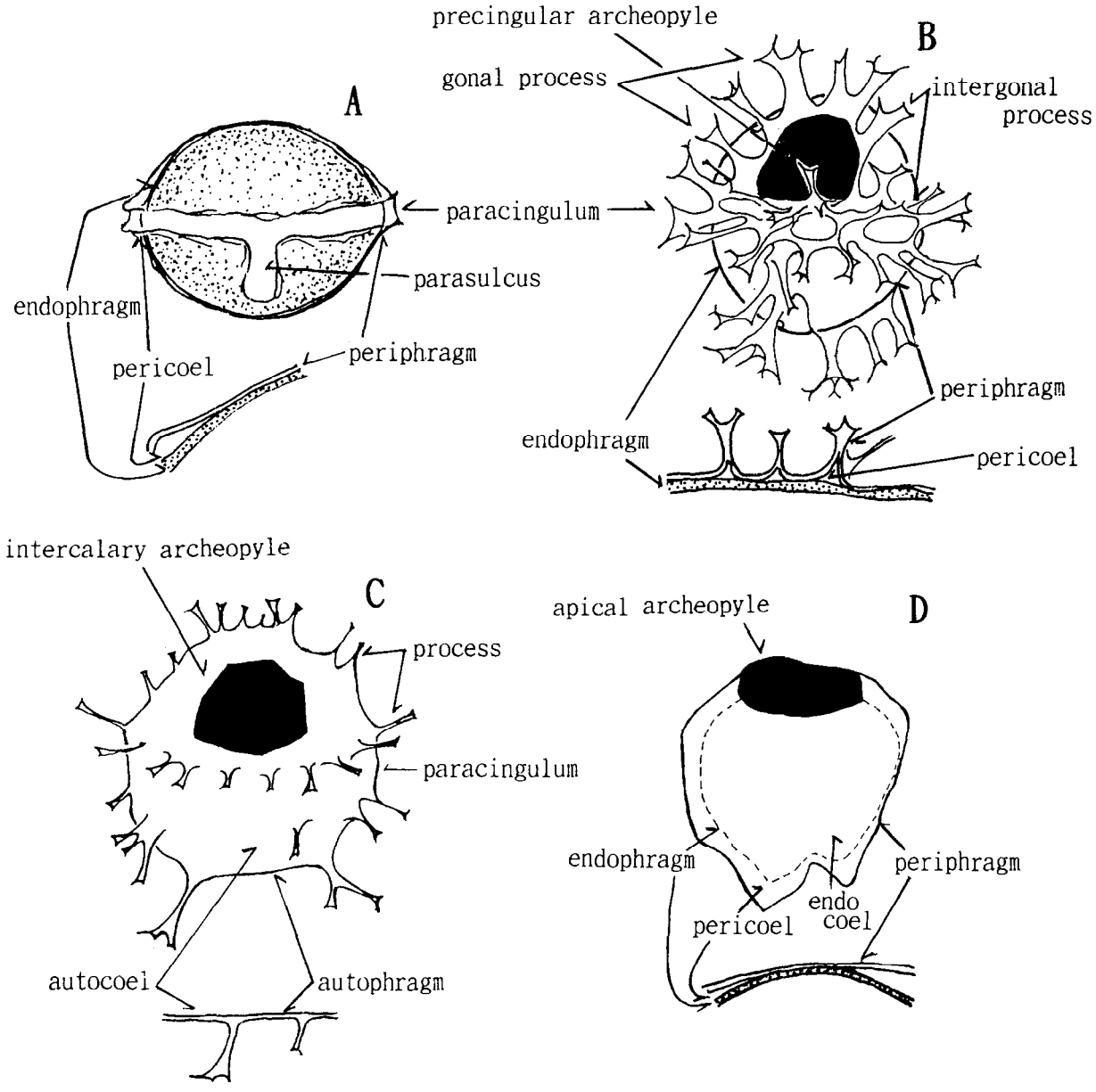

Figure 20.1. Wall structure and major groups in modern dinoflagellate cysts.
A: Cyst of *Zygabikodinium lenticulatum* (=*Dubridinium caperatum*), proximate (?) cyst having pericoel at paracingulum. B: Cyst of *Gonyaulax spinifera* complex (= *Spiniferites hyperacanthus*), chorate cyst with many gonal and intergonal processes. C: Cyst of *Protoperidinium divaricatum* (= *Xandarodinium* sp.), proximochorate cyst with short processes. D: Gen. et sp. indet., cavate cyst with apical archeopyle.

In general, the outlines of modern dinoflagellate cysts are relatively simple in comparison with fossil forms; that is, they are mainly spherical, subspherical, ovoidal, ellipsoidal, or peridinioid. Some brackish water cysts, however, vary in shape from simply spherical through peridinioid to cursiform. It is notable that even in a single species, environmental parameters such as are salinity, temperature and nutrient can produce remarkable variation in the cyst shape. This is well investigated for *Spiniferites cursiformis* Wall et Dale and *Tectatodinium pellitum* Wall et Dale found in Quaternary sediments of the Black Sea (Wall *et al.*, 1973).

Wall structure and color

The wall of modern cysts consists of one, two or three layers composed mostly of biopolymers, chemically similar to the sporopollenin of spores and pollen grains in higher plants, however rarely of calcium carbonate e.g. *Scrippsiella.* The cyst wall can be composed of up to three or four layers (Evitt, 1985); autophragm, periphragm, mesophragm, and endophragm (Fig. 20.2). The color of the cyst wall is also variable; mostly transparent, light yellow, pale brown, brown, or dark brown. Cysts which are partly composed of calcium carbonate such as *Scrippsiella trochoidea* are dark brown to black in living condition.

Morphology of surface ornamentation

For the description of surface ornaments of cysts, the terminology for pollen grains and spores is adopted. Some terms frequently used for modern cysts are shown in Fig. 20.3 and 20.4.

Archeopyle

The term "archeopyle" is defined by Evitt (1963) as an excystment opening formed at the germination stage of dinoflagellate cysts. In dinoflagellate cysts, apical, intercalary, precingular, hypocystal archeopyle types and combinations of these series have been recognized. However, it is not possible to use these definitions for cysts of gymnodinialean species which are not covered with typical thecal plates at the motile stage. Matsuoka (1985a) proposed new descriptive terms for the archeopyle of modern dinoflagellate cysts in both naked and thecate dinoflagellates, and divided them into saphopylic, theropylic and cryptopylic archeopyles (Fig. 20.5).

Saphopylic archeopyle

Archeopyle sutures correspond to paraplate boundaries and the operculum. Part of the cyst wall corresponding to the archeopyle is always detached from the cyst body. The archeopyle type can be subdivided into either apical, intercalary, precingular, "hypocystal" or a combination from one or more plate series. Most modern cysts belonging to the Peridiniales and Gonyaulacales have this archeopyle type.

Theropylic archeopyle

Archeopyle sutures follow paraplate boundaries, but the operculum is usually attached to the cyst. This is caused by incomplete development of archeopyle structures on the cyst body. This archeopyle can also be subdivided into several types on the basis of the position of the sutures.

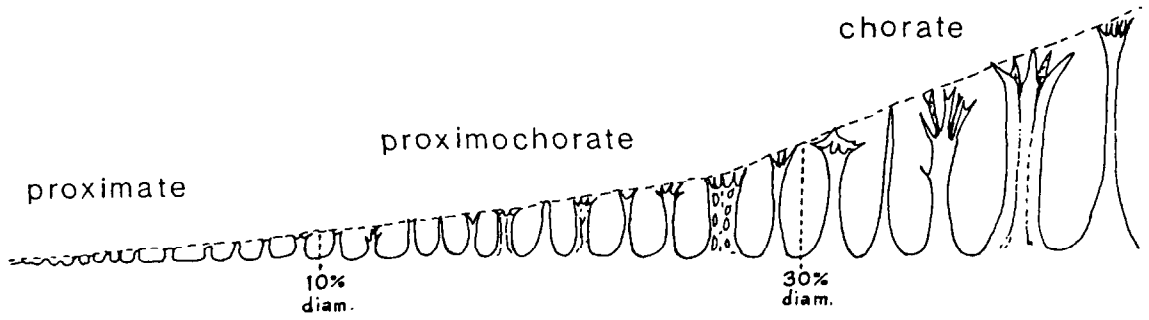

Figure 20.2. Major morphological groups in dinoflagellate cysts, defined on the basis of process length as a percentage of the shortest diameter of the central body; after Sarjeant (1982).

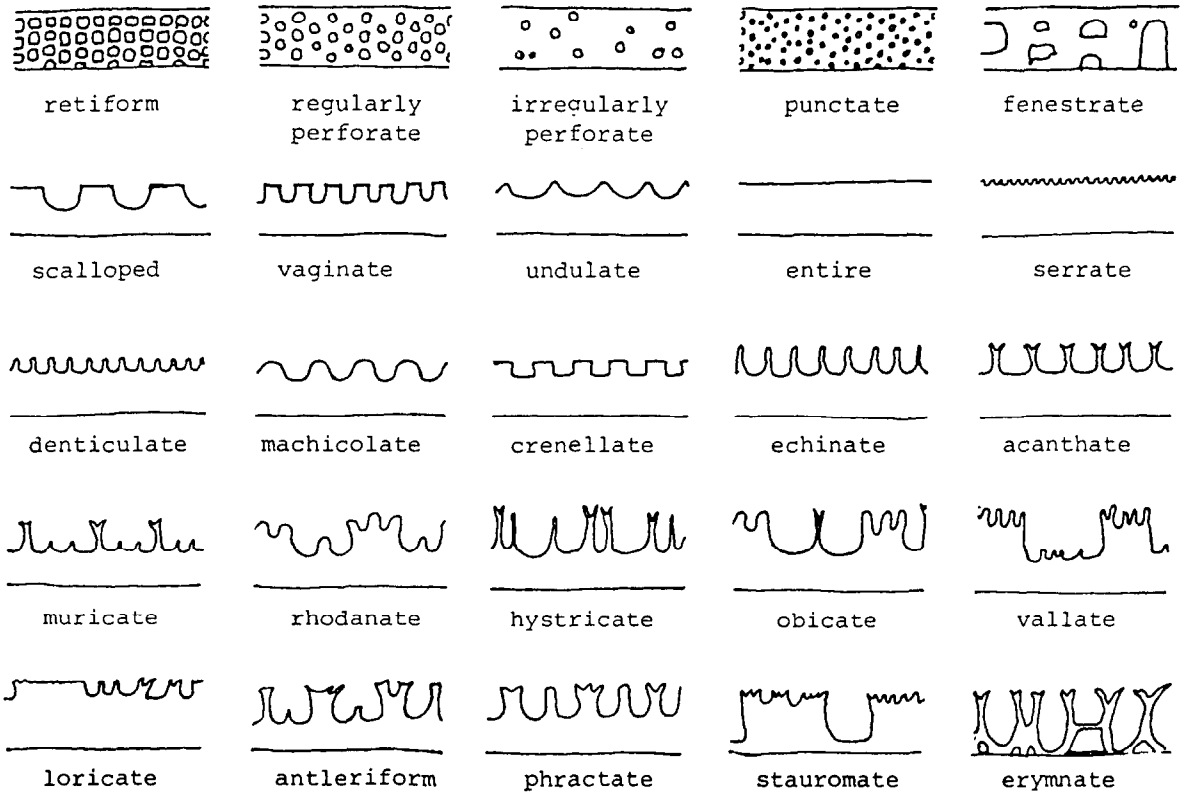

Figure 20.3. Terminology for crests and parasutures of dinoflagellate cysts after Sarjeant (1982). Parasutural morphology of modern dinoflagellate cysts is not so diverse as fossil cysts.

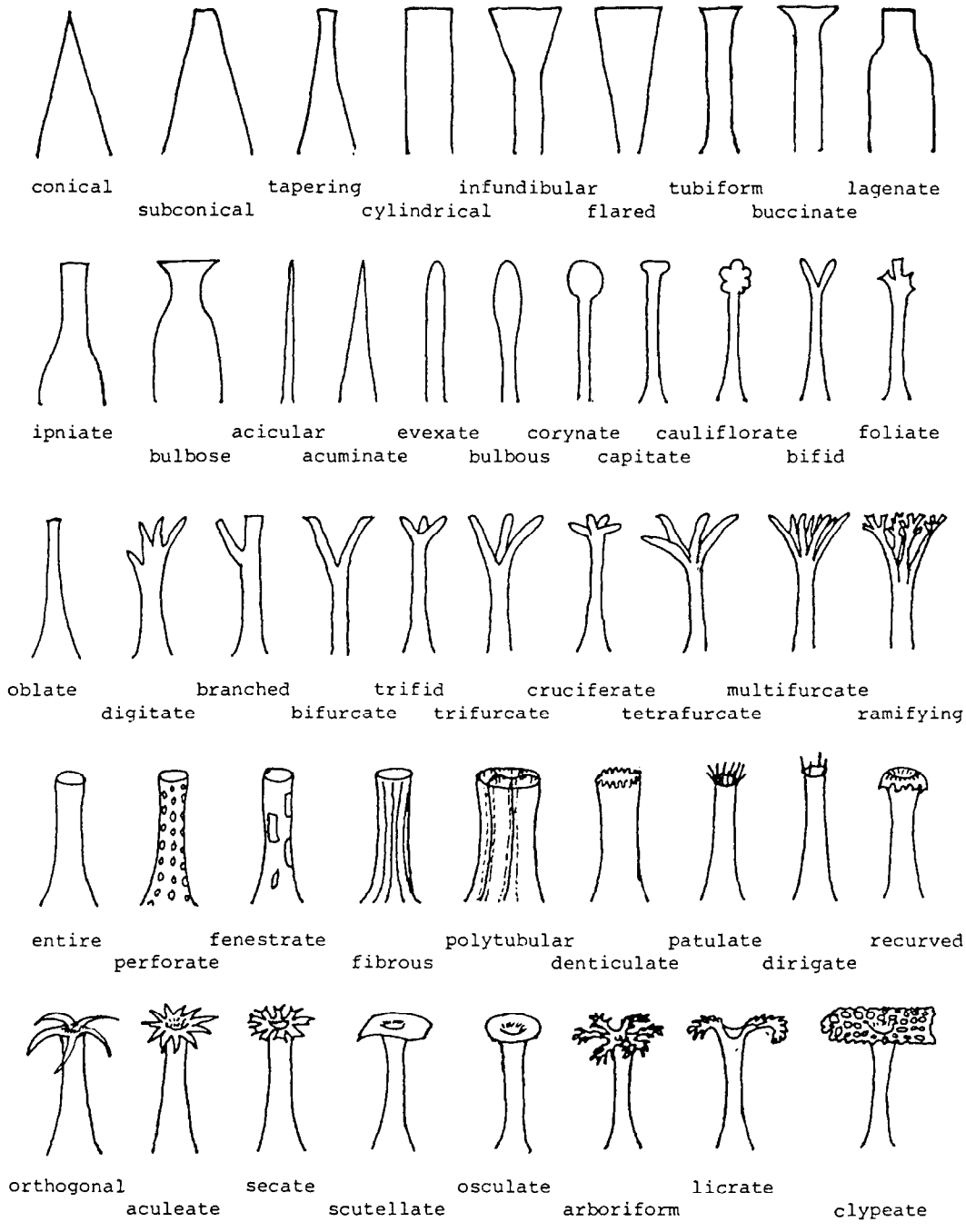

Figure 20.4. Terminology for processes of dinoflagellate cysts after Sarjeant (1982). Process morphology of modern dinoflagellate cysts is not as diverse as fossil cysts.

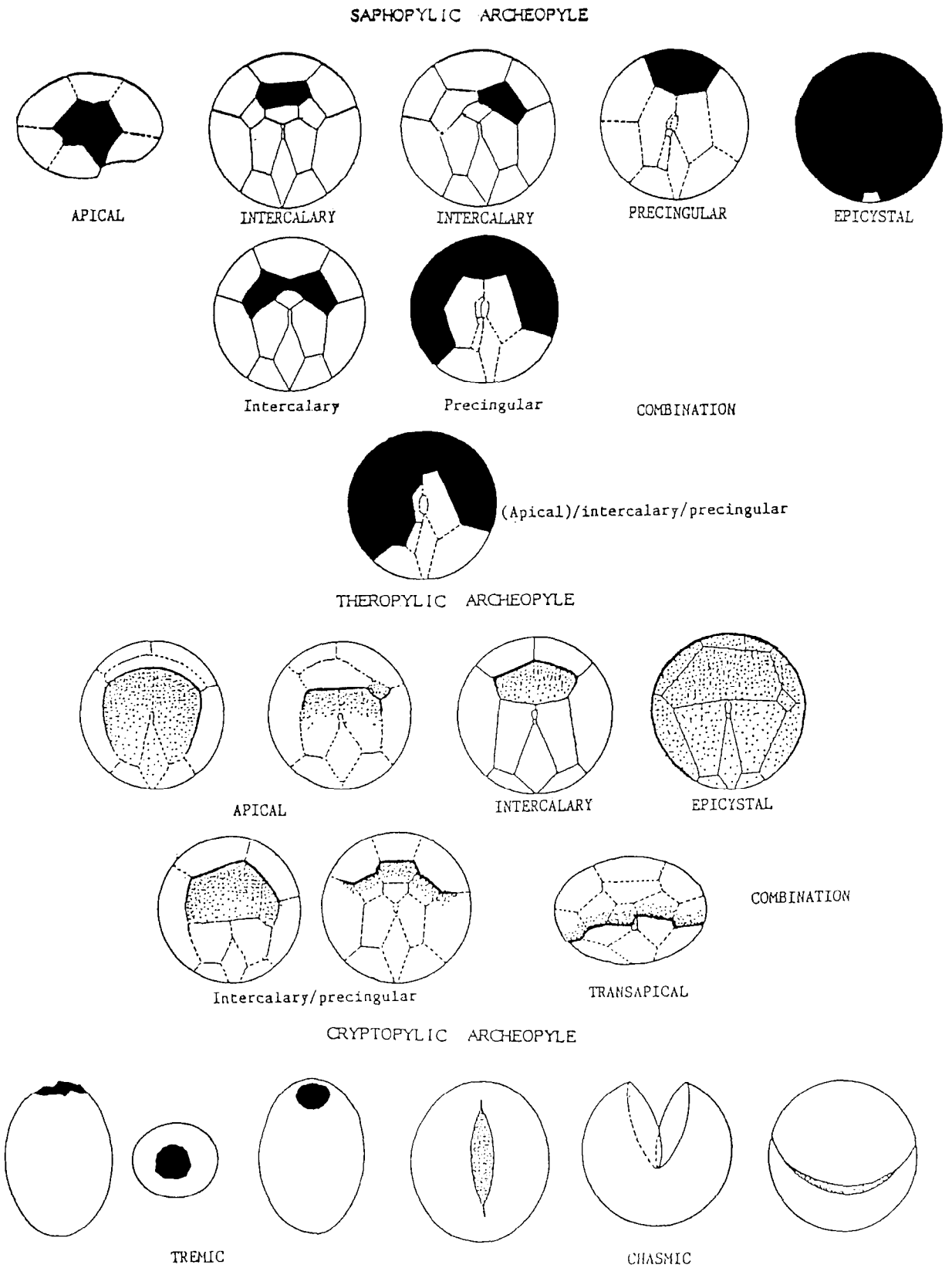

Figure 20.5. Archeopyle types in modern dinoflagellate cysts. Modified after Matsuoka *et al.* (1989).

Matsuoka *et al.* (1989) showed a provisional subdivision for this archeopyle, but more careful examination is needed to confirm this. Modern cysts produced by diplopsalid and calciodinellid species have this archeopyle type.

Cryptopylic archeopyle

Archeopyle suture does not reflect any plate boundary and the operculum is detached or free from the cyst. This is caused by the motile forms being composed of no thecal plates. Some modern gymnodinialian and gonyaulacacean cysts have this archeopyle type. On the basis of the shape of opening, this archeopyle type is classified into two forms, chasmic (slit-like opening) and tremic (hole-like opening) (Matsuoka, 1985a).

It is notable that some modern cysts do not show any distinct excystment aperture after germination, probably because of their fragile cyst wall. This archeopyle type includes most cysts of *Alexandrium* species, *Pentapharsodinium faeroense* Indelicato et Loeblich, and *Ensiculifera imariense* Kobayashi et Matsuoka.

DESCRIPTION OF HARMFUL MARINE DINOFLAGELLATE CYSTS

Prorocentrales cysts

Only two species of the Prorocentrales producing cysts have been observed. These are *P. lima* and *P. marinum* (Faust, 1990; 1993). These cysts are morphologically simple, spherical and similar to each other. As there have been no records in modern sediments, these cysts probably are not preservable.

Dinophysiales cysts

Sexuality of *D.* cf. *acuminata* has been documented on the basis of the presence of planozygotes possessing two trailing flagella by McLachlan (1993), but there is no evidence concerning hypnozygotes or resting cysts for this species. Two other species, *D. acuta* and *D. tripos* have been observed by Moita and Sampayo (1993) to produce resting cysts. These cysts have not yet fully been studied for their morphology including wall and archeopyle structures, and have never been recorded from modern sediments.

Gymnodinalian cysts

Shape of the cyst body is mostly spherical to ovoidal and sometimes ellipsoidal, with or without spinate or reticulate ornaments on the surface. The cyst wall is organic and pale brown, brown, rarely reddish brown in color; mostly composed of a single layer and sometimes two layers.The archeopyle type is cryptopylic, chasmic or tremic.

Species producing cysts: *Gymnodinium catenatum* Graham

Gonyaulacacean cysts

Shape of the cyst body is basically spherical to ellipsoidal and rarely discoidal, and seldom with or without process-like ornaments. The cyst wall is organic, colorless and sometimes transparent; rarely composed of a single or usually two layers. The archeopyle type is mostly saphopylic, precingular, but sometimes epicystal or combination type. In the cysts of *Alexandrium* spp. and *Gonyaulax verior*, no typical archeopyle is formed.

Species producing cysts:
Lingulodinium polyedra (Stein) Dodge
Alexandrium affine (Inoue et Fukuyo) Balech
Alexandrium catenella (Whedon et Kofoid) Balech
Alexandrium minutum Halim
Alexandrium tamarense (Lebour) Balech
Pyrodinium bahamense Plate var. *compressum* (Bohm) Steidinger, Tester et Taylor

Peridinialean cysts

Shape of the cyst body is mainly sperical, ellipsoidal, peridinioid, and rarely discoidal, mainly without process- like ornaments. The cyst wall is mainly organic and mostly brown in color, rarely transparent, and sometimes calcareous; mainly composed a single and rarely two layers. The archeopyle type is mainly saphopylic, of the intercalary and sometimes theropylic type, or apical, intercalary, epicystal and combination types.

Species producing cysts: *Scrippsiella trochoidea* (Stein) Loeblich III

CYSTS OF GYMNODINIALES

Gymnodinium catenatum Graham Fig. 20.6A

Gymnodinium catenatum Graham, 1943, p. 259-261, Figs 1, 2
Cyst form: *Gymnodinium catenatum*; Anderson *et al.*, 1988, p. 255-262, Figs 1-16.

Shape: Proximate, spherical without horns and spines. **Size:** Small to intermediate, 45-63µm in diameter. **Wall structure and color:** Autophragm; microreticulate ornaments which probably reflect the pattern of amphiesmal vesicles on motile stage. Shape and size of each reticulation is variable. Dark to reddish color. **Paracingulum:** Two rows of relatively smaller paravesicles reflect the cingulum of the motile cell. **Parasulcus:** Linear arrangement of smaller and larger reticulations show the sulcus of the motile cell. **Archeopyle and operculum:** Cryptophylic, chasmic type; slit opening encircling over the cell diameter; operculum adnate. **Other features:** Smaller paravesicles going up from the paracingulum to the apex and then encircling around the apex reflect the apical groove of the motile cell. The living cyst is dark brown and contains many food reserves of starch and oil drops, and a single red pigmented body.

Comment: When the cyst is filled with fresh protoplasm, it is very difficult to distinguish this from other round brown cysts belonging to *Brigantedinium*, and some cysts of the diplopsalid group. The microreticulate surface ornamentation and chasmic archeopyle are useful characters to distinguish this cyst.

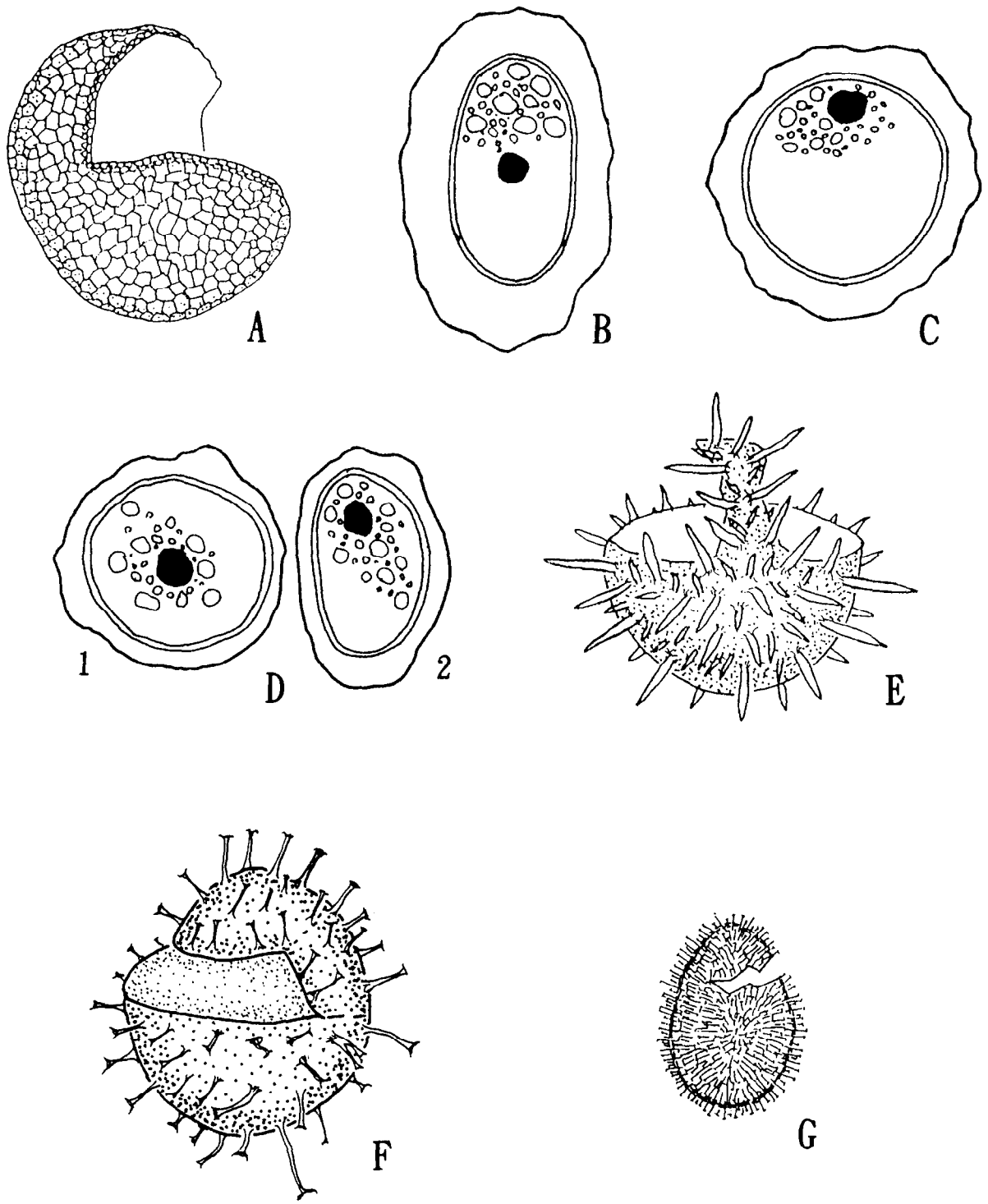

Figure 20.6. Ilustration of cysts of harmful modern dinoflagellate cysts.
A: *Gymnodinium catenatum*, B: *Alexandrium catenella* or *A. tamarense*, C: *Alexandrium affine*, D: *Alexandrium minutum*, E. *Lingulodinium polyedra* (= *Lingulodinium machaerophorum*), F: *Pyrodinium bahamense* var. *compressum* (= *Polysphaeridium zoharyi*), G: *Scrippsiella trochoidea*; scale bar: 20 μm.

CYSTS OF GONYAULACALES

***Lingulodinium polyedra* (Stein) Dodge** Fig. 20.6E

Gonyaulax polyedra Stein, 1883, p. 13, pl. 4, Figs 7-9.
Syn: *Lingulodinium polyedra* (Stein) Dodge, 1989, p. 291, Figs 1H, I, 34-38.
Cyst from: *Hystrichosphaeridium machaerophorum* Deflandre et Cookson, 1955, p. 274, pl. 9, Figs 4, 8.
Syn: *Lingulodinium machaeophorum* (Deflandre et Cookson) Wall, 1967, p. 109-110, pl. 15, Figs 16, 17.

Shape: Chorate; spherical with neither apical nor antapical bosses. As a large archeopyle is formed at germination, the shape is sometimes hemispherical. **Size:** Small to intermediate, 35-50 µm in diameter. **Wall structure and color**: Periphragm and endophragm strongly adpressed except for processes; granular on surface; colorless and transparent. **Processes:** Intratabular; variable in shape, hollow, and excavate, bullbose to acuminate with closed distal extremities; up to 17 µm in length. **Archeopyle and operculum**: Saphopylic, combination precingular type formed by loss of three to four precingular and sometimes an additional two anterior intercalary paraplates; operculum free. **Other features**: Important morphological characters for this species are a large spherical or hemispherical cyst body and hollow and excavate, bullbose to acuminate processes. The processes are variable in shape and length; rarely nodular forms are observed.

Comment: *Lingulodinium machaerophorum* (Deflandre et Cookson) is the paleontological name for this cyst. In surface sediments of Australian coastal waters, hemispherical specimens formed by loss of the whole epicyst were described as *Lingulodinium hemicystum* by McMinn (1990). However, as the motile form is yet unknown from an incubation experiment with living cysts, and as other morphological features of this cyst except for its epicystal archeopyle are similar, *L. hemicystum* is probably conspecific to *L. machaerophorum.*

***Pyrodinium bahamense* Plate var. *compressum* (Bohm) Steidinger, Tester et Taylor** Fig. 20.6F

Pyrodinium bahamense Plate forma *compressa* Bohm, 1931, p. 191, Fig. 4.; *Pyrodinium bahamense* Plate var. *compressa* (Bohm) Steidinger, Tester et Taylor, 1980, p. 329-334.
Cyst form: *Hemicystodinium zoharyi* Rossignol, 1962, p. 132-133, pl. 2, Fig. 10.
Syn.: *Polysphaeridium zoharyi* (Rossignol) Bujak, Downie, Eaton et Williams, 1980, p. 12, *Pyrodinium bahamense* Plate var. *compressum,* Matsuoka *et al.*, 1989, p. 301-304.

Shape: Chorate; spherical, covered with many processes. **Size:** Intermediate, 55-70 µm in diameter. **Wall structure and color:** Periphragm and endophragm strongly adpressd except for processes, and granular surface; colorless. **Processes:** Intratabular; long, slender, cylindrical to tubiform, and capitate distal extremities; 6-15 µm in length. **Archeopyle and operculum:** Epicystal; operculum composed of all paraplates of the free epicyst, but archeopyle sutures are often observed on the attached operculum. **Other features:** As the development of archeopyle sutures is incomplete, the number of opercular pieces is variable and also the shape of cyst body varies from spherical to hemispherical.

Comment: Another variety in this species, *Pyrodinium bahamense* var. *bahamense* also produces a spherical cyst with many and more slender processes. The surface ornament of the cyst of var. *compressum* is coarser than that of var. *bahamense*, but other morphological differences between these two varieties have not yet been fully described. This cyst differs from other spherical cysts with many processes such as *Protoceratium reticulatum* and

Lingulodinium polyedra in having a distinct epicystal archeopyle and slender tubiform to cylindrical processes.

Alexandrium minutum **Halim** Fig. 20.6D

Alexandrium minutum Halim, 1960, p. 102-105, Fig. 1.
Syn.: *Pyrodinium minutum* (Halim) Taylor, 1976, p. 13, pl. 2, Figs 1-5.
Cyst form: *Alexandrium minutum* Halim; Bolch *et. al.*, 1991, p. 218, Figs 1-2, 8-9.

Shape: Proximate; bean-shaped, circular in apical view (Fig. 20.6D1), and reniform in lateral view (Fig. 18-6D2), without any surface ornament. **Size**: Small, 20-30 μm in diameter. **Wall structure and color**: Thin periphragm and thick endophragm strongly adpressed without any surface ornament; colorless. **Archeopyle and operculum**: Chasmic ?, unknown in detail. **Other features**: The cyst is usually covered with a transparent gelatinous substance sometimes including fine mineral and detrital particles of diatoms, silicoflagellates and other microplankton. After germination, the empty cyst is rarely preserved in sediment, because of its thin and fragile cyst wall. The living cyst contains many colorless food reserves of starch and oil drops, and a single red pigmented body.

Comments: The cyst of *A. minutum* is similar to *A. lusitanicum*, but it differs in its smaller cyst body (Bolch *et al.*, 1991), and also differs from other cysts of *Alexandrium* in being reniform in lateral view.

Alexandrium affine **(Inoue et Fukuyo) Balech** Fig. 20.6C

Alexandrium affine (Inoue et Fukuyo) Balech, 1985, p. 38, Fig. 6.
Syn.: *Protogonyaulax affinis* Inoue et Fukuyo in Fukuyo *et al.*, 1985, p. 30, Figs 1E, 3A-C, 24- 29.

Shape: Spherical without any surface ornament. **Size**: Small, 30-35 μm in diameter. **Wall structure and color**: Thin periphragm and thick endophragm strongly adpressed without any surface ornamen; colorless. **Archeopyle and operculum**: unknown in detail. **Other features**: Almost the same as *A. minutum.* The empty cyst is not preserved in sediment, because of its thin and fragile periphragm.

Comments: It is very difficult to identify the cyst of *A. affine* in sediments, because of its simple, spherical cyst body that has no surface ornament.

Alexandrium catenella **(Whedon et Kofoid) Balech** Fig. 20.6B

Alexandrium catenella (Whedon et Kofoid) Balech, 1985, p. 37, Fig. 2.
Syn.: *Gonyaulax catenella* Whedon et Kofoid, 1936, p. 25-31, Figs 1-7, 14; *Alexandrium excavatum* (Braarud) Balech et Tangen, 1985, p. 338. *Gonyaulax tamarensis* var. *excavata* Braarud, 1945, p. 10-11, Pl. 2, Figs n, o.
Cyst form: *Protogonyaulax catenella* (Whedon et Kofoid) Taylor or *P. tamarense* (Lebour) Taylor; Fukuyo, 1985, p. 531-534, Fig. 2 o-p.

Shape: Proximate; elongate, cylindrical with rounded ends, and without any surface ornament. **Size**: Small, 38-56 μm in length, 23-32 μm in width. **Wall structure and color**: Thin periphragm and thick endophragm strongly adpressed without any surface ornament; colorless. **Archeopyle and operculum**: Chasmic?, unknown in detail.

Other features: Almost the same as *A. minutum.* The living cyst contains many colorless food reserves of starch and oil drops, and one to two red pigmented bodies.

Comments: The cyst of *A. catenella* is morphologically identical to *A. tamarense* (Lebour) Balech, and it is impossible to distinguish them from each other on the basis of cyst morphology. Germination experiments are required for this purpose.The cyst is also similar to that of *A. ostenfeldii* except for the cyst diameter.

CYSTS OF PERIDINIALES

Scrippsiella trochoidea (Stein) Loeblich III Fig. 20.6G

Scrippsiella trochoidea (Stein) Loeblich III, 1976, p.25.
Syn.: *Glenodinium trochoideum* Stein, 1883, p. 13, pl. 3, Figs 27-29; *Peridinium trochoideum* (Stein) Lemmermann, 1910, p. 673, Figs 14-17.
Cyst form: *Peridinium trochoidea* (Stein) Lemmermann; Wall et Dale, 1968b, p. 1401, pl. 172, Figs 1-4,27, text-fig. 2, 1-3

Shape: Chorate; spherical to ovoidal with many processes. **Size**: Small; 25-48 μm in length, 25- 44 μm in width. **Wall structure and color:** Thick and calcareous periphragm covered with many spines, and thin and transparent endophragm; dark brown in color with granular surface. **Processes:** Calcareous, nontabular, slender, cylindrical, and solid with capitate distal ends, densely distributed on the surface; length of processes variable. **Archeopyle and operculum**: Theropylic; archeopyle suture consisting of three to four sides of paraplate, however its location is not known in detail, probably anterior intercalary/precingular. **Other features**: After chemical treatment with acidic solutions such as HCl, calcareous parts of the cysts are removed, however the colorless organic endophragm still remains. Because of its calcareous wall and processes, the living cyst is dark brown to black in color, and usually contains a single red pigmented body.

Taxonomic note: Some other species of *Scrippsiella* such as S. *lachrymosa, S. trifida, S. rotunda, S. precaria* and *S. crystallina* also possess calcareous walls and processes. Among them, *S. trochoidea* and *S. precaria* are characterized by long and slender calcareous processes, and are very difficult to distinguish from each other.

CYSTS OF OTHER HARMFUL PHYTOPLANKTON

Some other phytoplankton species belonging to the Raphidophyceae (see chapter 18) also produce resting cysts in their life cycle. They include *Chattonella antiqua* (Hada) Ono, *C. marina* (Subrrahmanyan) Y. Hara et Chihara, *Heterosigma carterae (Hulburt) Taylor* and *Fibrocapsa japonica* Toriumi et Takano. The cysts produced by these species are generally small and spherical without any ornamentation on the surface. The morphology of their germination aperatures is not fully understood.

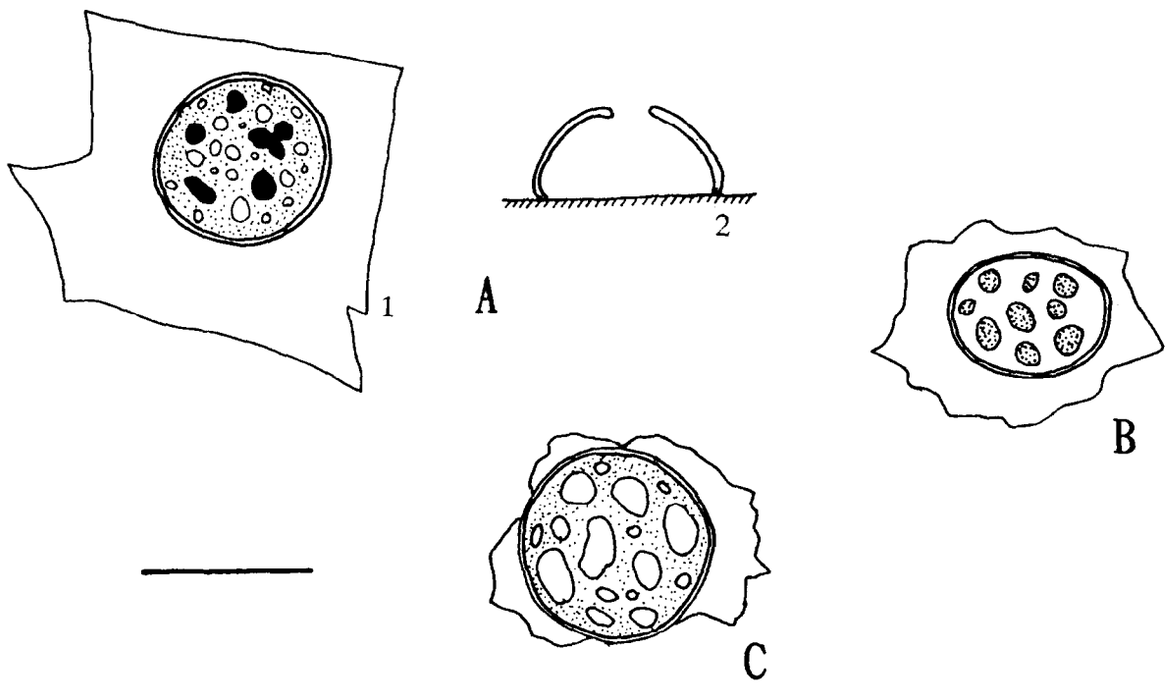

Figure 20.7. Ilustration of cysts of raphidophyte phytoplankton species.
A: *Chattonella antiqua*, 1, living cysts attached to a sediment particle; 2, empty cyst with circular opening, B: *Heterosigma akashiwo*, C: *Fibrocapsa japonica*; scale bar: 20 μm.

***Chattonella antiqua* (Hada) Ono** Fig. 20.7A

Cyst from: *Chattonella antiqua* (Hada) Ono et Takano, 1980; Imai et Itoh, 1988, p. 36-37, pl. 1, Figs A, C, E, G; pl. 2, Figs A-D; pl. 3, Figs A-D. *Chattonella* sp. Imai et Itoh, 1986, p. 62, pl. 1, Figs A-F.

Shape: Hemispherical when the cysts adher to the solid surface of diatom frustules and sand grains, and sometimes spherical when unattached. **Size:** Small; 25-35 μm in diameter, and 15-25 μm in height. **Wall relationship and feature**: Probably a single layer and transparent without any ornamentation on surface. **Opening for germination**: Circular and ca. 7μm in diameter, formed on the top of the cyst, with the cover completely detached after germination. **Other features**: Living cysts filled with fresh protoplasm are yellow-green to brownish in color, and contain several spots of dark brown or black materials. Under a fluorescence microscope, living cysts stained with DAPI, show the nucleus in blue white color.

Comments: Cysts of *Chattonella marina* (Subrahmanyan) Hara et Chihara have the same morphology as *C. antiqua.*

Heterosigma carterae (Hulburt) Taylor Fig. 20.7B

Cysts of *Heterosigma carterae (Hulburt) Taylor* 1967; Imai *et al.*,1993, p.1669-1673.

Shape: Spherical, usually covered with muculage **Size:** Very small; ca.10μm in diameter. **Wall relationship and feature:** Probably a single layer and transparent without any ornament on surface. **Opening for germination**: Unknown. **Other features:** Living cysts filled with fresh protoplasm are lightly yellow-green to brownish in color. With blue light exitation under a fluorescence microscope, living cysts show red fluorescence derived from chlorophyll.

***Fibrocapsa japonica* Toriumi et Takano** Fig. 20.7C

Cysts of *Fibrocapsa japonica* Toriumi et Takano, 1973; Yoshimatu, 1987, p. 28, pl. 1, Figs 2-7.

Shape: Spherical, but hemispherical when the cysts adher to the surface of diatom frustules, and probably covered with thin gelatinous layer. **Size:** Small; 15-20 μm in diameter. **Wall relationship and feature:** Probably a single layer and transparent without any ornament on the surface. **Opening for germination**: Unknown. **Other features:** Living cysts filled with fresh protoplasm are dark orange-brown in color, and lack a red pigmented body.

Comments: The cyst of *F. japonica* is similar to cysts of *Chattonella* in being small, transparent, and spherical to hemispherical, but differs from the latter in not having dark brown to black spots.

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APPENDIX

Table 20.1. List of species producing resting cysts in modern marine dinoflagellates (*harmful species).

Species	Reference
Prorocentrales	
Prorocentrum lima	Faust (1990)
Prorocentrum marinum	Faust (1993)
Dinophysiales	
Dinophysis acuta	Moita et Sampayo (1993)
Dinophysis tripos	Moita et Sampayo (1993)
Gymnodiniales	
Cochlodinium sp.	Fukuyo (1982)
*Cochlodinium*sp.	Matsuoka (1985a, 1987)
Gymnodinium catenatum *	Anderson *et al.* (1988), Matsuoka (1987),Hallegraeff *et al.* (1989)
*Gymnodinium*sp. 1	Bolch et Hallegraeff (1990)
Gyrodinium instriatum	Wall et Dale (1968a), Fukuyo (1982),Matsuoka (1985a) Kojima et Kobayashi (1992)

Gyrodinium resplendens	Dale (1983)
Gyrodinium uncatenum	Tyler *et al.* (1982)
Gyrodinium sp. 1	Bolch et Hallegraeff (1990)
Pheopolykrikos hartmannii	Fukuyo(1982),Matsuoka et Fukuyo (1986),
Polykrikos kofoidii	Matsuoka (1985a),Morey-Gaines et Ruse (1980), Fukuyo et Matsuoka (1983)
Polykrikos schwartzii	Wall et Dale (1968a), Matsuoka (1985a)
Woloszynskia sp. 1	Bolch et Hallegraeff (1990)

Gonyaulacales

Gonyaulax digitalis	Wall et Dale (1968a)
Gonyaulax scrippsae	Wall et Dale (1968a), Matsuoka (1984b)
Gonyaulax spinifera *	Wall et Dale (1968a)
Gonyaulax cf. *spinifera*	Dale (1983)
Gonyaulax verior	Matsuoka *et al.* (1988)
Gonyaulax sp.	Dobell et Taylor (1981)
Lingulodinium polyedra *	Wall et Dale (1968a),Kobayashi *et al.* (1981), Lewis(1988)
Protoceratium reticulatum	Wall et Dale (1968a)
Alexandrium affine *	Fukuyo *et al.* (1985), Hallegraeff *et al.* (1991)
Alexandrium catenella *	Yoshimatsu (1981), Fukuyo (1985)
Alexandrium cohorticula	Fukuyo *et al.* (1988)
Alexandrium globosum	Dale (1977b)
Alexandrium hiranoi	Kita *et al.* (1985)
Alexandrium leei	Fukuyo *et al.* (1988)
Alexandrium minutum *	Bolch *et al.* (1991)
Alexandrium monilatum *	Walker et Steidinger (1979)
Alexandrium pervianum	Fukuyo *et al.* (unpublished data)
Alexandrium pseudogonyaulax	Montresor*et al.* (1993a)
Alexandrium tamarense *	Dale (1977b), Fukuyo (1985)
Helgolandium subglobosum	Von Stosch (1969b)
Pyrodinium bahamense var. *bahamense*	Wall et Dale (1969)
Pyrodinium bahamense var. *compressum* *	Steidinger *et al.* (1980),Matsuoka *et al.* (1989)
Pyrophacus horologium	Wall et Dale (1971)
Pyrophacus steinii	Wall et Dale (1971), Matsuoka (1985b)

Peridiniales

Scrippsiella crystallina	Lewis (1991), Ishikawa et Taniguchi (1993)
Scrippsiella lachrymosa	Lewis(1991)
Scrippsiella pentagonica	Akselman et Keupp (1990)
Scrippsiella precaria	Montresor et Zingone (1988),Ishikawa et Taniguchi(1993), Kobayashi *et al.* (1994)
Scrippsiella rotunda	Lewis(1991),Ishikawa et Taniguchi (1993)
Scrippsiella trifida	Lewis(1991)
Scrippsiella trochoidea *	Wall et al. (1970), Bolch et Hallegraeff (1990), Lewis (1991)
Scrippsiella sweenyae	Wall et Dale (1968b)
Ensiculifera cf. *mexicana*	Wall et Dale (1968b)
Ensiculifera carinata	Matsuoka *et al.* (1991)
Pentapharsodinium dalei	Dale (1977a, 1978), Lewis(1991)
Pentapharsodinium tyrrhenicum	Montresor *et al.* (1993b)
Cachonina hallii	Von Stosch (1969a)
Hetrocapsa triquetra	Braarud et Pappas (1951)
Protoperidinium americanum	Lewis et Doge (1987)
Protoperidinium avellanum	Wall et Dale (1968a), Matsuoka (1984a),Lewis *et al.* (1984)
Protoperidinium antarcticum	Akselman (1987)
Protoperidinium claudicans	Wall et Dale (1968a), Akselman (1987)
Protoperidinium compressum	Wall et Dale (1968a)
Protoperidinium conicoides	Wall et Dale (1968a), Akselman (1987)
Protoperidinium conicum	Wall et Dale (1968a), Fukuyo (1980),Kobayashi et Matsuoka (1984), Bolch et Hallegraeff (1990)
Protoperidinium denticulatum	Wall et Dale (1968a)
Protoperidinium divaricatum	Matsuoka *et al.* (1982)
Protoperidinium excentricum	Wall et Dale (1968a), Lewis *et al.* (1984),Akselman (1987)
Protoperidinium latissinum	Wall et Dale (1968a)

Protoperidinium leonis	Wall et Dale (1968a)
Protoperidinium minutum	Wall et Dale (1968a), Fukuyo *et al.* (1977)
Protoperidinium nudum	Wall et Dale (1968a)
Protoperidinium oblongum	Wall et Dale (1968a),Akselman (1987),Bolch et Hallegraeff (1990)
Protoperidinium obtsum	Akselman (1987)
Protoperidinium pentagonum	Wall et Dale (1968a),Matsuoka (1982),Lewis *et al.* (1984), Inoue (1990)
Protoperidinium punctulatum	Wall et Dale (1968a)
Protoperidinium subinerme	Wall et Dale (1968a)
Protoperidinium thorianum	Lewis *et al.* (1984)
Protoperidinium cf. *divergens*	Dale (1983)
Protoperidinium sp.	Dale (1983)
Diplopelta parva	Matsuoka (1988),Bolch et Hallegraeff (1990)
Diplopelta symmetrica	Dale *et al.* (1993)
Diplopsalis lenticula	Wall et Dale (1968a)
Diplopsalis lebourae	Matsuoka (1988)
Diplopsalopis orbicularis	Wall etDale (1968a), Matsuoka (1988)
Diplopsalopsis latipeltata	Dale *et al.* (1993)
Gotoius abei	Matsuoka (1988)
Oblea rotunda	Lewis (1990)
Zygabikodinium lenticulatum	Wall et Dale (1968a), Akselman (1987),Matsuoka (1988), Bolch et Hallegraeff (1990)

Part III

Monitoring and Management

21. Environmental Monitoring

T.J. Smayda

INTRODUCTION

In designing an environmental monitoring program, selection of which factors to measure, the techniques and frequency of measurement, and siting of the sampling locations are major considerations. A design in which the types, frequencies and locations of measurement are too superficial may produce data inadequate for their intended use; too complicated a monitoring design may be needlessly labor intensive and costly. A well-defined objective is therefore essential to the development of a monitoring strategy: what do you wish to monitor?, to assess?, why?; are the data to be used for observational purposes, such as tracking blooms?; for "*early warning*", such as presence or absence of a potentially harmful species?; to document ecological damage or dysfunction?; to develop predictive capability?; to safeguard aquacultural farming?, etc. It is important to recognize that there is no fixed (= "*canned* ") monitoring protocol; the details of such programs will vary with the objectives, habitat and its HAB populations. Investigators will certainly benefit from considering the procedures and approaches of studies carried out elsewhere, but their monitoring program must match their own specific objectives and site-specific conditions. In doing so, decisions will probably have to be made based on such intangibles as the investigator's *intuition* and *experience,* beyond consideration of the existing data base. Moreover, progressive refinements of the monitoring program will probably have to be made with increasing experience, particularly during the early phases of monitoring.

The level of insight expected from a monitoring program, i.e., ***awareness, observational, quantitative,*** or ***predictive,*** also influences the design. Monitoring carried out for observational purposes probably will not require concurrent measurements of processes such as phytoplankton growth, grazing and nutrient uptake rates. Monitoring may also focus solely on nutrients and oxygen levels without accompanying biological measurements. If surveillence for presence or absence of potentially harmful species is the objective, i.e., "***early warning*** " or "*alert*" monitoring, sample collection can be restricted to the seasons when these taxa are expected to occur. Concurrent physical and chemical measurements are probably not needed in such monitoring, unless one hopes to find a physical or chemical variable for use as a ***proxy variable.*** Proxy variables are often easier and less expensive to monitor, but they are inexact predictors of HAB species' occurrences and blooms (Morel and Anderson, 1976), which are under multifactorial control. If proxy variables are to be used, the statistical correlation between the proxy variable and the HAB aspect of interest should be evaluated using locally collected observations made over a suitable time period; anecdotal relationships are to be avoided, as is use of proxy correlations established for other regions.

If monitoring is undertaken to assess the potential role of anthropogenic influences on HAB events, such as aquacultural activities or novel species' introductions from other regions through discharge of ballast water (Hallegraeff and Bolch, 1992), the required effort is quite different. In these cases, knowledge of the *normal* or *baseline* conditions, including their variability, prior to such influences is needed. Such monitoring will probably require a long-term effort. If such information is lacking, attainment of the monitoring objective is compromised. Even with a limited *a priori* data base, it may be very difficult to distinguish real changes induced by habitat or biological modifications from those which reflect natural variance.

Although monitoring programs have different objectives, they share *three* common design elements. First, reliable measurements of the monitored variables are needed. The need for quantification is not relaxed, for example, if one is interested in establishing long-term

patterns of HAB species' occurrences and blooms *vs.* developing predictive capability of HAB events; only the scope of the inquiry changes, not the rigor of measurement. *Second,* site-specific and organism-specific features also influence the monitoring design. Where upwelling is a factor, for example, upwelling events must be assessed. At sites where flushing is important to HAB events, this physical feature is to be evaluated; where nutrient loading increases, nutrients should be measured; where aquacultural deployments are made, bottom water oxygen should be measured. When meroplanktonic species are involved, their resting stage dynamics can not be ignored in sampling design, a life cycle aspect irrelevant when dealing with holoplanktonic taxa (e.g., ceratians). When motile species are involved, their diel migrations can not be ignored, unlike for non-motile species. The *third* common design element deals with *common attributes.* That is, the monitoring program must be designed in accordance with the major ecological and behavioral features representative of the phytoplanktonic life mode *per se* and those habitat factors which regulate occurrence, growth and blooms. These aspects transcend phylogenetic, organismic and site-specific differences. For example, in assessments of bloom regulation the availability of nutrients can not be ignored, but it would make little sense to measure silicate in favor of ammonia when non-siliceous phytoflagellates are the focus. The ***functional scales*** at which the HAB organisms respond to their environment and the scales of habitat variability which influence this biology must take precedence over the ***convenience scales*** of investigator perceptions, methods, and work ethic (Andrew and Mapstone, 1987). To ignore that variability is an intrinsic aspect of life in a fluid medium, or to ignore the spatial- and time-dependent patterns and processes which characterize HAB events, compromises both data collection and interpretation.

Some general characteristics of the phytoplanktonic habitat, life mode and bloom events relevant to designing monitoring programs are presented in the following sections.

SAMPLING DESIGN

Habitat

The planktonic habitat undergoes continuous physical and chemical change of variable frequency, duration and intensity. This variability is associated with daily tidal and flushing cycles, current movements, runoff delivery of nutrients and their *in situ* utilization and recycling, etc. Seasonal changes and interannual variations in temperature, irradiance, precipitation, runoff, nutrient delivery and concentrations, mixed-layer depth, etc. also occur. Meteorological and climatological events are major causes of habitat variability and seasonal and interannual variations in environmental conditions, stochastic events which complicate monitoring. Since irregular or anomalous responses can accompany unusual physical and climatic conditions, the investigator will be continuously confronted with the issue of *how unusual are unusual events?*, particularly when results based only on short-term or discontinuous monitoring are being evaluated. Dramatic events such as HAB outbreaks, fish kills, hypoxia or anoxia reveal themselves with little or no need for pre-existing data or subsequent observations; they are probably noteworthy departures from the locally prevalent ecosystem structure and dynamics. However, to establish whether such dramatic events are symptomatic of dramatic change or progressive chronic modication and have, or will become frequent properties of the local ecosystem, i.e., more *usual*, requires a major, long-term monitoring effort.

The chemical habitat may also be modified by cultural eutrophication, occurring either as episodic, acute events or chronically, which alters the nutrient and "water quality" conditions (Fig. 21.1). Watershed management practises, including deforestation and agriculture, alter runoff, salinity gradients, flushing rates, chemical conditions, and irradiance available for photosynthesis. Trophodynamic processes (i.e., *internal* regulation) accompanying foodweb dynamics also influence nutrient levels and light transmission *in situ.*

Aquaculture can modify the chemical and oxygenation characteristics. These daily, seasonal and interannual variations in physical-chemical conditions resulting from both external and internal control mechanisms, and reflecting both natural and anthropogenic events, are some examples of the types of environmental variations that influence HAB events that must be considered in the design of monitoring programs.

Physical and Chemical Habitat Measurements

Physical features

What measurements should be made? Of the physical factors, temperature and salinity, minimally, should be measured; ideally along a gradient, particularly in inshore areas subject to riverine discharge or incursions of offshore current systems and upwelled watermasses. From temperature and salinity, vertical density profiles can be calculated and the degree of vertical mixing/stratification of the water column, or loci of upwelling determined. A generally held paradigm is that HAB events often follow a period of intense rainfall and runoff which increase water-column stratification, possibly enclosing a patch of chemically-modified, surface-layer water favorable to phytoplankton growth, followed thereafter by a period of intense sunlight. Measurements of the vertical salinity profile can reveal such a condition. If watermass stratification is already well established and a HAB outbreak follows this runoff event, a change in chemical water quality may be the more significant bloom stimulus than a reduction in turbulence which is known to inhibit cellular division in dinoflagellates (White, 1976).

From the gradient of horizontal and vertical salinity distributions, the flushing rate can be established, and wind-induced stowing of water in arms or *cul-de-sacs* of the embayment detected. The ratio of a HAB species' cellular growth rate to flushing rate influences its population growth rate; HAB bloom events are progressively more favored as this ratio increases above 1:1 (Seliger *et al.*, 1970; 1971). HAB bloom accumulations in the inner reaches of an embayment often result from wind-driven stowing of watermasses, rather than from particularly favorable local growth conditions in those locations (Fig. 21.2). Thus, local meteorological conditions must also be considered during monitoring.

Well established physical events contributory to HAB outbreaks include entrainment of "seed" populations of HAB species in watermasses flowing into local habitats (Tester *et al.*, 1991), or within the circulation patterns of a given region (Fig. 21.3; Tyler and Seliger, 1978); regional incursions of more fully developed populations within currents subject to alongshore transport (Fig. 21.4, Lindahl, 1986; Franks *et al.*, 1989; Franks and Anderson, 1992); relaxations of upwelling events (Blasco, 1977; Fraga *et al.*, 1988); tidal fronts (Pingree *et al.*, 1975); and frontal zone dynamics (Seliger *et al.*, 1981; Le Févre, 1986). Monitoring of such physically-driven events is considerably more difficult, requiring a large inter-disciplinary research team, access to a wide variety of physical oceanographic instrumentation and ships, and is costly. The investigator should consider the use of satellites and other remote sensing detectors to establish the local incursions, frontal structure and regional movements of such watermasses based on their temperature and chlorophyll signatures. Such approaches have been successfully used in open coastal waters (Dundas *et al.*, 1989; Tester *et al.*, 1991; Keafer and Anderson, 1993).

Chemical features

Measurements of physical features are also essential because they partially control watermass chemistry. The chemical habitat is thought to influence HAB events in two major ways: in species' selection and bloom dynamics. However, field measurements of chemical conditions currently have little reliable, predictive value of whether, where, and when a HAB event will occur, or what species will be responsible. Nonetheless, there is a growing body of evidence which suggests HAB events tend to occur with greater frequency and magnitude in nutrient-enriched waters, with a further tendency for such blooms to increase progressively with nutrification (Fig. 21.5; Smayda, 1989; 1990; Hickel *et al.*, 1993; Honjo, 1993). The long-

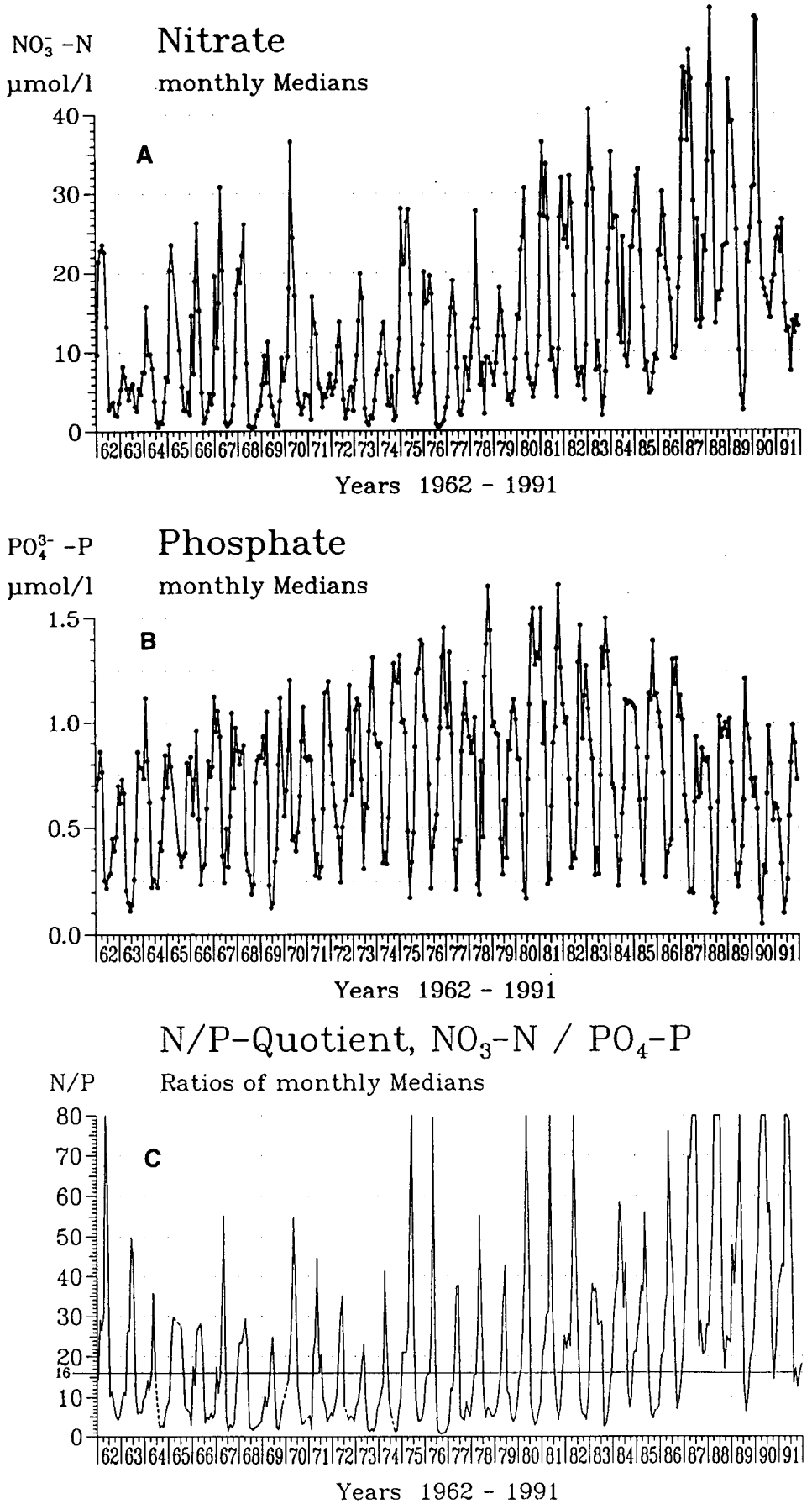

Figure 21.1. Dissolved nitrate and phosphate concentrations from 1962-1991 in the German Bight near Helgoland, as monthly median values, and the molar nitrate : phosphate ratio. (From Hickel *et al.*, 1993).

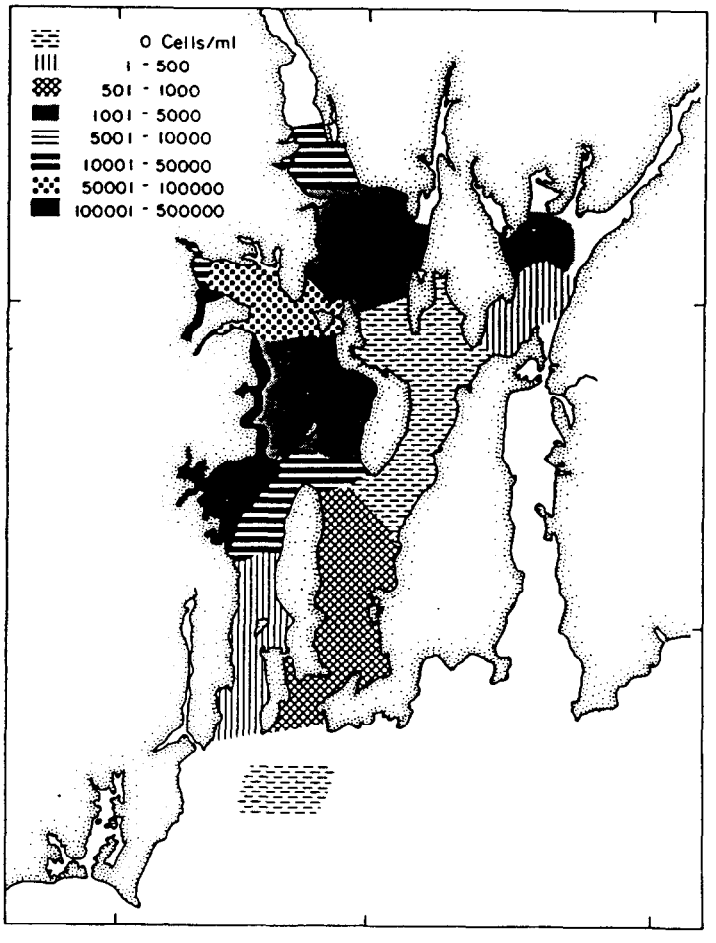

Figure 21.2. Regional abundance patterns of *Heterosigma akashiwo* during a bloom within Narragansett Bay showing elevated accumulations in *cul de sacs* and shallow embayments. **A** represents site of permanent monitoring station, and **B** location of the innermost station of a seven station transect extending from **A** to **B**, as further discussed in the text (From Tomas, 1980).

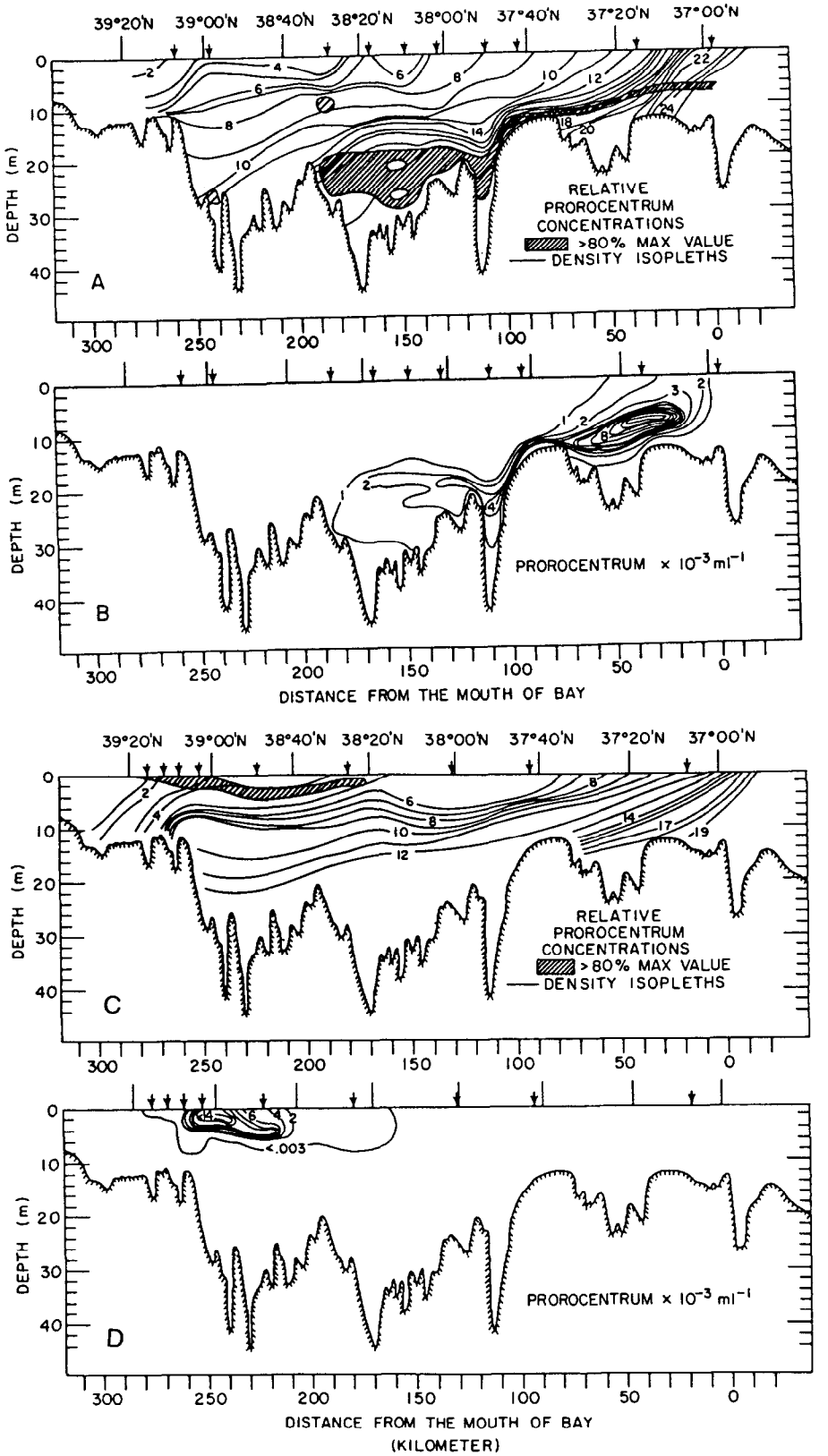

Figure 21.3. Sub-surface distribution (x 10^{-3} cells ml^{-1}) of *Prorocentrum minimum* and its transport within the Chesapeake Bay along with associated density isopleths (s_t x 10^3) during late winter (Panels A, B) and summer (Panels C, D). (From Tyler and Seliger, 1978).

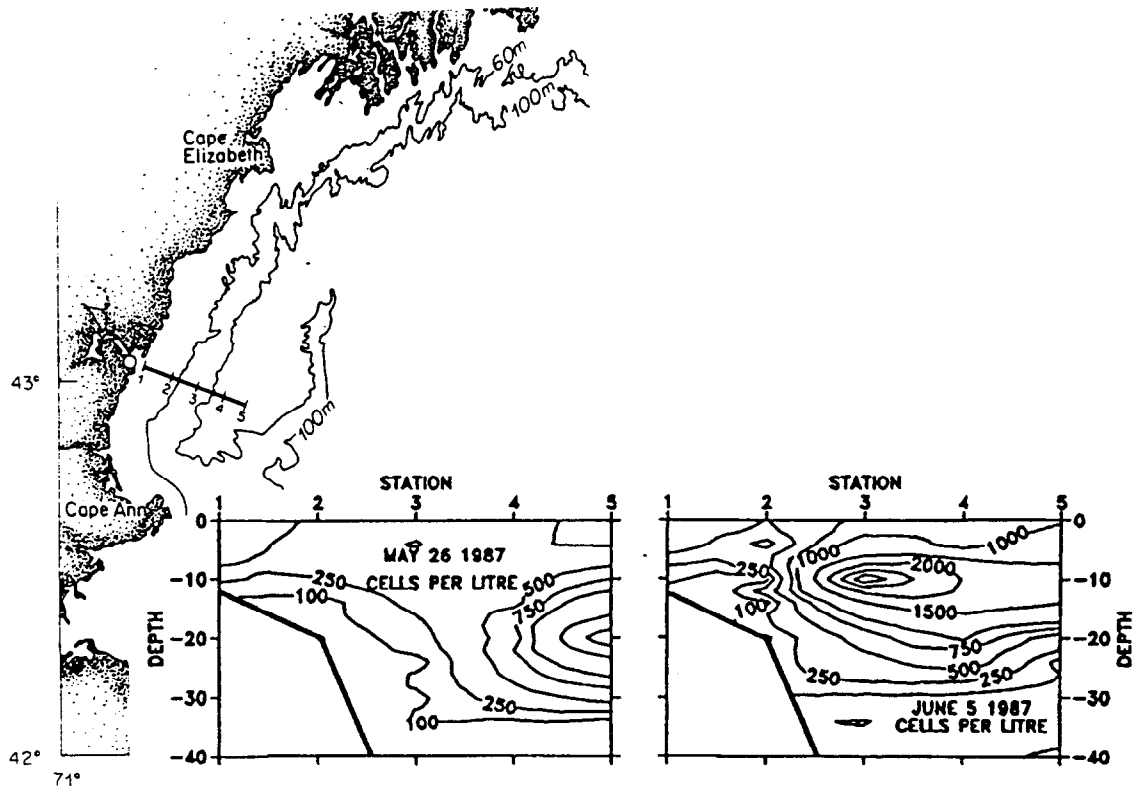

Figure 21.4. Vertical cell concentrations of the dinoflagellate *Ceratium longipes* in a southward flowing current along a 30 km onshore-offshore transect in the southwestern Gulf of Maine. Note differences in cellular concentrations, locations and depths of maximal abundance, apparently in response to wind-driven dynamics. (From Franks *et al.*, 1989).

term increases in blooms of *Phaeocystis pouchetii* in Dutch coastal waters (Cadée and Hegeman, 1986), *Prorocentrum cordata* in the Black Sea (see Smayda, 1990) and flagellates in the German Bight (Hickel *et al.*, 1993) accompanying progressive nutrient enrichment are some examples of this phenomenon. Failure to include nutrient measurements in the monitoring program will ordinarily seriously compromise such efforts, and result in significant loss of information vital to understanding the still unresolved linkages between the chemical environment and HAB events.

Major nutrients

The chemical milieu of the planktonic habitat is extremely complex, naturally variable, and subject to seasonal, regional and long-term modifications. Monitoring of macro-nutrient levels provides several insights. Long-term measurements of dissolved inorganic NH_4, NO_3 and PO_4 in riverine discharge and recipient waters can reveal the trends in riverine macro-nutrient delivery associated with watershed activities, and whether recipient waters are becoming progressively nutrient-enriched. The spatial heterogeneity in nutrient gradients within a region and onshore-offshore can reveal the potential for bloom outbreaks. Nutrient measurements prior to, and during aquacultural activities can establish the nutrient retention and bloom characteristics of the aquacultural site subject to increased nutrient excretion. The gradients in salinity plume structure driven by runoff can be expected to have associated changes in macro-nutrient levels leading to differences in actual or potential carrying capacity for HAB events. Investigators are, therefore, strongly encouraged to include chemical measurements in their monitoring program. The aforementioned, provocative, widespread accounts reported in the literature that HAB outbreaks often are triggered by a sequence of intense rainfall, runoff and high irradiance, is a phenomenon which is probably chemically mediated, in part. That is, runoff delivery of nutrients and humic substances may increase population carrying capacity, detoxify the habitat, or in some other way improve overall water quality leading to bloom development in the chemically-conditioned watermass (Granéli and Moreira, 1990). Investigators seeking cues as to the periods and locations of potential HAB outbreaks should consider the rainfall-runoff event during periods of watermass stratification and locations of the nutrient-enriched regions in designing their monitoring efforts. Chemical conditioning accompanying chronic, or episodic eutrophication events may select for HAB species through changes in nutrient ratios (Fig. 21.1C) which alter inter-specific nutrient resource competition (Smayda, 1989, 1990).

Measurements of inorganic nitrogen and phosphorus and oxygen are the minimal chemical monitoring measurements which should be made, particularly where anthropogenic nutrient modification is occurring. The investigator is reminded that measured nutrient levels represent only residual concentrations and provide no information on delivery and uptake rates. In seeking to establish the latter, process measurements are needed, as well as calculations of loading rates based on river flow and nutrient levels of the freshwater inputs. While micro-nutrients unquestionably influence HAB taxa, with the evidence for iron regulation and cupric ion sensitivity being best known (see Takahashi and Fukazawa, 1982; Doucette and Harrison, 1990; Anderson and Morel, 1978), the stringent sampling (="*clean techniques* ") and analytical requirements make routine monitoring of micro-nutrients difficult to achieve. The investigator would be advised to concentrate on macro-nutrient monitoring, at least during the initial stages of the program.

Oxygen

Monitoring of seasonal water-column oxygen levels, with emphasis on bottom water concentrations, is essential in shallower, poorly flushed coastal waters; at aquacultural sites; and in regions exhibiting environmental degradation. Routine monitoring in these regions may be needed, if seasonal oxygen concentrations decline [prior to HAB outbreaks] to low levels, and are potentially vulnerable to even further reduction to hypoxic or anoxic levels during deposition of ungrazed HAB events. A progressive decrease in bottom water oxygen levels accompanied by periodic dieoffs of animals has been noted in poorly flushed regions subjected to increased nutrient loading and phytoplankton abundance. Increased

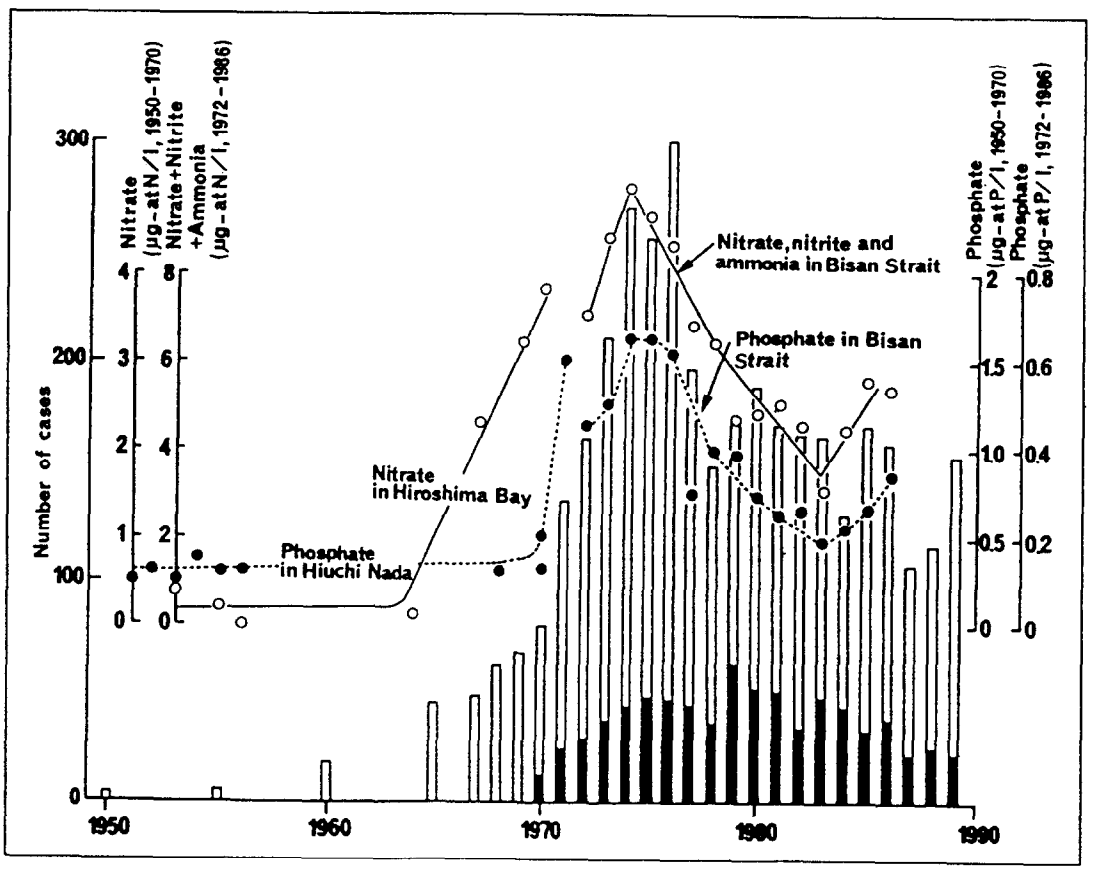

Figure 21.5. Concentrations of inorganic nitrogen and phosphate from 1950-1989 in Seto Inland Sea, and number of cases of red tide outbreaks (open histograms) and blooms of the rhaphidophyte *Heterosigma akashiwo*. (From Honjo, 1993).

phytoplankton biomass during specific, often poorly grazed bloom events, such as during *Ceratium* blooms (Falkowski *et al.*, 1980; Hickel *et al.*, 1989), becomes nutrient-limited, sinks to bottom-waters, and decomposes there leading to hypoxia or anoxia. In some instances, measurement of chemical oxygen demand (COD) or biological oxygen demand (BOD) might be warranted, an approach which was helpful in designing criteria to curb HAB outbreaks in the Seto Inland Sea (Okaichi, 1989).

HAB Organisms

The degree to which the eco-physiological requirements of the HAB taxa are matched by the physical-chemical habitat determines their occurrence and whether they bloom. The presence of a HAB species does not mean that it will bloom; that event is triggered by regulatory factors separate from those favoring survival of the seed stock. Moreover, if the species are not autochthonous in the region being studied, their seasonal and annual occurrence patterns may depend upon periodic seedings through watermass movements. Some introductions will be "sterile", since they may occur at a time when the recipient watermass will not support survival, let alone a bloom event, of the inoculated population. At other times, such seedings are major triggering events of a HAB event because the habitat growth properties then match the eco-physiological requirements of the HAB species. In still other instances, periodic introductions of a novel species may eventually lead to its obtaining a "foothold" within the local phytoplankton community, allowing it to survive year-round, thereby becoming indigenous, and eventually even to bloom; a sequence that may take many years. Spreading of the dinoflagellate *Pyrodinium bahamense* var. *compressum* in the Indo-Pacific region may have been accomplished by this stepwise emigration-colonization mechanism (Seliger, 1993).

Recorded absences of HAB species do not necessarily mean that they are permanently excluded from the region, or will not be a future problem. Effective design of monitoring programs requires that one consider the most likely candidate species of potential HAB events, and become familiar with their regional distribution, seasonal occurrence patterns and available eco-physiological data. The significant variability that generally characterizes the phytoplankton must also be considered in designing surveillence programs to detect HAB species' occurrences and bloom events. Such variability occurs in **various patterns:** as cycles, trends, fluctuations, unusual events, irregular pulses; at **various scales:** daily, seasonally, annually, decadally, etc.; and at **various frequencies.** The entire community may fluctuate (= respond) as a unit, or as individual species. In monitoring for a given species, whether as an "early warning" system or in following its bloom dynamics, the principal organizational features of the phytoplankton community, which pose serious methodological problems, also must be dealt with. These are: *a phytoplankton community is a polymixture of species having different abundances; each species may be in a different phase of its own bloom cycle; and collectively these species are transitional components in the progression to the next successional stage.*

If the monitoring objective is only to detect presence or absence, even though quantitative sampling to establish abundance level is not needed, the sampling procedure *must* be adequate to detect very low levels of occurrence. Should the taxa be members of the "***hidden flora*** ", their collection may require processing a m^3, or more of sample. If the organism is large enough, a net tow of suitable mesh size should suffice. Net tows can even be made for nanoplanktonic-sized taxa. However, when using net tows the investigator should be prepared to apply suitable sub-sampling procedures and to spend a lot of time finding the *"needle in the haystack"* among the significant amounts of other material collected. Counting for the presence or absence of potential HAB species is the fastest method of "counting" because of the rapidity with which it can be determined, whether or not the observed specimen is the species of interest. However, the speed with which this can be done depends on the relative abundance of that taxon in the sample and how large a sample will be searched. In order to establish the absence of a species from a sample at some significant level (a), one needs to count (1 - a) per cent of the sample (Venrick, 1978a). Thus, to establish

absence at a 5% significance level in a sample of 1,000 specimens, it would be necessary to examine 950 of them. But although this technique is relatively easy, mere *presence/absence* assessments are undesirable because of the considerable loss of information; quantitative estimates of abundance are the more desirable index, particularly if ancillary data on physical and chemical environmental variables are being collected. The *presence/absence* approach has compromised assessments of HAB events in another way. To a surprisingly large degree, investigators reporting such events often merely identify the apparently dominant HAB species without accompanying information on their abundance or that of other members of the phytoplankton community. This thwarts quantification of HAB events and dynamics. For information on how many cells to count, Venrick (1978a) should be consulted. The manual of sampling and methodological techniques edited by Sournia (1978) also presents material useful to monitoring HAB events, and should be consulted.

In the case of tracking bloom species' dynamics, the opposite monitoring problem is confronted. The super-abundance of the HAB species tends to deflect the investigator from considering the co-occurring species and their abundances. The impression usually given in the numerous reports of HAB blooms is that they are "*mono-specific*" events; but where detailed community analyses have been carried out (see Smayda and Villareal, 1989), this is hardly the case (Fig. 21.6). This methodological shortcut becomes an issue where monitoring programs are designed to evaluate the regulation of HAB dynamics; these are obviously under collective internal, interactive physical-chemical and meteorological control. That is, inter-specific competition for nutrients, allelochemical effects and differential grazing are involved in the HAB event, and unless these are evaluated, focus on the HAB species alone compromises the intended application of that particular monitoring effort.

To summarize, the common plankton attributes also characterizing HAB taxa must be considered in monitoring design strategies: the taxa occur in communities of polymixtures of species; have variable abundance, seasonal preferences, and exhibit interannual variations in occurrence, abundance and bloom events. These may be entrained in rhythmic cycles (White, 1987) or show long-term trends (see Smayda, 1989, 1990). Within a given HAB event several HAB species may be exhibiting concurrent blooms, with their bloom stages being either in, or out of phase with each other. Termination of one HAB event may be followed by another bloom resulting in a series of HAB outbreaks during a given year, such as found in Narragansett Bay (Fig. 21.6) and in Tolo Harbour, Hong Kong (Wong and Wu, 1987), for example. Monitoring programs must be prepared to handle such situations.

Special Characteristics Influencing Sampling Design

In situ distributions of phytoplankton are *not* spatially uniform, nor do their distributional patterns exhibit smooth gradients of abundance (Cassie, 1963). Considerable spatial discontinuity occurs at every level of community organization and dynamics; in species occurrences and co-occurrences; in abundance; successional stage; community diversity, and dynamics. Tests for random spatial distribution of phytoplankton based on analyses of departure from *Poisson* occurrence, which describes the distribution of random, rare events (Venrick, 1978a), have proven to be negative (Cassie, 1963). Non-random distributions (= *over-dispersion)* characterize phytoplankton, and are a consequence of spatial heterogeneity (= *patchiness*) in growth variables, community processes, and physical effects. The designation of this phenomenon as *over-dispersion* does not refer to the dispersal or physical spreading of phytoplankters within their habitat. Rather, it is a mathematical term referring to the spread of numerical abundance estimates about the mean abundance estimate for samples collected within a given sampling grid over time. A vivid demonstration of such over-dispersions is the water discolorations (=*swarming*) and their patterns which sometime accompany HAB outbreaks. Even if water discoloration is not evident, contagion, aggregation or clumping of the cellular distributions contributing to non-randomness still occur. Two of the major environmental factors contributing to over-dispersion which need to be considered in designing monitoring programs are as follows.

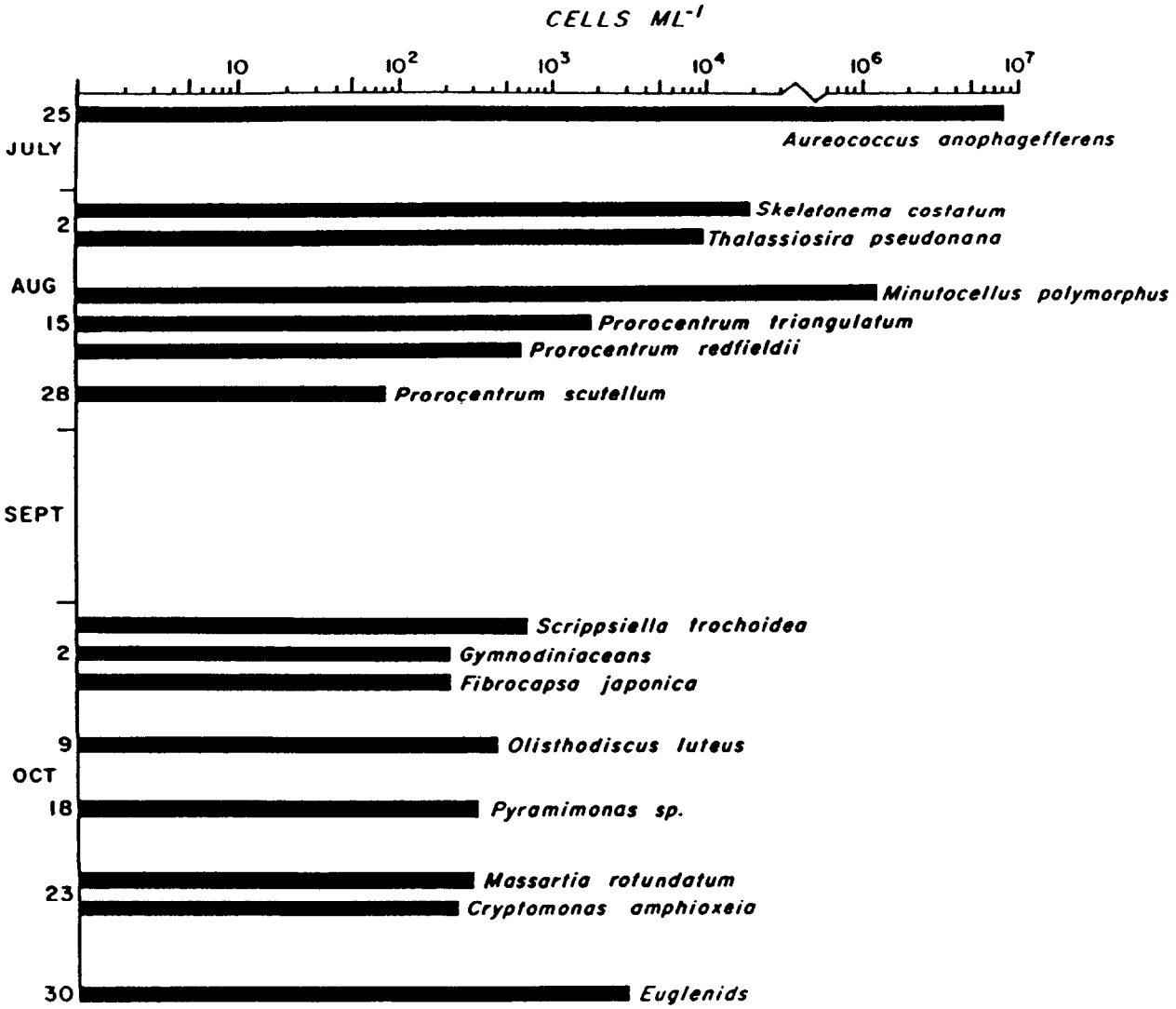

Figure 21.6. Successional pattern of the major bloom species during a 1985 "brown tide" event in Narragansett Bay, including the time and magnitude of their maximal abundance. Note: 15 different taxa bloomed during this five month HAB event. (From Smayda and Villareal, 1989).

Static factors

Small-scale, micro-distributional patterns resulting from slightly different growth-promoting conditions occur. This is illustrated in Table 21.1A which shows the surface distributions of three dinoflagellate species and accompanying temperature and salinity at four horizontal space intervals along a 250 m transect sampled at two locations. Along transect #1, characterized by uniform temperature, but a ca. 3‰ range in salinity, a 5- to 10-fold range in abundance characterized each species. Along transect #5, characterized by nearly uniform temperature and salinity, abundance of two of the species was similar, but varied ca. 3-fold for the more abundant *Prorocentrum micans.* If it had been decided to sample only at 5 m, a similar 10-fold variation in abundance would have been found, but the population at this depth is significantly lower than that at the surface. Moreover, if the decision had been made to collect only one sample along this transect, and 5 m at site 1d was selected, the population estimate for *Prorocentrum micans* would have been 540 cells liter^{-1}; but if the sample was collected at the surface only 25 m away (site 1c), the population estimate would have been 33,100 cells liter^{-1}, ca. 60-fold greater.

Small-scale, micro-distributional patterning of HAB cells into ephemeral bands, streaks and patches of variable size resulting from wind-induced filaments of circulating water, and which complicate sampling, also occur (Barstow, 1983; Ryther, 1955). The chapter by Franks (this volume) should be consulted for further discussion of such Langmuir Circulation effects.

Phototaxic migrations

HAB flagellates often exhibit significant diel variations in vertical distribution because of their phototaxic behavior or semidiurnal entrainment in internal tides (Kamykowski, 1976). Some species exhibit a "night rise - day descent" strategy; others the opposite diel migratory pattern (Hasle, 1950, 1954a; Blasco, 1978; Kamykowski, 1981). Diel abundance at different depths in the water-column can vary significantly, therefore. In the example given in Table 21.1B, abundance of *Prorocentrum micans* (sampling hours unknown) was fairly uniform in the upper 10 m at transect site 1a, whereas at sites 1c and 1d the population was highly aggregated at the surface. The consequence of vertical migratory behavior is that no two samples, unless they represent the entire water column (i.e., pooled samples) and are collected at the same time of day under the same light conditions, are strictly comparable. Diel migratory behavior poses significant sampling problems that have been traditionally neglected in field studies; among other requirements, it is highly demanding in terms of labor. However, if models for prediction and mitigation application are to be developed using the results of the monitoring program, then knowledge of diel migrational behavior is essential (Kishi and Ikeda, 1989). Diel migration also influences retention within an estuary (Anderson and Stolzenbach, 1985). Daily variations in vertical distribution patterns are not only consequences of diel migration. Internal wave dynamics (Kamykowski, 1976) and directed motility along pycnoclines (see Pingree *et al.*, 1975) also contribute to over-dispersion. Cassie (1963) concluded that given the over-dispersion characterizing phytoplankton, the ideal sampling strategy of being able to collect only one, or relatively few samples to estimate accurately phytoplankton population composition, abundance and dynamics is not realizable. Rather, to achieve a more accurate estimation of these features a large number of smaller samples (for example, 1-liter samples) will have to be collected than a smaller number of larger volume samples (for example, 5-liter samples). This translates into sampling a greater number of stations and depths. This effort can sometimes be reduced by using an integrated sample over the water column or mixed layer composited by pooling the samples collected at the discrete sampling depths.

Tables 21.1A, B. Small-scale and Meso-scale Horizontal Patchiness at Surface, and Vertical Patchiness in Distribution of Dinoflagellates (as cells 50 ml^{-1}) in Oslofjord [distance between a and b; c and d = 25 m; b and c = 250 m; stations 1 and 5 = 20 km] (modified from Hasle, 1954b).

A. Small-scale and Meso-scale Horizontal Patchiness:

Station:	1a	1b	1c	1d
Temperature	17.7	17.5	7.5	17.9
Salinity (‰)	11.67	12.74	13.37	14.42
Ceratium furca	6	45	18	31
Dinophysis acuta	2	8	4	10
Prorocentrum micans	148	441	1655	1240
Pror. micans at 5 m	153	218	149	27
Station:	5a	5b	5c	5d
Temperature	18.1	17.9	17.9	17.9
Salinity (‰)	16.44	16.20	16.62	16.38
Ceratium furca	107	131	96	80
Dinophysis acuta	82	66	56	75
Prorocentrum micans	450	1170	1386	922

B. Vertical Patchiness:

Depth (m)		0	5	10	25
Prorocentrum micans:					
Station	1a	148	153	110	2
	1b	441	218	16	0
	1c	1655	149	19	0
	1d	1240	27	14	0

Monitoring the HAB Effects of Environmental Perturbation

If chemical or other environmental perturbations from agro-industrial-domestic waste discharge, seafloor mining, aquacultural deployments, mangrove destruction, or other habitat modifications are suspected of stimulating HAB events, it is essential that *control* sites free from such contamination be available and sampled during the monitoring program. Without controls, suspected effects of habitat modifications will not be distinguishable from stochastic processes, natural variance, or localized events and factors operative within the sampling grid (Andrew and Mapstone, 1987). Controls may be of two forms: a specific, control sampling site, or a temporal (i.e., time-series) data set at a given sampling site within the perturbed region, with the retrospective time-series begining prior to initiation of environmental modification.

SAMPLING

General Aspects

Whether the goal of the HAB monitoring program is purely descriptive, or to seek statistical correlations between specific HAB characteristics and the presumed environmental regulatory variables, these different objectives have similar, basic *in situ* sampling requirements and constraints. The investigator must first define the ***target variables***, i.e., a HAB species,

temperature, a nutrient, etc., about which information is sought, and also ensure that the conclusions derived from this information will be applicable to the HAB species, event, its habitat, etc. This requires that representative samples be collected; to achieve this requires a sampling strategy which considers the characteristics each of the target variables possesses. Following selection of the target variables and identification of their relevant characteristics, there are *three* additional requirements: identify the sampling unit (=gear) needed; select the sampling grid [if the sampling units differ with the variable to be analyzed, these must be combined into a useful framework]; and set up a sampling schedule (Venrick, 1978b). The sampling unit may be a surface bucket sampler, a water-bottle sampler, a vertical pump, or a net-tow, etc. If, for example, both 20 μm (or finer) mesh net-tows to sample larger and/or "hidden flora" taxa (see Turner *et al.*, 1995) and measurements of nutrients (which require water-bottle samples) are needed, then different sampling modes will be part of the field program. This requires appropriate orchestration to ensure the data can be compared.

Selection of Sample Collection Sites

In selecting the sample collection sites, there are two options: use of *randomly selected stations*, or *fixed-station* sites. Randomly selected stations might facilitate "early warning" monitoring programs for potentially problematic species, but this sampling strategy has the disadvantage that comparisons of changes between sampling dates are compromised. The use of fixed-station locations, which is recommended for more quantitative studies, diminishes this problem. The investigator must next determine the number and locations of the sampling stations [see below], and the *duration* and *frequency* of sampling; two aspects highly dependent upon the objectives of the program. If there is need to establish long-term patterns in HAB events, the monitoring program obviously must continue over several years. If the objective is to monitor for potential HAB events during a given year within a region, then sampling should be carried out during the "HAB season". It should be possible to define this period from previous HAB or benign "red tide" events described for that location, or contiguous waters. If such information is lacking, then in temperate and boreal waters the period of watermass stratification, usually from late-spring to early-fall, could be targeted for such monitoring. In tropical and sub-tropical regions, stratification may occur nearly year-round [sometimes interrupted by localized upwelling], facilitated both by seasonal monsoons and temperature conditions. This compromises *a priori* selection of the "HAB season" in unexplored tropical and sub-tropical regions, but it does appear that these regions may, in fact, have year-round HAB potential, i.e., a wider annual window of occurrence than in other biogeographical regions (Fig. 21.7), which the monitoring program should be prepared to address. A close watch on meteorological conditions may help establish situations when the potential for a HAB event increases, either in consequence of localized growth, or from wind-driven accumulations of noxious species, such as *Trichodesmium* windrows. With regard to *duration* of monitoring, HAB events may vary in duration from several days to several years. As with all aspects of monitoring design strategies, the objectives of the program will determine the duration of sampling needed.

Frequency and Duration of Sampling

With regard to *frequency* of sampling, the general experience of investigators is that HAB events are basically fast-moving, ephemeral outbreaks. The intrinsic growth properties and environmental regulation, together with meteorologically induced disruptions and accumulations, combine to influence the transitory occurrences of HAB outbreaks. Even though the actual growth kinetics may be slow, physical environmental disturbances may determine the speed with which the HAB event appears and disappears. *The basic monitoring strategy, therefore, is for a high sampling frequency,* particularly when an understanding of environmental control is the objective. Ideally, samples should be collected on a daily basis,

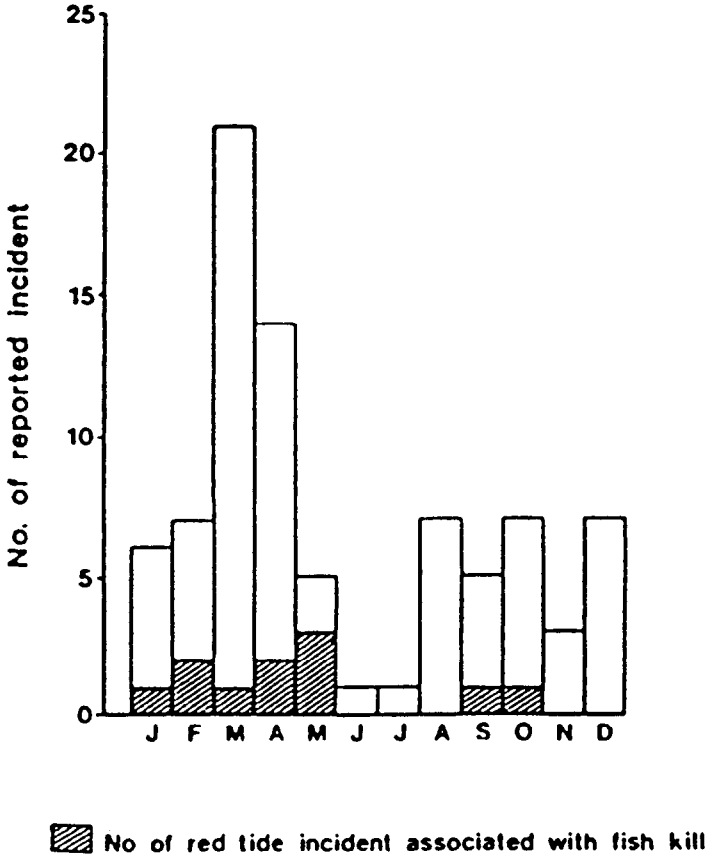

Figure 21.7. Seasonal variation in frequency of occurrence of red tide and fish kill incidents reported between 1975-1984 in Hong Kong waters. (From Wong and Wu, 1987).

but certainly not less than once per week during longer lasting blooms. The logistics of this sampling requirement obviously are more easily met in coastal regions, from which land-based monitoring operations can be launched, than in continental shelf waters and inland seas requiring expensive ship-based operations and large spatial sampling grids. There are also instances when diel sampling will be desirable to unravel the basic features of the phototaxic, migratory behavior of HAB flagellates (Blasco, 1978; Hasle, 1950; 1954a; Kamykowski, 1981). In "early warning" programs one might sacrifice frequency of sampling for increasing the number of stations sampled to achieve greater regional coverage. In some instances, because of the size of the sampling grid and workload it might not be possible to visit all stations on a given sampling date. Every effort should be then made to sample the remaining stations as soon as possible thereafter (preferably the next day) to achieve more accurate typification of the HAB outbreak during that phase of its bloom cycle.

The duration of the sampling period to be followed for any given HAB event is not fixed; it will vary with the bloom species and growth conditions. HAB events lasting less than a week are not unusual, but have lasted for five months (Smayda and Villareal, 1989) and, as in the case of a "*brown tide* " in Laguna Madre, Texas, even years (Stockwell *et al.*, 1993).

Sample Collection

Excepting the special circumstances when net tows may be needed to sample larger HAB species, if they are then components of the "hidden" pre-bloom flora (Turner *et al.*, 1995), the preferred sampling device is a standard water-bottle sampler. The total sample volume needed will reflect the sub-sample needs for the plankton and chemical variables being monitored; but, at least a 1-liter sample should be collected. Selection of the collection depths at the sampling stations is not a trivial decision, nor is there a pre-set protocol. To set this depth at 5 m, for example, would define target phytoplankton community HAB events based on dynamics only at 5 m. The investigator is then confronted with the issues: how representative of the water-column at this station are the observed dynamics and measured environmental variables, and how representative of the entire site are these local observations? The need for suitable representation influences the number of depths and stations to be sampled in the grid. For very localized blooms, the number of stations needed will probably be less than if a very widespread HAB event occurred. A useful procedure is to divide the region into *zones* that differ in some major physical, chemical, geo-morphological or biological features, and then locate stations within each zone. Preliminary sampling to define these zones, based on their spatial variability and gradients in environmental and plankton properties, may have to be carried out. Clearly, the objectives of the monitoring program will influence the station grid.

With regard to *sampling depths*, the investigator has three options: sampling at a fixed depth; at multiple depths within the euphotic zone; or pooling a sample made up of equal volumes collected from several depths within the water column. Often, single depth sampling is employed, usually at, or near the surface. The disadvantage is that relevant sub-surface dynamics and habitat conditions important in the HAB event, particularly for migratory flagellates, are excluded. Multiple depth sampling is the strategy of choice, accordingly. Use of pooled samples made up with equal portions from several depths partially circumvents the limitations of single depth sampling. While measurements are fewer, less labor intensive and less expensive for pooled samples than discrete, multiple depth sampling, this compromises statistical analyses of linkages between the HAB event itself and its environmental regulation. The parameters measured in pooled samples represent only *average* water-column conditions, a statistic that does not reflect the vertical habitat zonation in, distribution of, and gradients in the physical, chemical and biological variables which influence HAB dynamics.

Population Analyses

The design and selection of procedures to assess environmental regulation of HAB events in monitoring are influenced by certain general properties of community dynamics and mode of inimical action of the HAB taxa. Presently, ***cell number*** is the preferable measure of HAB population abundance. Although microscopic identification and enumeration of phytoplankton species are tedious and time consuming, they provide high quality, and otherwise inacessible information (provided the taxonomic identifications are reliable) needed to evaluate HAB events. Chlorophyll measurements are routinely used to estimate total phytoplankton community biomass, but it is difficult to establish the population level and bloom dynamics of the HAB species from such measurements. Use of specific pigment measurements unique to the HAB species as a quicker, equally reliable measure of abundance requires technique development (Bidigare, 1989; Gieskes and Kraay, 1983, 1986). Even in virtually mono-specific blooms, chlorophyll levels may be an inadequate or incomplete parameter. For example, a "*brown tide* " outbreak of the 2 μm non-motile, picoplankter *Aureococcus anophagefferens* in Narragansett Bay, during which chlorophyll levels were similar to the winter-spring bloom maximum, was harmful to some trophic components because its exceptional population abundance of 2 x 10^9 cells liter^{-1} reduced light transmission suitable for macroalgal photosynthesis and growth, and also blocked filter feeding in mussels, leading to a dieoff of both trophic components (Smayda and Villareal, 1989). During this same event, however, *Aureococcus* was antagonistic to the herbivorous copepod *Acartia tonsa,* either because of its small size or secretion of substances inhibitory to grazing (Durbin and Durbin, 1989). At the other extreme [*at abundance levels six orders of magnitude lower!*], *diarrhetic shellfish poisoning* of humans associated with the dinoflagellate genus *Dinophysis* may occur at very low population densities of the latter, < 10^3 cells liter^{-1}, and without effect on the shellfish (Belin, 1993).

Thus, inimical effects during a HAB episode may result from very high or very low biomass bloom events; occur at very low or very high cellular abundances; be due to very small or to very large species' size; and within this variance the same HAB species may be simultaneously inimical to different trophic, or within trophic components for different reasons. Thus, the anecdotal perception of a "*red tide* " bloom or HAB event as being one of exceptional cellular abundance leading to watermass discoloration is not only incorrect, but if this visual display [note: *benign blooms can also discolor* !] stimulates the monitoring effort, then the investigator must understand that the factors triggering this bloom are no longer accessible; much information is lost; and monitoring initiated at that point will be a description of bloom dynamics and associated variations in the measured environmental parameters at an unknown point in the *growth phase,* or later stage in the bloom cycle. Given these factors, formal definitions of what makes a bloom (pulse) a harmful algal bloom (pulse) are not needed, practical, nor very helpful in the design and initiation of monitoring of HAB events. A more relevant aspect is whether a species is a HAB taxon and, if so, what are its life cycle and eco-physiological properties.

In interpreting the associations between the HAB event and the other biological (i.e., non-phytoplankton) and chemical variables monitored, the investigator must recognize that the observed HAB dynamics are influenced by overall community structure and interspecific competition among the phytoplankton species and among grazers. The presence of each species, for example, reduces the capacity of the environment to support all other species competing for a common pool of available nutrients. Such interactions cannot be ignored (see Barnes and Hasle, 1957). If observations on herbivorous zooplankton are lacking, for example, conclusions regarding HAB species' selection or bloom regulation based on statistical correlations derived using this incomplete data base on relevant regulatory variables must be tempered by the knowledge that no information on the role of grazing in regulating observed bloom dynamics is available. This is not to discourage efforts of statistical correlation; indeed, they are currently much needed and encouraged for hypothesis formulation and testing experimentally.

A Phytoplankton Monitoring Program

Table 21.2 summarizes an ongoing monitoring program in Narragansett Bay which may be helpful to those seeking to design such an effort. Although designed to describe patterns, trends and variability in habitat and phytoplankto dynamics and relationships, this program has proven to be suitable for monitoring of HAB events (Smayda, 1984; Karentz and Smayda, 1989; Smayda and Villareal, 1989). The variables measured and sampling design elements in the Narragansett program are common to phytoplankton monitoring generally; but the number of stations, selection of sampling depths, etc. will vary with site-specific properties and monitoring objectives. The use of continuous vertical profiling instruments will also influence the monitoring protocol.

In this monitoring program, a permanent station is sampled at weekly intervals (since 1959), located at a representative site (A in Fig. 21.2). This station is also the end member of a 7 station transect (ending at site B in Fig. 21.2) extending ca. 40 km inwards within the bay along an increasing nutrient and decreasing salinity gradient. The transect stations are periodically sampled for special studies not resolvable by sampling at the permanent station only. The transect is also reactivated during HAB events, with the sampling frequency increased to 2 - 3 times weekly. Except for seasonal stratification in its innermost reaches, Narragansett Bay is well-mixed year round. This feature and its relatively shallow mean depth (9 m) has led to the selection of three depths (top, mid and bottom) for routine sample collection. Mid and bottom sampling depths are selected at the time of each sampling, after on-station measurement of water column depth, which varies with tidal phase. Samples are collected without regard to tidal phase. A 5-liter sample is collected at each depth by water bottle; from this, a 2-liter sub-sample is taken for further sub-sampling for the individual nutrient and phytoplankton analyses (Table 21.2). A 3-liter "pooled" sample is also composited (when needed) by mixing equal volumes of seawater collected from the three sampling depths. "Pooled" samples are used as an alternative option to discrete samples to determine average or integrated water column levels of phytoplankton abundance and species composition, for measurements of primary production, and fractionation of the phytoplankton community into its various size classes (Table 21.2). Since the causative species of HAB events usually falls within a narrow size-class band, or is potent at relatively low levels of abundance, i.e., *Dinophysis* spp., size fractionation can facilitate its assessment. Studies on the size-classes comprising the phytoplankton communities are also carried out when the desired information cannot be accessed from total community analyses. The pore or mesh sizes used for size fractionation vary with size of the phytoplankters of interest. Of the variables listed in Table 21.2, only size fractionation is not a routine measurement. For zooplankton collections, oblique tows of 153 μm aperture nets (30.5 cm diameter), fitted with a calibrated flowmeter, are made from bottom to top of the water column while underway. Vertical, top-to-bottom net tows of various mesh sizes are used to collect phytoplankton or microzooplankton for qualitative analyses.

In the Narragansett program, the time between completion of the sampling and return to the research laboratory is usually several hours. The samples are stored within darkened coolers and transported live for onshore processing. When necessary, filtration, deep freezing, sample preservation, and related processing procedures are carried out on shipboard.

Of the variables listed in Table 21.2, a routine monitoring program should minimally include the meteorological, nutrient and physical measurements listed. For phytoplankton monitoring, species composition, numerical abundance and chlorophyll biomass are essential variables. Routine measurement of primary production, particularly in a HAB monitoring program, is not essential, unless the monitoring program requires information on processes and rates. In the Narragansett program, sub-samples from the "pooled" sample are spiked with ^{14}C and incubated in an outdoor tank at five light levels (100, 60, 25, 10 and 3% of incident irradiance) for generation of photosynthesis vs. irradiance curves and *in situ* water column production rates. Incident irradiance is recorded continuously during the experiments at a nearby pyranometer. For zooplankton, measurement of dry weight biomass based on quantitative collections is essential. Measurements of species composition and numerical

Table 21.2. A Phytoplankton Monitoring Protocol Carried out in Narragansett Bay at a Long-term, Permanent Station and at Transect Stations Sampled Periodically During "Red-tide" Blooms.

Variables Measured:

Meteorological	Physical	Nutrients	Phytoplankton	Zooplankton
River runoff	Water °C	NO_3	Species Composition	Species Composition
Precipitation	Salinity	NH_4	Numerical Abundance	Numerical Abundance
Wind Speed	Secchi Disc	PO_4	Chlorophyll Biomass	Dry Weight Biomass
Irradiance		SiO_3	Primary Production	C, N Biomass
			Size Fractionation*	

Stations: 1 permanent station; 7 transect stations sampled periodically.

Sampling Depths: 3; samples from these discrete depths also used to prepare a "pooled" sample for measurements of average water column conditions; for zooplankton, oblique tows of entire water column are made.

Sampling Frequency: Weekly at permanent station and (when sampled) at transect stations; during bay-wide 'red tide' blooms, samples collected 2 - 3 times weekly at all stations.

Sample volume: 2-liter discrete samples collected for measurements of salinity, nutrients, phytoplankton species composition, numerical abundance, chlorophyll.

3-liter pooled sample prepared for measurements of primary production, or size fractionation analyses, and analyses of nutrients and phytoplankton variables.

* Total, 20-60 μm, < 20 μm and < 10 μm size-classes fractionated for measurements of species composition, numerical abundance, chlorophyll and primary production.

abundance, where desirable, will require participation of a zooplankton specialist. In contrast, the nutrient measurements and suggested minimal phytoplankton and zooplankton dry weight measurements can be carried out by a trained phytoplankton specialist requiring (exclusive of field collection) ca. two days of effort for each permanent station sampling date. The inclusion of primary production measurements and processing of seven transect stations, however, is relatively labor intensive and requires at least two trained personnel ca. one week to complete the analyses for each sampling date.

Meteorological measurements (Table 21.2), very helpful to phytoplankton monitoring efforts, are usually available at no cost from agencies which routinely monitor and record weather data and river flow. The Narragansett program measures nutrients by Autoanalyzer, using standard, well documented procedures; in programs lacking such instrumentation the need to use non-automated procedures should not deter making these essential measurements. Phytoplankton samples are usually counted live by microscope in the Narragansett program using 1 ml Sedgwick Rafter counting chambers; during size-fractionation analyses concentrated samples are often enumerated using the techniques described in Durbin *et al.* (1975). The various chapters in UNESCO's *Phytoplankton Manual* edited by Sournia (1978) providing information on phytoplankton sample preservation techniques, counting chambers, estimating cellular abundance, conversion of cell numbers into cell volume, etc. are recommended to those seeking to determine some of the available techniques and options for use in monitoring the recommended phytoplankton variables (Table 21.2). Chlorophyll is measured using Yentsch and Menzel's (1963) techniques using Lorenzen's equations (1966).

For measurement of zooplankton dry weight biomass, the zooplankton collections returned live to the laboratory are split into two equal aliquots, with one aliquot used to determine dry weight after drying at 60°C; where needed, the carbon and nitrogen content of this biomass is then determined following Sharp (1974). The other aliquot is preserved and zooplankton species composition and abundance determined using standard techniques.

SOME SPECIFIC TYPES OF MONITORING SURVEYS

Several types of monitoring surveys are worthy of special comment.

Global Environmental Monitoring and HAB Events

The growing concern internationally over the extent to which the increasing reports of HAB events represent an actual global increase in such blooms requires a global network of monitoring sites to resolve this important issue. Since such a network is presently lacking; the design of the monitoring program need not presently conform to an online global standard, i.e., an inter-calibrated network. However, the investigator is strongly encouraged to design a program which would be suitable for retrospective inclusion in a global monitoring network. This effort, which could then be upgraded, if necessary, to meet any future global environmental monitoring design, would require quantitative sampling and measurements to be carried out at appropriate frequency and duration to provide representative information on the monitored habitat. *The establishment of at least one permanent, long-term, fixed-station sampling site is therefore strongly encouraged (if a multiple series can not be managed), independent of the specific local monitoring needs.* Measurements at this station should include minimally: temperature, salinity, light transmission (Secchi disc, or other measurement), NH_4, NO_3, PO_4 and SiO_2, phytoplankton species composition and numerical abundance, chlorophyll and some measure of zooplankton abundance. This approach would not only provide a mechanism of long-term surveillence within the sampled habitat. HAB events fall within a class of biological oceanographic problems whose description and solution require inter-regional, comparative ecological analyses based on detailed site-specific studies. Resolution of whether a global increase in HAB events is occurring and, if so, the nature of its planetary biogeochemical regulation must be based on the collective patterns revealed by local, regional and sub-regional monitoring efforts.

Large Scale Regional Monitoring

An onshore-offshore gradient occurs in whether physical or chemical parameters will be of greater potential significance in regulating HAB events. In shallower, inshore regions, nutrient dynamics appear to be more significant. In deeper, offshore waters, i.e., along the continental shelf, and within Inland Seas physical factors appear to be more significant, such as upwelling events, frontal zone dynamics and alongshore currents. The nutrient delivery mechanisms in such physically driven systems differ from those inshore where riverine influences may predominate. The logistics of monitoring offshore regions and Inland Seas are more difficult and costly for a variety of reasons (see Kelley, 1976). Fairly long intervals between surveys will probably be needed, which will compromise sampling at the time scales appropriate for quantifying HAB dynamics in offshore waters.

Numerous reports in the literature indicate that HAB events in Inland Seas are associated with both nutrient enrichment and physical processes, with a progressive extension of enrichment and HAB outbreaks spreading into offshore watermasses, as found in the inner Adriatic Sea (see Justic *et al.*, 1987), Baltic Sea (Kononen, 1992), Black Sea (Bodeanu, 1993), North Sea (Hickel *et al.*, 1993; Reid *et al.*, 1990), Seto Inland Sea (Okaichi, 1989). Monitoring in such regions therefore requires a combination of approaches used for systems which are chemically dominated *vs.* physically dominated. Moreover, given the large spatial scales and multi-national borders usually characterizing Inland Seas, multi-institutional and

multi-national collaborative monitoring efforts are desirable. The impressive HAB monitoring and information exchange systems developed for the Seto Inland Sea (Fig. 21.5) illustrate the effectiveness of this approach (Okaichi, 1989).

Local HAB events may be under meso-scale influences, probably meteorological and climatological phenomena, and even be part of an undetected regional scale bloom. For example, a 1985 "brown tide" event, which lasted five months in Narragansett Bay, began synchronously over a 500 km stretch of coastline (Smayda and Villareal, 1989). The mechanisms of such large scale regional HAB events are difficult to understand, and both their detection and explanation will be an uncertain aspect of all monitoring programs. But the significance is that local HAB events may be part of a large scale phenomenon under meso-scale regulation, with the HAB response modified by site-specific, local regulation. The investigator during the design and/or implementation of the monitoring program must not ignore regional scale phenomena, particularly meteorologic and hydrographic events, and concurrent reports of phytoplankton dynamics in contiguous waters.

Another type of regional scale event relevant to monitoring programs are widespread, unexpected occurrence and spreading events of HAB taxa, such as reported for the dinoflagellates *Gyrodinium aureolum* within the North Sea, since its first sighting in 1966 (Dahl and Tangen, 1993; Partensky and Sournia, 1986) and the tropical Indo-West Pacific spreading of *Pyrodinium bahamense* var. *compressum* (Hallegraeff, this volume); and the dispersal of *Chrysochromulina polylepis* in Scandinavian waters (Granéli *et al.*, 1993). Such dramatic large scale expatriations may be unusual, but must be anticipated. Therefore, in regions lying in the potential line of dispersal of novel HAB species being spread through current systems, initiation of a monitoring effort designed to detect such taxa, if introduced, should be considered, particularly if aquacultural activities are being carried out. Establishment of the fixed, permanent monitoring station recommended above would provide such coverage.

Special Case of Aquacultural Site Monitoring

There is diffuse, but growing evidence that the sites of aquacultural deployments eventually begin to exhibit HAB events associated somehow with such sea farming. In the case of caged culture of finfish (Horner *et al.*, 1990), this may simply result from enclosure preventing the penned fish from escaping damage; the HAB event might otherwise have been a normal bloom event in those waters. "Early warning" monitoring might detect presence of harmful taxa sufficiently early to allow their temporary removal to safer waters, such as done with penned salmon culture enclosures during a *Chrysochromulina polylepis* bloom in Norwegian coastal waters (Underdal *et al,* 1989). But there is another aspect to aquaculture of special interest to HAB events and environmental monitoring, particularly where filter-feeding shellfish are cultured. There are three major environmental modifications associated with such culturing which may stimulate a HAB event and/or lead to dieoff. Filter-feeding shellfish ingest the natural phytoplankton community selectively; this significant predation pressure influences phytoplankton community structure, succession and abundance. The latter may also be modified if disease-control agents are applied. Considerable nutrient excretion also accompanies filter-feeding. [Leaching and remineralization of nutrients from unused food provided caged fish cultures also occur.] Decomposition of waste material deposited onto the sediments underlying the culturing rafts, which may be supplemented by ungrazed HAB biomass, reduces oxygen levels. The potential for hypoxic or anoxic conditions leading to dieoffs then becomes of concern. The extent to which these factors may combine to favor HAB outbreaks is influenced by the degree of flushing which varies seasonally and inter-annually. Increased flushing will contribute to better oxygenation of bottom waters; wash out slow-growing HAB taxa; replenish the water-column with the more "natural" phytoplankton community associated with that season and location; and dilute HAB growth promoting factors resulting directly from the aquacultural activity. Hence, aquacultural activities in some locations may result in environmental modifications seemingly favorable to HAB events, creating the need for a "*product safety* " monitoring program.

Miscellaneous

This chapter has focused on some of the more general aspects of environmental monitoring. Margalef (1976) presents some examples of specific types of surveys and their sampling requirements useful in designing monitoring programs. Experimental approaches, including use of species' bioassays, and the use of toxins, molecular probes, and sentinel species in environmental monitoring have all been used, but their evaluation falls outside the scope of this chapter. Those who are newly interested in environmental monitoring, and are unfamiliar as yet with HAB events in their region of interest, may find information useful in the design of their monitoring programs in the various proceedings of the international conferences convened on HAB events in the sea (LoCicero,1975; Taylor and Seliger, 1979; Anderson *et al.*, 1985; Okaichi *et al.*, 1989; Granéli *et al.*, 1990; Smayda and Shimizu, 1993; Lassus *et al.*, 1995), and in Cosper *et al.* (1989).

CONCLUSION

The admonition " *to design with Nature* " is an appropriate guideline; monitoring program design should not be based on scales of investigator convenience or methodological considerations only. In regions of aquacultural activities, environmental monitoring should be a considered a necessary complement to such enterprises, if only to ensure product safety and facilitate resource protection. There is a growing impression that a global increase in HAB events may be in progress, and this may be linked jointly to anthropogenic environmental modifications and climatic trends. Resolution of these issues would be facilitated by the implementation of a global network of inter-calibrated environmental monitoring (= surveillence) sites. As a step in that direction, it is recommended that fixed, permanent, long-term monitoring stations be established (independent of local needs for environmental and HAB monitoring) in representative coastal regions to form the basis of a global monitoring network.

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22. Management of Shellfish Resources

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Interactions between marine biotoxins and shellfish are complex, dynamic and vary between species and even within subpopulations. For detailed reviews the reader is directed to the following: Shumway (1990; 1994), Shumway *et al.* (1988, 1990), Shumway and Cembella (1993). In addition to commercial harvest areas, aquaculture facilities are adversely and often unpredictably affected by toxic blooms. Public health officials as well as harvesters, processors and dealers of shellfish must remain alert to outbreaks of toxic algal blooms to protect human health as well as preserving a high standard of quality assurance. Recent reviews of particular interest to managers include those by Maclean (1993), Cembella and Todd (1993) and van Egmond *et al.* (1992, 1993, 1994).

The following chapter provides a summary of available information on rates of loss of toxins by bivalve molluscs, regulations regarding paralytic (PSP), diarrhetic (DSP), amnesic (ASP) and neurotoxic (NSP) shellfish toxins worldwide and a detailed description of the United States Food and Drug Administration (USFDA) Interstate Shellfish Sanitation Commitee (ISSC) and National Shellfish Sanitation Program. In addition, the monitoring program of the State of Maine (USA) is described as an example of an efficient and highly reliable system. The appendix in Fig. 22.2 provides an example of a comprehensive Red Tide Response and Management Framework as developed by the ASEAN-Canada Cooperative Programme on Marine Science (Seagel, 1994).

INTOXIFICATION AND DETOXIFICATION OF SHELLFISH

Shellfish (bivalve molluscs, gastropods, crabs, lobsters and others) accumulate phycotoxins either by direct filtration of the plankton cells or by feeding directly on contaminated organisms (e.g. carnivores and scavengers). Rates of intoxification and detoxification of filter-feeding shellfish by toxic algae are species-specific and are, in most cases, directly related to the number of cells available to the animals (Sribhibhadh, 1963; Gilfillan *et al.*, 1976; Prakash *et al.*, 1971; Saunders *et al.*, 1982). Toxicity of individual shellfish in any given area is highly variable (see White *et al.*, 1993; Chebib *et al.*, 1993). The rate of loss varies with season (Prakash *et al.*, 1971) and low water temperatures apparently retard toxin loss; however, the degree to which temperature affects the uptake and release of toxins is not clearly understood (see Madenwald, 1985). Further, the rate of detoxification is highly dependent on the site of toxin storage within the animal i.e., toxins in the gastrointestinal tract (e.g. *Mytilus*) are eliminated much more readily than toxins bound in tissues (e.g. *Placopecten*, *Spisula*, *Saxidomus*), and on initial or peak level of toxicity. Few data are available for the retention times of toxins by crabs and carnivorous gastropods; however, the general trend appears to be towards long-term retention (Desbiens and Cembella, 1994; Shumway, 1994; Shumway, unpublished data).

The majority of information available concerns bivalve molluscs (see also Shumway, 1990), and as these are the species most commonly reared in aquaculture, they will be focussed upon here. Table 22.1 summarizes existing data on toxin retention for a number of bivalve species. Mussels (e.g. *Mytilus* spp., *Modiolus* spp., *Perna* spp.) are known to accumulate PSP toxins faster than most other species of shellfish and also to eliminate the poison quickly. While oysters do not accumulate the toxic species as readily as mussels, they take considerably longer to detoxify (Neal, 1967; Shumway *et al.*, 1990). In contrast, some species (e.g. *Saxidomus giganteus*, *Spisula solidissima*) may remain toxic for extended periods (e.g. in excess of 3

years) (Quayle, 1965; Blogoslawski and Stewart, 1978; Chambers and Magnusson, 1950; Cembella and Todd, 1993; Shumway *et al.*, 1994). In addition to the clams, scallop tissues (not adductor muscles) also store toxins for periods in excess of 2 years (Shumway and Cembella, 1993).

Some species of bivalves are known to avoid toxic dinoflagellates (see Shumway and Cucci, 1987). One species of particular interest is the northern quahog or hard clam, *Mercenaria mercenaria*. During an outbreak of a bloom of *Alexandrium tamarense* (=*Gonyaulax* =*Protogonyaulax tamarensis*) in 1972, the entire coastline of Massachusetts came under interdict. Monitoring of the coast indicated that some 2800 acres of shellfish harvesting areas were contaminated. Bioassays of shellfish samples showed toxin in the range of 3000-5000 µg per 100 g with the most heavily contaminated shellfish being the mussel (*Mytilus edulis*), soft-shelled clam (*Mya arenaria*) and bay scallops (*Argopecten irradians*). It was specifically noted that **no** quahogs (*M. mercenaria*) or oysters were affected. *Mercenaria mercenaria* was reported to be toxic in the Bay of Fundy and St. Lawrence regions by Bond and LaChance (1959). Studies in our laboratory have shown that in the presence of *A. tamarense* the quahog first retracts its siphons and then completely isolates itself from the external environment by means of shell valve closure. The animals did not re-open their shell valves until after the addition of clean sea water. Efforts to induce toxicity by feeding *A. tamarense* were unsuccessful. It is also possible that *Mercenaria* responds to the presence of other dinoflagellates in the same manner. Castagna (personal communication) has observed that quahogs exposed to red-tide blooms in Virginia (non-toxic) bury themselves very deep in the experimental trays. He further noted that 'wild' populations were found at depths of up to 35cm below the sediment surface as opposed to their usual 15cm during these blooms.

Subsequent studies by Bricelj *et al.* (1991) have shown, however, that the quahogs will ingest toxic dinoflagellates if fed mixtures containing both toxic dinoflagellates and other suitable food items, e.g. the diatom, *Thalassiosira weissflogii*. Perhaps more importantly, these workers have shown in both mussels (*M. edulis;* Bricelj *et al.*, 1990) and quahogs (*M. mercenaria*; Bricelj *et al.*, 1991) that ingestion rate of toxic cells, maximum body burden of toxin and initial rates of detoxification vary markedly in response to differences in cell toxicity and/or toxin composition of the dinoflagellate cells consumed.

Phycotoxins other than those associated with paralytic shellfish poisoning are also accumulated by filter-feeding molluscs and are also a threat to public health. Diarrhetic shellfish toxins (e.g. okadaic acid and its derivatives, dinophysistoxin-1 and dinophysistoxin-3; pectenotoxins and yessotoxins) associated with *Dinophysis* spp. and *Prorocentrum* spp. are readily accumulated by shellfish and little is known of the retention time of the toxins. Marcaillou-Le Baut *et al.* (1993) compared DSP depuration rates of mussels reared in an aquaculture pond and in the laboratory and found that the highly toxic (3 MU g^{-1}) mussels dropped to acceptable levels more quickly in the culture ponds than in the laboratory. They suggested that the quality of food available to the mussels during detoxification may affect the rate at which toxins are eliminated.

More recently, domoic acid has been identified as a potentially lethal phycotoxin associated with various species of the diatom, *Pseudo-nitzschia* (see Chapter 17).

Generalizations regarding the uptake and retention of phycotoxins by shellfish should be avoided. Differences in rates of toxin accumulation and retention are dependent upon the shellfish and algal species under consideration and these differences should be taken into account before choosing a species to be reared in areas prone to toxic algal blooms.

DETOXIFICATION - DEPURATION

Various attempts have been made at detoxifying shellfish contaminated with paralytic shellfish poisons in an effort to reduce the duration of 'off market' times. The most obvious method is to transfer shellfish to waters free of the toxic organisms and allow them to self-depurate. While

Table 22.1. Approximate times of toxin retention for various species of bivalve molluscs (represents time taken for toxin levels to fall below either quarantine or detection levels). Algal species are as given in original publications[1].

Species	Toxin source	Retention time	Reference
Ameghinomya antiqua	probably *Dinophysis acuta*	6 months	Lembeye *et al.* (1993)
Anadara maculosa	*Pyrodinium bahamense*	6 weeks	Worth *et al.* (1975)
Arctica islandica	*Protogonyaulax tamarensis*	2 months *in vivo*	Shumway, unpublished
Aulacomya ater	probably *Dinophysis acuta*	6 months	Lembeye *et al.* (1993)
Choromytilus meridionalis	*Gonyaulax catenella*	3 months	Popkiss *et al.* (1979)
Clinocardium nuttalli	*Gonyaulax acatenella*	9 weeks	Quayle (1965)
Crassostrea cucullata	not specifed, probably *Pyrodinium bahamense*	2 months	Karunasagar *et al.* (1984)
Crassostrea echinata	*Pyrodinium bahamense*	3 weeks in closed system; longer periods *in vivo*	Maclean (1975)
		4 months	Worth et al. (1975)
Crassostrea gigas	*Gonyaulax acatenella*	1-9 weeks	Quayle (1965; 1969); Sharpe (1981)
		1 month	Sribhibhadh (1963)
Crassostrea iridescens	*Gymnodinium catenatum*	>1 month	Mee *et al.* (1986)
Crassostrea virginica	*Gymnodinium breve*	2-6 weeks	Morton and Burklew (1969)
Meretrix casta	not specified, probably *Pyrodinium bahamense*	1 month	Karunasagar *et al.* (1984)
Modiolus auriculatus	*Pyrodinium bahamense*	6 weeks	Worth *et al.* (1975)
Modiolus modiolus	*Gonyaulax tamarensis*	up to 60 days[2]	Gilfillan *et al.* (1976)
Mya arenaria	*Gonyaulax acatenella*	5 weeks	Quayle (1965)
	Gonyaulax tamarensis	4-6 weeks	Prakash *et al.* (1971); Bicknell and Collins (1973)
		up to 45 days[2]	Gilfillan *et al.* (1976)
Mytilus californianus	*Gonyaulax catenella*	< 1 month	Sommer and Meyer (1937); Sharpe (1981)
Mytilus edulis	*Protogonyaulax tamarensis*	10 days- 7 weeks	Oshima *et al.* (1982);
		up to 50 days	Gilfillan *et al.* (1976); Prakash *et al.* (1971)
	Gonyaulax acatenella	11 weeks	Quayle (1965)
		4 weeks	Sharpe (1981)
	Gonyaulax excavata	2-3 weeks	Gaard and Poulsen (1988)
	Dinophysis spp.	1 week	Haamer *et al.* (1989)
	Dinophysis spp.	8 weeks	Marcaillou-le Baut *et al.* (1990)
	Dinophysis spp.	8->42 days[2]	Marcaillou-le Baut *et al.* (1993)
	Dinophysis spp. (?) *Prorocentrum* spp. (?)	≈10 days	Quilliam *et al.* (1993)
	probably *Dinophysis acuta*	6 months	Lembeye *et al.* (1993)
Patinopecten yessoensis	*Protogonyaulax tamarensis*	6 weeks- 5 months	Oshima *et al.* (1982); Iioka *et al.* (1964)
*Perna canaliculus**	*Nitzschia pungens* f. *multiseries*	2 days	MacKenzie *et al.* (1993)
Placopecten magellanicus	*Protogonyaulax tamarensis*	6 month in closed system; can be toxic year round *in vivo*	Bourne (1965); Shumway *et al.* (1988)
Protothaca staminea	*Protogonyaulax acatenella*	5 weeks	Quayle (1965)
Saxidomus giganteus		2 years +	Quayle (1965); Anonymous (1974)
Saxidomus solidissima	*Gonyaulax catenella*	<1 month	Sommer and Meyer (1937)
Siliqua patula	*Pseudonitzschia* spp.?	>2 years	Wekell et al. (1993); Drum *et al.*(1993); Horner *et al.* (1993)
Spisula solidissima	*Protogonyaulax tamarensis*	up to one year	Medcof *et al.* (1947); Blogaslowski and Stewart (1978)
Spondylus sp.	*Pyrodinium bahamense*	still highly toxic after months	Worth *et al.* (1975)
Tresus capax	*Gonyaulax acatenella*	11 weeks	Quayle (1965)
Venerupis japonica	*Gonyaulax acatenella*	5 weeks	Quayle (1965)

[1] Note: *Gonyaulax* and *Protogonyaulax* = now*Alexandrium*; *Nitzschia* = now *Pseudo-nitzschia*

[2] Dependent on initial level of toxicity.; * laboratory study only; toxic organisms not identified in natural habitat

this may appear to be a satisfactory method for many species of shellfish, rates of detoxification vary considerably between species (See Table 22.1) and some species remain toxic for extended periods of time. Further, transferring large quantities of shellfish is labor-intensive and costly. Desbiens and Cembella (1993) investigated the potential use of vertical displacement of mussels in the water column as a means of minimizing PSP toxin accumulation. While they were able to demonstrate that transfer of mussels had an ameliorating effect on the toxin accumulation, they pointed out that the potential applications of vertical relaying for mussel culture are restricted by high levels of PSP toxicity observed in the region (eastern Canada). Detoxification of PSP-toxins using temperature or salinity stress has also been tried with marginal success (Gilfillan *et al.*, 1976; Blogoslawski and Neve, 1979). Instantaneous electrical shock treatments accelerated toxin excretion in scallops (Kodama *et al.*, 1989). Reduced pH has been tried as a means of detoxifying butter clams, but with no success (Anonymous, 1966; Neal, 1967). Chlorination has been used in France; however, this process alters the flavor of the shellfish and thus decreases marketability. Emergency relocation areas have been set aside in Tasmania (September 1994) to reduce the impact of upcoming paralytic shellfish poison blooms on shellfish stocks (Hallegraeff, personal communication).

Ozone has been touted as an effective means of reducing toxicity; however, its usefulness is questionable. Several early studies reported ozone to be effective in the inactivation of PSP toxins in shellfish exposed to *Alexandrium tamarense*, *A. catenella* and *Gymnodinium breve* blooms (Thurberg, 1975; Blogoslawski *et al.*, 1975, 1979; Dawson *et al.*, 1976; Blogoslawski and Stewart, 1978). Blogoslawski *et al.* (1973) also suggested that ozone could be used to inactivate *Gymnodinium breve* toxins. More recently, preliminary studies by Gacutan *et al.* (1984, 1985) demonstrated that both ozone gas and PVP-iodide-iodine may effectively inactivate PSP toxins from *Perna viridis* contaminated by *Pyrodinium bahamense*. However, a subsequent study by White *et al.* (1985) gave results totally contradictory to previous studies in that no detoxification occurred in *Mya arenaria* exposed to ozone treatments.

In a review (Blogoslawski, 1988), it was again suggested that ozonised seawater can be of value in detoxification of shellfish contaminated recently by the vegetative cell phase of toxic (PSP) dinoflagellates. In a study during a red tide outbreak, it was shown that ozone treatment of the seawater does prevent shellfish (*Mytilus edulis, Mya arenaria* and *Guekensia demissus*) from accumulating paralytic shellfish poison. Blogoslawski concluded that inactivation could be achieved in bivalves exposed to and contaminated by motile dinoflagellate cells bearing PSP without measurably altering the physical state of the treated bivalves and that this inactivation could be achieved in a marketable species such as *Mya* within an economically feasible time frame (Blogoslawski *et al.*, 1979). Ozone is useless in detoxifying cysts or in bivalves that have ingested cysts or have the toxins bound in their tissue over long periods of time. Further, detoxification of algal toxins, especially paralytic shellfish poisons, over long periods of time is not economically feasible. We do not recommend ozone as a practical or safe means of eliminating algal toxins from shellfish. At present the economic feasibility of efficiently detoxifying shellfish on a large scale in artificial systems is not promising. In areas prone to regular outbreaks of toxic algal species, culturists and commercial fishermen alike must still depend on reliable monitoring systems to warn of toxic shellfish and plan their activities accordingly. Through the combined efforts of an intensive monitoring program and culture of 'rapid release' species (e.g. *Mytilus edulis*), species known to avoid toxic dinoflagellates (e.g. *Mercenaria*, most oysters) or scallops (adductor muscles rarely if ever toxic), economic losses can be kept to a minimum (see also Shumway *et al.*, 1988).

Cooking has also been touted as a possible means of detoxifying shellfish contaminated with paralytic shellfish poisons. **Cooking does not eliminate the danger of intoxication**; however, it may reduce levels of toxins. If initial levels of toxicity are low, cooking may effectively reduce toxicity to safe levels. Pan frying seems to be more effective than other methods of cooking (Medcof *et al.*, 1947; MacDonald, 1970). When clams or mussels are steamed or boiled, toxins lost from the tissues are contained in the cooking liquid rendering the fluids extremely toxic.

Commercial canning has been shown to reduce toxicity (paralytic shellfish poisons) of soft shell clams, *Mya arenaria*, by as much as 90%. A toxicity level of 160 µg STX equiv.100

g^{-1} for soft-shell clams and mussels (*Mytilus edulis*) to be canned was established in the 1950's in Atlantic Canada and remains in effect today (Cembella and Todd, 1993). (Note: canning of soft shell clams contaminated by paralytic shellfish poisons in Canada is confined to those clams that were harvested prior to a closure. This is a method which allows a clam dealer to utilize these clams. No harvesting is permitted in any closed area.) Noguchi *et al.* (1980) (see also Nagashima *et al.*, 1991) showed that toxicity levels of PSP-infested scallops could be reduced during canning processes. They demonstrated that during retorting (110°C, 80min, or 122°C, 22min) most of the PSP-toxins could be eliminated (maximum initial level 102MU g^{-1} digestive gland) whereas heating (70°C, 20min) followed by washing was less effective in reducing the toxicity below the quarantine limit of 4MU g^{-1}. Recent efforts in Spain (Berenguer *et al.*, 1993) have demonstrated that toxicity levels of Mediterranean cockles (*Acanthocardia tuberculatum*) may be significantly reduced via the canning process. Total toxicity of cockles (initial levels of approximately 800 µg STXequiv.$100g^{-1}$) was reduced to <35 µg STXequiv $100g^{-1}$ after cooking. These authors believe that the decreases in PSP toxicity obtained by commercial processing is sufficient to warrant canning as a practical means of obtaining a legal and acceptable product. Similar attempts have been made to detoxify surfclams (*Spisula solidissima*) via the canning process, but results are thus far inconclusive. The effectiveness of canning as a means of reducing PSP-toxicity levels below quarantine levels is dependent upon the initial level of toxicity and should be approached with great caution.

With the exception of the study by Berenguer *et al.* (1993), there have been no useful methods devised for effectively reducing phycotoxins in contaminated shellfish. All methods tested to date have been either unsafe, too slow, economically unfeasible or yielded products unacceptable in appearance and taste. Given the apparent global increase in harmful algal blooms and the continually growing interest in culture of bivalve molluscs, further efforts are needed to develop effective means of detoxifying shellfish contaminated with phycotoxins. Failing the development of any such methods, increased efforts will need to be expended in monitoring shellfish for the presence of phycotoxins.

REGULATION AND MONITORING PROGRAMMES

There appears to be general, worldwide agreement on the need for measurements to control shellfish toxins in seafood and many countries have taken legal action to ensure that phycotoxin-contaminated shellfish do not reach the consumer.

Factors that may influence the regulation of shellfish toxins

Various factors may play a role in establishing regulatory criteria and limits for phycotoxins. These include:

- the availability of survey data
- the availability of toxicological data
- the distribution of phycotoxins throughout sampled lots and the stability of the toxins in the samples
- the availability of methods for analysis of toxins
- regulation in force in other countries.

Data on the occurrence of toxic algal species may indicate which toxins may be expected during periods of algal blooms and which seafood products should be considered for analytical monitoring. A problem is that certain algal species, which have never occurred in a certain area, may suddenly appear and then rapidly cause problems, e.g. the intoxication episode with

domoic acid in Canada (Wright, 1989) and recent, unprecedented outbreaks of neurotoxic shellfish poisoning in New Zealand (Jasperse, 1993).

Without toxicological information there can be no hazard assessment, one of the basic ingredients of risk assessment. Although there are many reported cases of human intoxications due to shellfish toxins, it is difficult to obtain reliable human toxicity data. For example, variations in PSP toxicity to humans may be due not only to variable sensitivity between people, but also to the composition of individual toxins in the samples. Toxin profiles can vary according to the species of shellfish consumed and the area of harvest (Krogh, 1988). In addition, toxic doses are often estimated from left-over toxic seafood, which may not be representative of the ingested food. Data from animal experiments are rather restricted.

The distribution and composition of the multiple toxins that make up many of the shellfish toxins throughout the sampled lots and within the individual shellfish, as well as the (in)stability of the toxins in the samples may pose certain difficult problems in establishing criteria. A few mussels may not be representative for the whole catch. For example, the composition of DSP toxins varies throughout the world, and sometimes in some areas from year to year. Mussels collected in Europe contain okadaic acid as the major toxin (Edebo *et al.*, 1988; Kumagi *et al.*, 1986); however, in Norway in 1985, mussels collected in one area, contained okadaic acid as the major toxin constituent while mussels collected in another area of Norway in 1986 contained dinophysistoxin-1 as the major toxin and yessotoxin as well as pectenotoxin-like compounds as minor toxins (Lee *et al.*, 1987). Scallops from Japan show the most complicated toxin profile; pectenotoxins have been detected and confirmed only in shellfish harvested there (Yasumoto and Murata, 1990). In addition, a high level of variability (±48%) in toxin concentration has been demonstrated in some species of shellfish collected from the same sample in the Gulf of Maine (White *et al.*, 1993). The risk to both consumer and producer must be considered when establishing sampling criteria to protect not only the public health, but also the fishery resources and the coastal economy.

Accurate methods of analysis have to be available, because legislation calls for methods of control. A big problem with many of the phycotoxins is that reliable, validated chemical methods of analysis are not yet available, or cannot be easily operated because of the lack of standards and reference materials (Van Egmond *et al.*, 1993). Most countries still rely on animal bioassays to detect PSP and DSP (see Tables 22.2 and 22.3). A main disadvantage of bioassays is the ethical aspects of these tests, which have led to growing resistance from animal welfare groups. It should also be borne in mind that it is not realistic to establish a tolerance level lower than the actual limit of detection, although this might be desirable from the toxicity point of view. Should the analytical methodology improve as desired, then it may be necessary to reconsider the tolerances.

Regulations presently in force in other nations, especially those of trading partners, should be considered when new domestic regulations are being put into place. Differences between nations in tolerances set for shellfish toxins can result in chaos and inconsistencies in the protection of public health. Differences can also raise unnecessary barriers to international trade.

Weighing the various factors that play a role in the decision-making process of establishing shellfish toxin tolerances may not be easy. Despite the dilemmas, there are a number of countries that have established limits and regulations for shellfish toxins.

Current limits and regulations for phycotoxins

Within the framework of a project of the International Union of Pure and Applied Chemistry (IUPAC) a project was initiated in 1990 to obtain a global overview of current legislation on phycotoxins and plant toxins. A substantial part of the information concerned shellfish toxins. The Agricultural Attachés or Counsellors of the Dutch Embassies were approached with the request to collect up-to-date information on the state-of-affairs of national regulations for these toxins in as many countries of the world as possible. For that purpose, enquiry forms together

Table 22.2. Phycotoxin - producing marine algae for which monitoring programs exist worldwide.

Country	Algal species monitored	Closure of fishery product harvesting area
Australia	*Alexandrium catenella*	$>5x10^4$ cells l^{-1}
	Gymnodinium catenatum	based on level of toxin
Canada	*Alexandrium* spp.	when toxin levels in shellfish
	Pseudonitzschia pungens spp.	exceed tolerable limits
	Dinophysis spp.	
	Prorocentrum spp.	
Denmark	*Amphidinium* spp.	at approx. $5x10^5$ cells l^{-1}
	Dinophysis spp.	depending on species
	Alexandrium spp.	
	Gyrodinium aureolum	
	Gymnodinium spp.	
	Noctiluca scintillans	
	Prorocentrum spp.	
	Protogonyaulax catenella	
	Dictyocha speculum	
	Chrysochromulina polylepis	
	Prymnesium spp.	
	Heterosigma cf. *akashiwo*	
	Pseudonitzschia pungens	
France	*Dinophysis* spp.	based on toxicity level
	Alexandrium spp.	in shellfish
	Gambierdiscus toxicus	not applicable
	Ostreopsis lenticularis	not applicable
	Prorocentrum lima	not applicable
Ireland	*Dinophysis* spp.	>200 cells l^{-1}
	Alexandrium spp.	$>5x10^4$ cells l^{-1}
Italy	*Dinophysis* spp.	1000 cells l^{-1} and presence of DSP in mussels
	PSP and DSP producing species	simultaneous presence of algae in water and toxin in mussels
The Netherlands	*Dinophysis acuminata* and other *Dinophysis* spp.	when DSP toxins detected in shellfish
Norway [1)]	*Prymnesium parvum*	when detected
	Chrysochromulina polylepsis	during routine check
	Dinophysis spp.	around shellfish farm
	Alexandrium spp.	
Singapore	*Cochlodinium catenatum*	not applicable
	Chattonella	
	Heterosigma	
	Mesodinium	
South Korea	*Chaetoceros* spp.	$>10^6$ cells l^{-1}
	Skeletonema spp.	$>10^6$ cells l^{-1}
	Thalassiosira spp.	$>10^6$ cells l^{-1}
	Cochlodinium spp.	$>10^4$ cells l^{-1}
	Heterosigma spp.	$>10^5$ cellsl^{-1}
	Prorocentrum spp.	$>10^5$ cells l^{-1}
	Protogonyaulax spp.	$>10^5$ cells l^{-1}
USA (Florida)	*Gymnodinium breve*	$>5x10^3$ cells l^{-1}

1) only in special situations (unusually large algal blooms)

Table 22.3. Regulations for paralytic shellfish poisons in various countries.

COUNTRY	PRODUCT	TOXIN(S)	TOLERABLE LEVEL[1,2]	RESPONSIBLE AUTHORITY	METHOD OF ANALYSIS	REMARKS	REFERENCE
Australia	shellfish	saxitoxin	80 µg $100g^{-1}$	State authorities under supervision of the Australian Quarantaine and Inspection Service	mouse bioassay		
Austria	shellfish	saxitoxin	40 µg $100g^{-1}$	Ministry of Public Health and provincial authorities	Spectrophotometric method; mouse bioassay for confirmation		Sauler (1990)
Canada	molluscs	PSP	<80 µg $100g^{-1}$	Dept. of Fisheries & Oceans; Dept. of Health & Welfare	mouse bioassay	Products having levels between 80-160µg $100g^{-1}$ may be canned	Fish Inspection Regulations (1978)
European Union[3] (EU)	bivalve molluscs	PSP	80 µg $100g^{-1}$	Various	(Mouse) bioassay in association if necessary with a chemical method for detection	If the results are challenged, the reference method is the biological method	Council of the European Communities (1991)
Guatemala	molluscs	saxitoxin	400MU $100g^{-1}$	Ministry of Public Health; Fisheries	mouse bioassay		Rosales-Loessener *et al.* (1989)
Hong Kong	shellfish	PSP	400 MU $100g^{-1}$	Dept. of Health; Agriculture and Fisheries Dept.	mouse bioassay		Wong and Wu (1987)
Japan	bivalves	PSP	400 MU $100g^{-1}$	Ministry of Health & Welfare; Bureau of Environmental Health	mouse bioassay		Yasumoto (1991)
Korea	bivalves	gonyautoxins	400 MU $100g^{-1}$	Ministry of Health & Social Affairs	mouse bioassay HPLC method		Il-Yong Ha (1990)
New Zealand	shellfish	PSP, NSP, DSP ASP		Department of Health; Ministry of Agriculture and and Fisheries; Fishing Industry Inspection and Certification Council		regulations being developed	Jasperse (1993)
Norway	all types of mussel	PSP	40-80 µg $100g^{-1}$	Food Control Authority	mouse bioassay	40-80 µg $100g^{-1}$: single case assessment; >80 µg $100g^{-1}$: banned	Yndestad (1989)
Panama	bivalves	PSP	400 MU $100g^{-1}$	Ministry of Public Health	mouse bioassay	proposal	De Solis (1990)
Singapore	bivalves	saxitoxin	80 µg $100g^{-1}$	Ministry of National Development (Primary Production Department) Ministry of the Environment	mouse bioassay		Lim Lian Chuan (1989)
Sweden	molluscs	PSP	80 µg $100g^{-1}$	Ministry of Public Health; Ministry of Agriculture	mouse bioassay	tolerance expressed as saxitoxin equivalents	Hagelom (1989)
United States	bivalves	PSP	80 µg $100g^{-1}$	Interstate Shellfish Sanitation Conference (ISSC); Food and Drug Administration	mouse bioassay	ISSC coordinates the Shellfish Programs, administered by the individual states	NSSP (1990)

1) For reasons of uniformity in presentation, all tolerances are expressed as MU $100g^{-1}$ or µg $100g^{-1}$; 2) MU = mouse unit;
3) European Union countries include:Belgium, Denmark, France, Germany, Greece, Ireland, Italy, Luxembourg, The Netherlands, Portugal, Spain, United Kingdom

with some background information about the phycotoxin problem were sent out in four languages (English, Spanish, French, and Dutch). The questions concerned:

- measures taken to restrain the presence of phycotoxins;
- surveillance programs to check for occurrence of toxic algal species in areas where shellfish are grown;
- types and concentrations of algal species leading to closure of harvesting areas;
- types of phycotoxins and fishery products for which legislation is in force, together with the maximum permissible levels;
- the rationales for the regulations;
- the authorities responsible for the control of phycotoxins;
- the use of official and published methods of sampling and analysis;
- the disposal of consignments containing inadmissible amounts of phycotoxins.

About half (47) of the countries that were approached, responded to the inquiry. For a few countries information (also) came from other sources such as publications and personal communications. Altogether at least 24 countries, including the 12 member States of the European Union (EU), seemed to have regulations or detailed proposals for regulations on toxin-producing marine algae or marine phycotoxins. Figure 22.1 illustrates where the countries with marine phycotoxin legislation are geographically situated (black areas), and where such regulation does not exist (white areas). The regulations are summarized in Tables 22.3-22.5 and they are further discussed in the following sections. Other responding countries (Bolivia, Brazil, Burkina Fasso, Cameroun, Chile, Colombia, Ecuador, Egypt, Ethiopia, Guinée Bissau, Honduras, Hungary, India, Jordan, Kenya, Malawi, Mexico, Peru, Romania, San Salvador, Sudan, Switzerland, Syria, Yemen) indicated they had not (yet) specific regulations for marine phycotoxins. The fact that the law is silent on the phycotoxin topic does not necessarily mean that the problem is non-existent or ignored. Several countries rely on general food legislation in this respect, and may take specific action as each case arises in respect of phycotoxin problems. Regulations are continually changing, e.g. the EU Commission recently decided that Morocco may be included in the list of countries which satisfy the "equivalency" conditions of Third Countries able to export live mollusc bivalves to the Community (Directive 387, June 1993 of the EU Commission). The Directive 397 also specifies the particular sanitary conditions requested for the import of these products.

Several of the countries surveyed indicated they have monitoring programmes to check for occurrence of (toxic) phytoplankton species in areas where shellfish are grown. Table 22.2 gives an overview of these programs. In 25 countries tolerances have been established or proposed for one or more phycotoxins in shellfish. Regulations exist for: PSP and specifically for saxitoxin and gonyautoxins; DSP and specifically for okadaic acid; domoic acid (one of the amnesic shellfish poisons [ASP]); brevetoxin (one of the neurotoxic shellfish poisons [NSP]); ciguatera toxins are currently limited to finfish and will not be discussed here. In Tables 22.3-22.5 tolerances for these toxins, responsible authorities, methods of analyses used, and relevant references are summarized. The data presented in Tables 22.3-22.5 may not be complete or fully correct in a number of cases, due to problems experienced with language, terminology and the interpretation of the authors of the responses in the inquiry forms. Moreover, new or modified regulations are continuously being established.

Monitoring programs for toxic algal species

Some countries monitor only for one or two algal species, others have a long list of species monitored for, Denmark being the most active in this respect (see Table 22.2). In some countries the shellfish harvesting areas are closed when the number of cells of certain algal species exceeds certain concentrations (Australia, Denmark, Ireland, South Korea, USA, State of Florida) the latter depending on the species. Other countries (Canada, The Netherlands) close their harvesting areas only when the toxic phytoplankton species have been detected in the

Table 22.4.Regulations for diarrhetic shellfish poisons in various countries.

COUNTRY	PRODUCT	TOXIN(S)	TOLERABLE LEVEL[1,2]	RESPONSIBLE AUTHORITY	METHOD OF ANALYSIS	REMARKS	REFERENCE
Canada	shellfish	DSP	0.2µg g^{-1}	see Table 2	mouse bioassay	not official Todd (1993)	Cembella and
European Union[3] (EU)	bivalve molluscs	DSP	not detectable	various	customary biological testing methods		Council of the European Communities (1991)
Japan	bivalves	DSP	5 MU $100g^{-1}$	See Table 2	mouse bioassay		Yasumoto (1991)
Korea	shellfish	DSP	5 MU $100g^{-1}$	See Table 2	mouse bioassay		IL-Yong Ha (1990)
Norway	all types of shellfish	DSP	5-7 MU $100g^{-1}$	See Table 2	mouse bioassay		Yndestad (1989)
Sweden	molluscs	DSP	40-60 µg $100g^{-1}$	See Table 2	HPLC; mouse bioassay for confir-mation	tolerance expressed as okadaic acid equivalents; 40 µg $100g^{-1}$: tolerance. 60 µg $100g^{-1}$: domestic tolerance	Hageltorn (1989)

1) For reasons of uniformity in presentation, all tolerances are expressed as MU $100g^{-1}$ or µg $100g^{-1}$.
2) MU- mouse unit. A mouse survival time equal to 5 hours corresponds to about 0.2MU g^{-1} digestive gland.
3) European Union countries include: Belgium, Denmark, France, Germany, Greece, Ireland, Italy, Luxembourg, The Netherlands, Portugal, Spain, United Kingdom

Table 22.5. Regulations for phycotoxins other than PSP and DSP.

COUNTRY	PRODUCT	TOXIN(S) LEVEL	TOLERABLE	RESPONSIBLE AUTHORITY	METHOD OF ANALYSIS	REMARKS	REFERENCE
Canada	molluscs	domoic acid	2mg $100g^{-1}$	See Table 22.2	HPLC		Bureau of Chemical Safety (1988)
Portugal	shellfish	domoic acid	2 mg $100g^{-1}$	See Table 22.2		proposal	
Italy	shellfish	NSP	n.d.	See Table 22.2			Ministerio della Sanità (1990)
United States	bivalves	domoic acid	2 mg $100g^{-1}$	See Table 22.2	HPLC	not official	
	bivalves	NSP	n.d.		mouse bioassay	only in certain states (Florida)	NSSP (1990)
viscera	cooked crab	domoic acid (mg kg^{-1})	30ppm	See Table 22.2	HPLC	(1992)	HHE NO. 2937

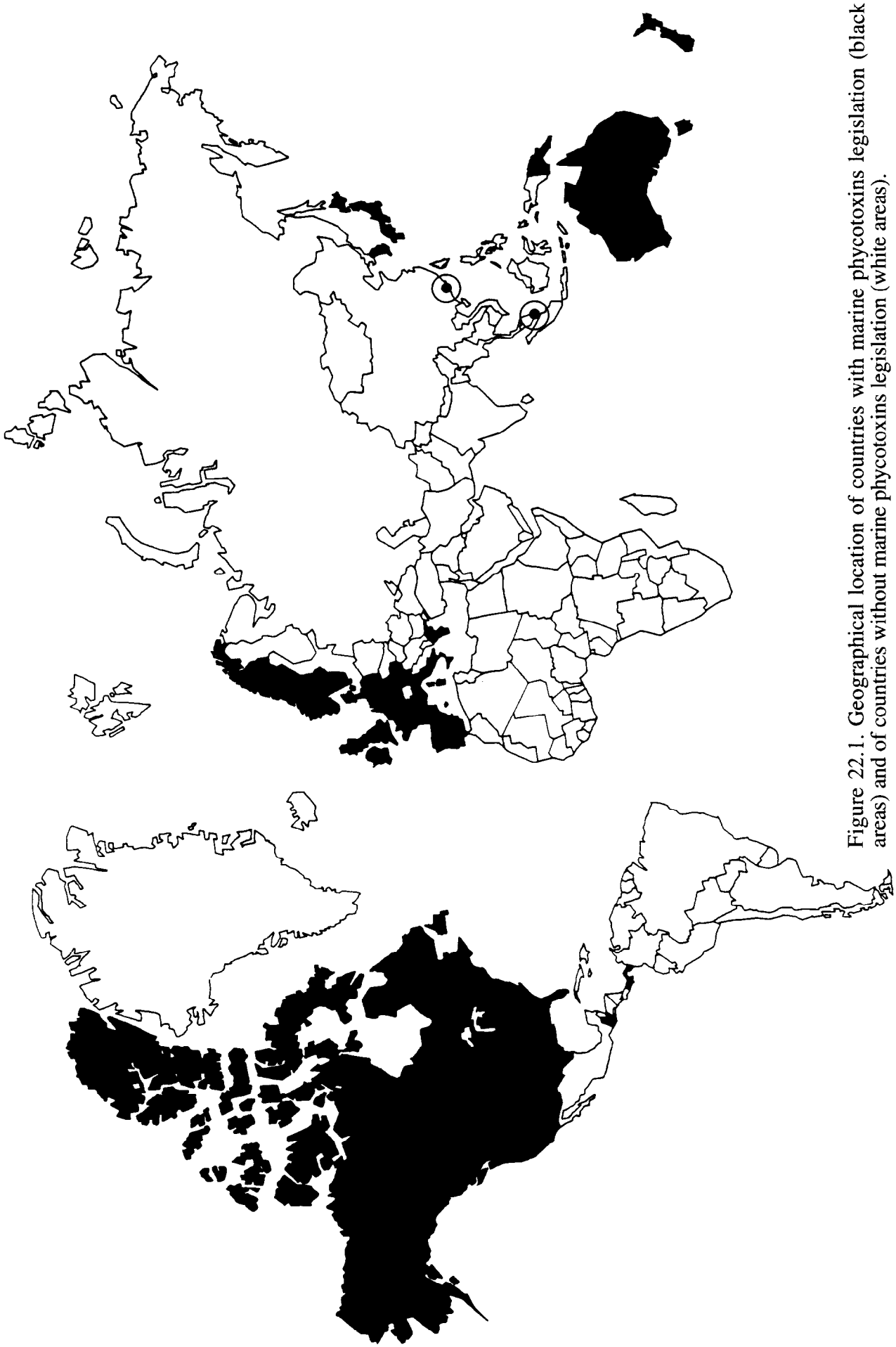

Figure 22.1. Geographical location of countries with marine phycotoxins legislation (black areas) and of countries without marine phycotoxins legislation (white areas).

shellfish. In Italy closure of harvesting areas occurs when simultaneous presence of toxic algae in water and toxin in mussels is noticed.

Regulations for paralytic shellfish poisons

The number of countries known to have in-force or proposed regulations for PSP was 25 at the time of writing (Table 22.3). Most regulations are set for paralytic shellfish poisons as a group. Some countries indicated specific regulations for one of the PSP toxins, mostly saxitoxin. In most cases the regulations concerned shellfish, but some countries mentioned more generally molluscs, or more specifically bivalves, as the types of products for which maximum permissible levels of PSP toxins were set.

Many countries indicated use of the standard mouse bioassay of the Association of Official Analytical Chemists International (AOAC) (Hollingworth and Wekell, 1990) as the method of analysis. A (non-selective) spectrophotometric method is applied in Austria (Hellwig and Petuely, 1980) and in Germany (Bundesgesundheitsamt, 1989). A few other countries apply an HPLC-method (Sullivan *et al.*, 1985), sometimes in addition to the mouse bioassay. In the EU a new directive came into force 1 January 1993 (Council of the European Communities, 1991), stating that the total PSP content in molluscs has to be determined according to the "biological testing method in association, if necessary, with a chemical method for detection of saxitoxin. If the results are challenged, the reference method shall be the biological method."

Different concentration units are used to express the tolerance level: mouse unit g^{-1} (MU g^{-1}) and $\mu g\ g^{-1}$ (incidentally $\mu g\ ml^{-1}$). The latter unit currently seems to be less appropriate in the countries that use the mouse bioassay, because they actually test for toxicity in the mouse. Expression of a tolerance level for PSP in $\mu g\ g^{-1}$ would be valuable, if the various PSP toxins exhibit the same toxicity, which is not the case (Krogh, 1988). If the unit $\mu g\ g^{-1}$ still would be preferred above MU g^{-1}, one might consider application of a toxic equivalence factor and expression of concentration of the various PSP (if these can be selectively measured) in concentration units of saxitoxin. The development of analytical-chemical methodology (HPLC) shows promise in selective toxin measurements.

The application of HPLC for regulatory purposes has been hampered by the lack of validated analytical methodology, pure analytical standards of the various PSP toxins, and reference samples for analytical purposes (Van Egmond *et al.*, 1993). Recent advances by the National Research Council of Canada (Marine Analytical Chemistry Standards Program, NRC, 1411 Oxford Street, Halifax, Nova Scotia, Canada B3H 3Z1) have made available certified solutions for paralytic shellfish poisoning poisons (standards for domoic acid and diarrhetic shellfish toxins are also available). To date, the AOAC mouse bioassay remains the only method of detection accepted by the United States Food and Drug Administration and members of the NSSP/ISSC agreement.

All countries that have a limit for toxicity apply a level of 400 MU 100 g^{-1} (which corresponds to approximately 80 μg STXequiv 100 g^{-1}). For those countries that apply tolerances expressed in physico-chemical concentration units, the situation is less uniform. As demonstrated in Table 22.3, the following variants occur: 80 μg PSP 100 g^{-1}, 40 μg PSP 100 g^{-1}, 80 μg saxitoxin 100 g^{-1}, 40 μg saxitoxin 100 g^{-1}. This situation cannot be beneficial to public health, nor to international trade, and it would be desirable to harmonize these limits on a commonly accepted basis.

Information was requested in the international inquiry as to the rationales that national authorities had applied in establishing their tolerance levels. Some type of response to the rationale question for PSP was received from 12 countries, but none of these indicated the risk considered acceptable. Australia indicated that tolerance levels are generally based on toxicity, taking account of analytical possibilities. The regulations of trading partners (e.g. the USA) also played a role in the establishment of the Australian limit for PSP. Canada mentioned that the rationale used to establish their PSP-tolerance was currently being re-addressed. France referred to the PSP bioassay of the AOAC (Hollingworth and Wekell, 1990), in which the threshold is

clearly indicated at 80 µg 100 g^{-1} and to the recommendations of a WHO Expert Consultation on PSP (Halstead and Schantz, 1984). Guatemala and Hong Kong referred to risk assessment studies based on WHO reviews of Halstead and Schantz (1984) and Wood (1976) respectively. Ireland referred to EU-documents and working group reports of the International Council for Exploration of the Sea (ICES) on harmful effects of phytoplankton, without further details. Japan set the PSP regulation level in accordance to that used in the USA and Canada, as this level was considered to be successful in both countries. The Netherlands indicated that a low tolerance for PSP of 40 µg kg^{-1} was desirable in view of the fact that a toxicological evaluation of the whole PSP-mixture was not available, and that most toxicological data relate solely to saxitoxin. The establishment of the desired Dutch tolerance was also influenced by the low Italian tolerance. Norway indicated that PSP limits were based on international risk studies and recommendations from a Norwegian expert group, without providing further details. In South-Korea, the risk had been determined in accordance with international specifications, supposedly those of the U.S.A. and Japan. Portugal also referred to international norms and standards, without giving specifics. Singapore referred to the AOAC standard mouse-bioassay, thereby obviously following the U.S. rationale in this respect. The U.S.A. indicated that no formal risk assessment had been completed and that the limit was primarily established on the basis of epidemiogical data and the capability of analytical methodology. However, should the methodology improve and the limit of determination decrease, this would not change the regulatory level in the U.S., because regulations in the U.S. were said to based on human health consequences and not on the idea that possible harmful substances must be avoided at any level.

In the European Union, where originally divergent tolerances for PSP-toxins were administrated, the limits were recently harmonized at 80 µg PSP/100 g mollusc flesh (Council of the European Communities, 1991), following the advice of the Scientific Veterinary Committee. This Committee agreed that levels up to 80 µg total PSP per 100 g mollusc flesh have not been shown toxic for consumers and that a tolerance of 80 µg total PSP per 100 g shellfish would ensure that no single component, such as saxitoxin, exceed 30 µg/100 g shellfish. Despite the preference of some EU-countries to go as low as 40 µg/100 g with their limit (Germany, Italy, The Netherlands) they follow the common EU-directive.

It might be appropriate for international authoritative bodies such as the World Health Organization (WHO) and the International Life Sciences Institute (ILSI) to re-evaluate current knowledge about toxicity of PSP and give guidance as to safe intake levels for PSP (Van Egmond *et al.*, 1993).

Regulations for diarrhetic shellfish poisons

There were 18 countries that had regulations for DSP at the time of writing, Canada and New Zealand being the most recent additions (Table 22. 4). Products for which limits were set were indicated with different degrees of specificity, e.g.: molluscs; shellfish; bivalves; mussels. The toxins covered by the regulations were mostly identified as DSP toxins and sometimes, more specifically okadaic acid. The tolerance levels for DSP were generally set at the limit of detection of the analytical method used, most often a mouse bioassay, originally developed in Japan (Yasumoto *et al.*, 1978). A few countries relied on a rat bioassay (Kat, 1983). HPLC was used in addition to the rat bioassay in Ireland and as the main method of detection in Sweden (Lee *et al.*, 1987). New regulations in the EC (Council of the European Communities, 1991) state that the normal biological methods of analysis may not lead to positive results for DSP in consumable parts of molluscs (whole animal or every separate consumable part). As with PSP toxins, analytical determination of DSP toxins has been limited. Efforts are underway in several countries to improve the situation. Neither the bioassays, nor the HPLC-procedure have been validated in international collaborative studies. Pure analytical standard and reference materials have not been readily available, and there is a great difference in performance of the mouse bioassay (toxicity criterion: animal death) and the rat bioassay (toxicity criterion: soft stool, diarrhoea and feed refusal), resulting in differences in specificity and detectability.

Presumably the mouse bioassay detects all DSP components, and probably also other toxins, whereas the rat bioassay detects only okadaic acid and the dinophysis toxins, because only these acidic components are known to cause diarrhoea in animals (Krogh, 1989). A new and promising analytical development is a solid phase immuno-bead assay, initially designed for ciguatera toxins. This assay cross reacts with other polyether compounds such as okadaic acid (Park, 1991). The recent development of certified calibration solutions of okadaic acid (OACS-1) and mussel tissue reference material for diarrhetic shellfish poisoning toxins (MUS-2) by the National Research Council of Canada (ibid.) should provide a viable means of standardizing methods of detection for DSP toxins worldwide.

The questions of rationale for established tolerances were only partially and superficially answered. Ireland and the Netherlands indicated that the limit should be established by the limit of the analytical method selected, as to avoid possible harmful substances in food at any level. In Italy the DSP-limit was said to be based on the experience of other countries, in particular France. Japan based its limit for DSP partly on a poisoning case study (Yasumoto *et al.*, 1978) and partly on the pracatical limit of detection of the mouse bioassay. In Sweden, risk assessment based on domestic experiences on the National Food Administration was the basis for the limit. South-Korea, Norway, and Portugal gave the same answers as for PSP.

As for PSP, the non-uniformity of tolerance levels for DSP requires harmonization for the benefit of public health and international trade. It might be desirable if international organizations would evaluate the hazards caused by DSP, and provide a basis for a common rationale for the establishment of DSP limits. Such an evaluation could include other adverse effects, such as the tumor-producing effect of okadaic acid, in addition to the acute effects of some DSP toxins on the gastro-intestinal tract.

Regulations for other marine phycotoxins

At the time of writing, only a few countries had established regulations for shellfish toxins other than PSP and DSP (Table 22.5). The countries that have (proposed) regulations for domoic acid (ASP) have a uniform tolerance level of 2 mg 100 g^{-1} product, based on a Canadian risk assessment (Bureau of Chemical Safety, 1988). In contrast to PSP and DSP toxins, the analytical determination of domoic acid is fairly straightforward. An HPLC method exists, which has been evaluated in a collaborative study (Bureau of Chemical Safety, 1989), and a certified instrument calibration solution and a certified mussel tissue reference material have been developed (Hardstaff *et al.*, 1990). The National Research Council of Canada has also developed a certified calibration solution for domoic acid (DACS-1B).

Regulations exist for neurotoxic shellfish poisoning (NSP), specifically for brevetoxin, and for ciguatera toxins in finfish. The acceptable level of brevetoxins (NSP) in shellfish is administrative zero, i.e. undetectable by bioassay. The United States and New Zealand are currently the only countries directly affected by NSP; however, as with PSP, ASP and DSP, all countries conforming to the NSSP/ISSC regulations adhere to the same limit.

Actions taken, when products contain unacceptable levels of toxins

Most countries surveyed indicated they did not allow entry of consignments of shellfish products containing an inadmissible amount of toxin. A few countries (Austria, United Kingdom) mentioned that condemned imported goods would be destroyed.

In the case of domestic produce, several countries (Canada, Guatemala, Ireland, Norway, Korea, Sweden, United Kingdom and USA) stop harvest of fishery products if levels of toxins exceed the limits and a waiting period is established until the concentrations of toxins are below acceptable limits. Harvested products containing too much toxin are usually destroyed. Canada mentioned that products containing PSP levels ≥ 80 µg 100 g^{-1} and ≤ 160 µg 100 g^{-1} may be canned and retorted.

CONCLUSIONS

Currently, limited information exists about limits and regulations on marine phycotoxins in 25 countries, located in North and Central-America, Western Europe, and Australasia. These regulations concern environmental surveillance for toxic algae and analytical monitoring of shellfish products for one or more of the toxins associated with PSP, DSP, ASP, and NSP.

The acceptable levels for the major toxins of concern (PSP, DSP) may differ between countries. It would be desirable if international organizations would (re-)evaluate the hazards caused by marine phycotoxins, to provide a common basis for risk assessment.

Moreover, further development and validation of analytical methodology (and reference materials) for marine phycotoxins is highly desirable, because the enforcement of phycotoxin legislation is ultimately based on the ability of analysts to identify and quantify accurately these toxins in seafood products.

INTERSTATE SHELLFISH SANITATION COMMITTEE AND NATIONAL SHELLFISH SANITATION PROGRAM (ISSC/NSSP, USA)

In the United States, the Federal Food and Drug Administration (USFDA) is charged with the responsibility of assuring that all food items shipped in interstate commerce are safely prepared, packed, and always held under sanitary conditions. Items must be correctly labeled and the conditions in which the food is prepared and held must also be safe and sanitary. In 1925, the F.D.A. was authorized to receive aid from State and local authorities in order to enforce laws to prevent and suppress communicable disease transmission. This led to the creation of the National Shellfish Sanitation Program (NSSP). The NSSP includes members of the F.D.A., State control agencies, and shellfish industry which set the standards to insure that sanitary conditions exist in the production and interstate shipment of shellfish, on a voluntary basis. The Interstate Shellfish Sanitation Conference (ISSC) was formed in 1982 and consists of members of State and Federal control agencies, the shellfish industry and the academic community. It is a voluntary organization and is open to all persons interested in assuring that shellfish reach the consumer under safe, sanitary conditions. The ISSC provides up-to-date sanitation guidelines for the regulation of harvesting, processing and shipping of shellfish. It also provides a forum for all interested persons to air their concerns regarding shellfish sanitation, and disseminates information of recent developments via publications, meetings and working with academic institutions as well as trade associations. Any country shipping shellfish to the United States must comply with these regulations (Hurst, personal communiation).

Within the United States, the State of Maine has one of the most comprehensive monitoring programs for paralytic shellfish poisons and will be described here as an example of a successful and effective monitoring system (see NSSP Manual - Part 1 Section C, Appendix A January 1990). This program was necessitated by yearly occurrences of toxic shellfish and has been used as a template for establishing monitoring programs in many other regions worldwide. The purpose of this program is to assure that only safe shellfish are harvested. Years of practical experience have afforded the opportunity to continually modify the sampling program, to better reflect increasing knowledge of potential toxic areas as well as the changing utilization of shellfish. Instead of "primary-key stations", areas of similar toxic patterns are used. At the beginning of the PSP testing year, shellfish samples are collected from each of these areas to determine the background level of toxicity. Sampling stations from these areas are sampled each week from April-October regardless of toxin patterns. When shellfish show any toxicity sampling is expanded until stations of no toxicity are found. This sampling program allows for closures to be made in a safe manner. Maine's law and regulation require the immediate closure of toxic shellfish harvest areas, embargo or confiscation of all suspect

shellfish. When necessary, licenses and certificates may be suspended. Administrative actions are accomplished on a same day basis.

THE SAMPLING PLAN

The Maine Department of Marine Resources is the state agency responsible for marine biotoxin monitoring. Personnel of the department located coastwide report any unusual occurrences such as bird or fish kills, water discoloration, and abnormal behavior of shellfish to the Marine Science Laboratory at Boothbay Harbor.

Channels of communication concerning fluctuations in shellfish toxicity have been actively established to provide updated information on shellfish toxicity with Canada, New Hampshire, Massachusetts and the FDA.

LAWS AND REGULATIONS

Paralytic Shellfish Poison Monitoring Program Section 6076

1. Purpose: A comprehensive Paralytic Shellfish Poison Monitoring Program is established to protect the public health while providing for the harvest of susceptible species of marine mollusks in areas not shown to be affected by contamination.
2. Responsibilities: The department shall be the state agency responsible for implementing the program. The department may adopt rules under section 6172 as may be warranted to provide for adequate protection of the public health.

Contaminated or Polluted Flats Section 6172:

The Commissioner may examine the coastal waters and intertidal zone and adopt regulations to close coastal waters or intertidal zone areas if he determines that any marine mollusks are or may become contaminated or polluted. The commissioner may adopt or amend regulations as he deems necessary, setting forth standards for contaminated or polluted areas, giving consideration to established state water quality standards, the most recently adopted federal sanitation standards, the most recent generally accepted research data and known sources of pollution in any area, in a manner so as to protect the public health and safety while allowing reasonable use of the state's shellfish. The commissioner may adopt or amend regulations under the emergency procedures, if immediate action is necessary to prevent the taking of polluted or contaminated marine mollusks.
SECTION 6192: *Notwithstanding any provisions of the Maine Administrative Procedure Act, an emergency regulation authorized by Section 6172, subsection 2 or 3 shall be effective immediately upon signature by the commissioner or his authorized designee. Upon promulgation of such an emergency regulation, the commissioner shall give oral notice of such an emergency closure to local governmental authorities and shall publish notice of closure as soon as possible in a newspaper of general circulation in the area of the State affected. Marine Patrol Officers shall take action to prevent taking of shellfish from that area, including the embargo of contaminated shellfish under section 6856, subsection 6 and the arrest of any person violating the emergency regulation.* SECTION 6856: *The commissioner or his agent, shall indefinitely embargo, condemn or order to be destroyed any shellfish product in any establishment whenever it is determined that the product is of unsound quality, contains any filthy, decomposed or putrid substance, or may be poisonous or deleterious to health, or otherwise unsafe. The commissioner and his agent shall cooperate with those state and federal*

agencies, having similar responsibilities, in the protection of public health and in enforcing the order to embargo or destroy.

Closure of Contaminated Areas Chapter 23.30

A. An area shall be closed to the harvest of shellfish immediately if the meats of shellfish harvested from that area contain 80 micrograms of Paralytic Shellfish Poison toxins per 100 grams of shellfish meats or contain concentrations of other toxins or contaminates known to be harmful to consumer health. The commissioner may also close surrounding areas of lower toxicity levels to provide a margin of safety in the event of rapidly changing toxicity levels.
B. The commissioner may close areas or fisheries if sufficient current information is not available to assure above conditions do not exist or current information does not permit prediction that the above conditions are unlikely to occur.

Chapter 23.40 Repeal of Polluted or Contaminated Area Closures

The Commissioner shall repeal polluted or contaminated area closure regulations when sanitary surveys reveal that pollution or contamination conditions no longer exist and that shellfish may be harvested from the area without threat to the public health.

PARALYTIC SHELLFISH POISON MONITORING PROGRAM

Maine's PSP monitoring program is continually modified to reflect increasing knowledge as to where toxic shellfish are likely to occur as well as changing utilization of shellfish. Shellfish sampling locations may be changed throughout the sampling year to reflect unusual conditions.

Maine's PSP monitoring program is conducted on a yearly basis from April until October. Based upon areas of similar toxin patterns the coast of Maine has been divided into 18 areas from southwest to northeast with Area 10 the most southerly and area 27 in Cobscook Bay the most northerly. At the beginning of the PSP testing year shellfish samples (mussels, *Mytilus edulis* and clams, *Mya arenaria*) are collected weekly from "primary" sampling stations from these areas. Early season shellfish samples determine the background level of toxicity in an area (hopefully below the limit of detection sensitivity of the test). Whenever there is any rise of toxin in an area the sampling of shellfish is increased to adjacent sampling stations so that when a closure is necessary adequate data is available to make the proper public health oriented closure. Closures are made with safety zones in place and frequently are made on the basis of previous PSP closures for that area. Shellfish collectors are trained by experienced staff on sample collection methods as well as how to locate the sampling stations. To aid in sampling all stations have been listed by area as well as which species of shellfish are present. Shellfish are returned to the lab under refrigeration and assayed as soon as possible under the standard mouse bioassay. Whenever toxin levels are found approaching the quarantine level of 80 micrograms STX equiv per 100 g of shellfish, the area is closed to shellfish harvesting. Closures are made on a species basis if adequate information is available to demonstrate that not all species are toxic. Species closures require increased sampling of the "toxin-free" shellfish. In areas where it can be justified, increased sampling will permit a partial opening of an area where part of the area is proven not toxic. Shellfish such as the ocean quahog, *Arctica islandica*, are sampled from contract fishing boats by department personnel. Areas where quahogs and similar species cannot be sampled are closed because they cannot be regarded as safe without sampling.Reopening of areas closed is dependent upon continued toxin levels less than 80 micrograms. Openings are made after evaluations of current and historical records. Areas in which historical data indicates that several rises of toxin may be expected remain closed throughout the PSP season, as making multiple openings and closures in these areas reduces the credibility of the PSP program. Areas in which there is a high value shellfish resource and

where there is reason to believe that there will not be another rise of toxin may be reopened after at least two weekly samplings below 80 micrograms. Reopenings are at best a judgment call and must be made upon the ability to assure that the area is safe.

TOXINS OTHER THAN PSP COVERED UNDER NSSP MANUAL REQUIREMENTS JANUARY 1990

The Department of Marine Resources (DMR) acknowledges that there may be toxins other than PSP in shellfish. DMR conducts a limited sampling program for Amnesic Shellfish Poison (ASP), domoic acid, in conjunction with it's PSP sampling program. Information from adjacent Canada concerning domoic acid is available on a up to date basis. Closures will be made whenever domoic acid levels reach 20ppm. Diarrhetic Shellfish poisoning (DSP) has not been reported from North America. Due to the presence of *Dinophysis* spp DMR recognizes that cases are likely to occur. DMR cannot assay for DSP toxins at this time.

Any area suspected of containing ASP, DSP or any other toxin defined or undefined will be closed under Chapter 23.30 until such time that area is deemed toxin free.

In order to sell shellfish to countries to of the EU shellfish must be accompanied by a Health Certificate. The USFDA has indicated that they will issue these certificates if the dealer involved is in compliance with applicable laws and regulations. This will involve giving consent for FDA to access quality control, production, and other relevant records. If the dealer is in compliance with NSSP regulations they will meet the EU requirement other than for DSP.

DSP may or not be a problem if the dealer has any records at all concerning the presence of toxic algae in the harvest waters.

There is at present no test for DSP in the US. The requirement for DSP in EU countries is a negative biological test. The FDA is assuming the responsibility until December 31, 1994 for issuing Health Certificates for molluscan shellfish to be exported to EU nations, they cannot certify them to be DSP free. It is reasonable to expect and suspect that DSP may at times be present in Maine mussels. The credibility of any certification of compliance with a biological standard without documentation of tests being run must be deemed questionable. Until the FDA can give us a standard testing method for DSP we should insist that *they* not the State laboratory certify the safety of these shellfish with respect to DSP.

There is a possible positive solution for determining the presence of DSP in mussels. A quick reading of the EU requirements indicates that knowledge of plankton in the harvest area is a important consideration. It is reasonable to assume that documented plankton absence is a good indication of the absence of DSP. We must recommend that anyone contemplating shipping shellfish to EU countries to collect data in their harvest area's on the presence or absence of potentially toxic plankton.

It is reasonable to expect the FDA to make the States assume the responsibility of determining any safety standards which is realistically theirs. If and when a Maine dealer(s) asks for certification to the EU market it must be pointed out that there is no way of determining a negative DSP assay.

Contingency Plan(s) for Toxic Shellfish Emergencies

Regardless of the overall success of a shellfish toxin monitoring plan designed to avoid toxin shellfish emergencies, they will occur, thus contingency plans should be developed to resolve these emergencies.

Contingency plans for toxin shellfish emergencies that affect the public health and safety should be developed so there will be no misunderstanding of what actions to take. It is very important that adequate legal authority to act immediately be in place and understood by all.

Updated lists of the person(s) responsible for decisions should be known to all persons involved so that proper actions can be taken in a timely manner. Emergencies by their very nature can be expected to occur at inopportune times, thus the chain of command must be defined in advance.

The extent of the toxin emergency should be defined if at all possible. Small local emergencies can be concluded quickly by the embargo and destruction of product and a closure of the harvest area(s). Attempts to salvage portions of confiscated shellfish should be discouraged, as the sampling method will always be in question.

Listed below are some of the information needed to evaluate a shellfish emergency:
Location of the shellfish in question:

Positive identification of harvest area(s)
Species of shellfish under question
The amounts of shellfish involved
Is the toxin identified?

In emergencies that occur due to unexpected rises of known toxins, quick positive action to close harvest areas and embargo and destroy shellfish in the market should be expected to solve the problem.Rises of unknown toxins require quick action to prevent real serious problems. Shellfish must be removed from the market and harvesting prohibited until the toxin is identified. It must be assumed that if the safety of shellfish is in doubt, they are not safe.

Public health and safety requires the as complete as possible removal of any toxic shellfish from the market and closure of any suspect harvest area. Economic value of the shellfish resource is secondary to public health and safety.

Unknown toxins present special problems as testing procedures have to be developed to determine when the shellfish will be safe. Modifications of shellfish monitoring plans must be made to include these new toxin(s).Cooperative agreements between laboratories to address the appearance of new toxins are desirable. These agreements should be general in nature as what expertise and needed equipment cannot be determined in advance.Contingency funding for shellfish emergencies while desirable cannot be anticipated in advance thus some idea of where funds can be obtained should be given some thought.

Shellfish Certification

Persons or Cooperations interested in selling shellfish in the International market should contact their respective National Health agency and their National Natural Resource agency for their current regulations concerning shellfish. In order to market safe shellfish, regulations must be in place and enforced concerning the sanitary suitability of the harvest area(s) and the safe processing of the shellfish. The exporting nation is responsible for determining the safety of shellfish being exported.If the potential shellfish market is the United States, there must be in place a Memorandum of Understanding (MOU) between the National Agency responsible for Shellfish Safety and the USFDA. Nations that have current MOU's with USFDA are: Australia, Canada, Chile, Japan, The Republic of Korea, Iceland, Mexico, England and New Zealand. The MOU's may restrict harvest areas and selected species of shellfish.Shipments of shellfish to nations that are members of the European Union (EU) - France, Germany, Italy, United Kingdom, Spain, Portugal, Greece, Netherlands, Belgium, Denmark, Ireland and Luxembourg must meet the EU requirements of sanitation under Council Directive (91/492/EEC) (Commission of the EU, 1992) which requires a Certificate of Health by a competent authority from the nation of origin. This certificate must address measures that assure that the shellfish meet the EU standards for shellfish sanitation. Nations not listed above can also be expected to require a MOU from the nation of origin concerning safety of shellfish being imported.The

USA regulations are carried out under the National Shellfish Program which is similar to other National Programs.

Toxin Management

The overall management of toxins in shellfish is in the developmental stage, and should be considered to be limited at best. Management of toxic shellfish requires extensive monitoring of the harvest areas and as demonstrated by the example of the State of Maine (USA) it can successfully combine public health assurances and commercial harvest realities. It must be understood that defining safe harvest areas is the real goal of management of shellfish resources as related to toxic shellfish. Monitoring of the toxic harvest areas allows for the reopening of these areas but does not protect the public health and safety.

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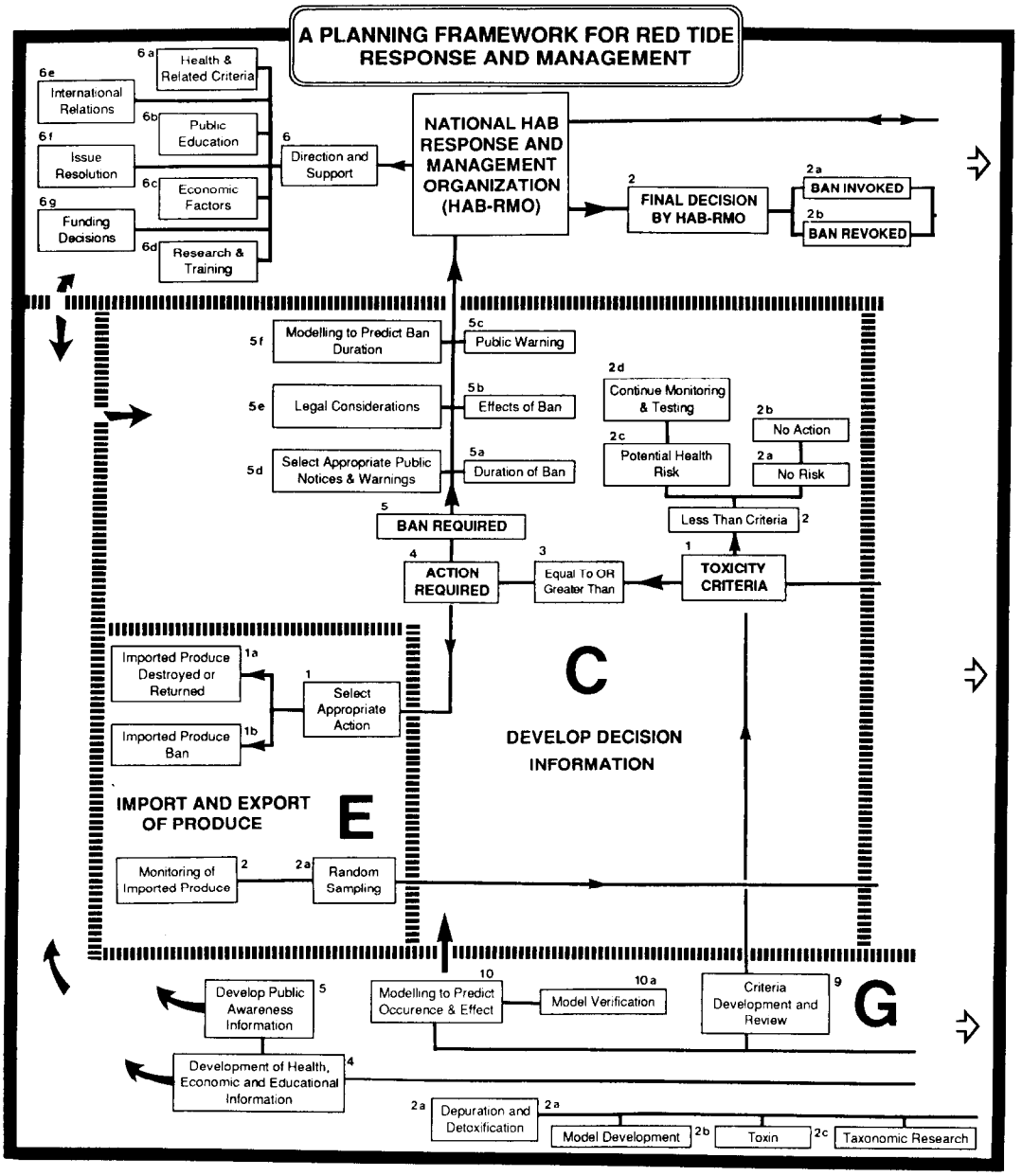
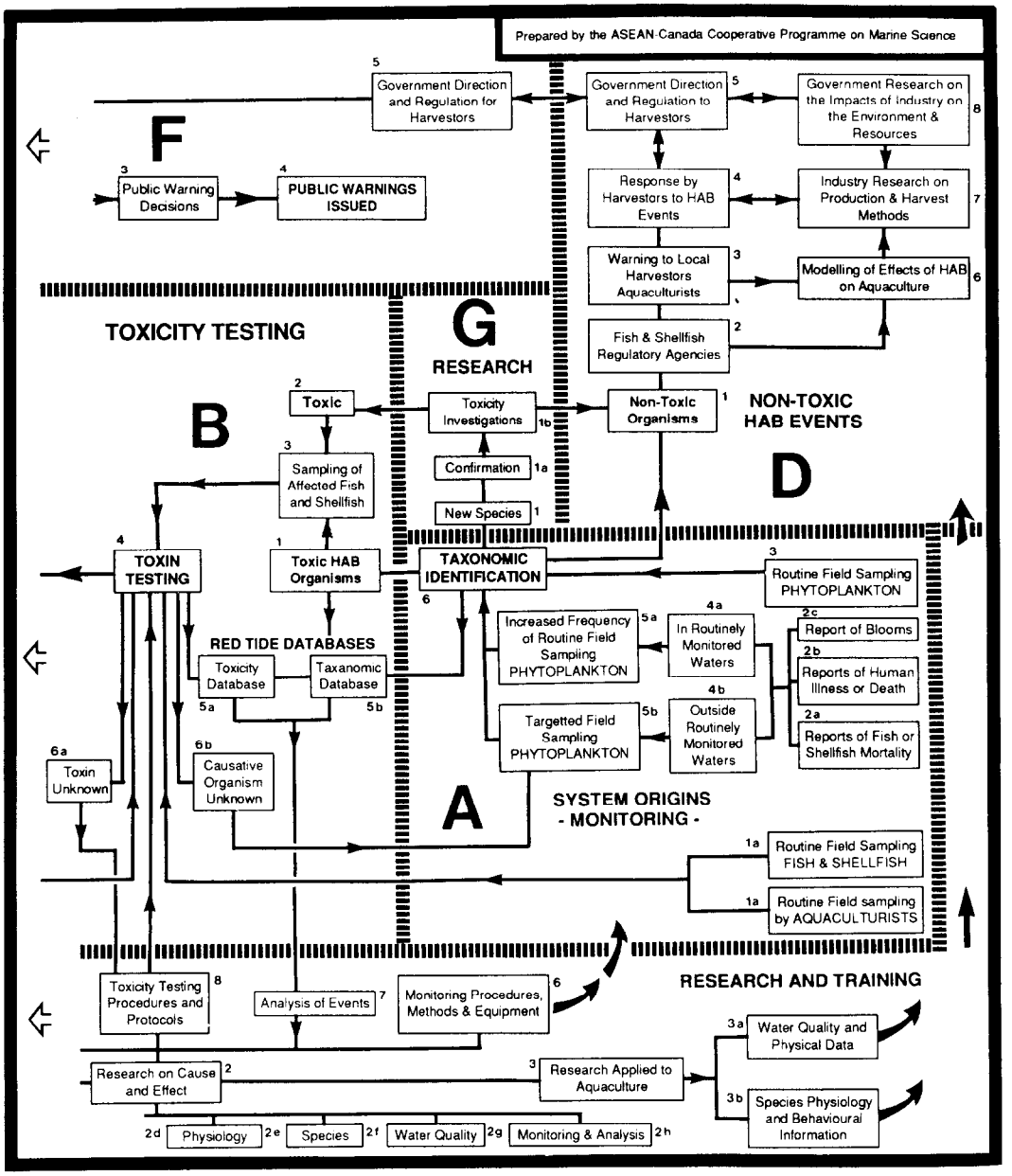


Figure 22.2. Red Tide Response and Management Framework, prepared by the Red Tide Technical Working Group under the ASEAN-Canada Cooperative Programme on Marine Science, as a tool for assessing and developing red tide response and management systems in ASEAN and its member states.


Prepared by the ASEAN-Canada Cooperative Programme on Marine Science
F
Government Direction and Regulation for Harvestors
Government Direction and Regulation to Harvestors
Government Research on the Impacts of Industry on the Environment & Resources
Public Warning Decisions
PUBLIC WARNINGS ISSUED
Response by Harvestors to HAB Events
Industry Research on Production & Harvest Methods
Warning to Local Harvestors Aquaculturists
Modelling of Effects of HAB on Aquaculture
Fish & Shellfish Regulatory Agencies
TOXICITY TESTING
G
RESEARCH
B
Toxic
Toxicity Investigations
Non-Toxic Organisms
NON-TOXIC HAB EVENTS
D
Sampling of Affected Fish and Shellfish
Confirmation
New Species
TOXIN TESTING
Toxic HAB Organisms
TAXONOMIC IDENTIFICATION
Routine Field Sampling PHYTOPLANKTON
RED TIDE DATABASES
Toxicity Database
Taxanomic Database
Increased Frequency of Routine Field Sampling PHYTOPLANKTON
In Routinely Monitored Waters
Report of Blooms
Reports of Human Illness or Death
Targetted Field Sampling PHYTOPLANKTON
Outside Routinely Monitored Waters
Reports of Fish or Shellfish Mortality
Toxin Unknown
Causative Organism Unknown
A
SYSTEM ORIGINS - MONITORING -
Routine Field Sampling FISH & SHELLFISH
Routine Field sampling by AQUACULTURISTS
RESEARCH AND TRAINING
Toxicity Testing Procedures and Protocols
Analysis of Events
Monitoring Procedures, Methods & Equipment
Water Quality and Physical Data
Research on Cause and Effect
Research Applied to Aquaculture
Species Physiology and Behavioural Information
Physiology
Species
Water Quality
Monitoring & Analysis

23. Management of Finfish Aquaculture Resources

J.E. Rensel

Worldwide there is a growing awareness of the importance of harmful algal blooms (HABs) and their impact on wild and aquaculture finfish. Recent expansion of marine aquaculture in both the northern and southern hemispheres has revealed several species of microalgae as fish killers. Catastrophic losses of aquaculture fish have occurred in recent years due to some of these HAB species, but it is likely that other microalgal species will be implicated in future losses of aquaculture fish, as the geographic range of aquaculture expands. Some of the most important fish-killing microalgal forms that involve aquaculture are listed in Table 23.1.This chapter focuses on HABs and marine culture of salmonids, yellowtail tuna and red sea bream because of their economic importance Several other fish-killing microalgae such as *Gymnodinium breve* (=*Ptychodiscus brevis*) are not discussed here because their involvement has been mostly with wild fish stocks.

OVERVIEW OF HARMFUL ALGAL BLOOM EFFECTS AND MITIGATION STRATEGIES

1) A variety of physiological mechanisms may be involved in HAB-caused fish kills. The microalgae which cause these effects may be grouped into two broad categories that reportedly:

a) physically damage fish gills to the point of compromising osmoregulation and/or inhibiting oxygen uptake. The mechanisms may include abrasion of the gill epidermis, physical clogging of the gill filaments with excess mucous copiously produced in response to some irritants, or in some cases stripping of the protective mucous layer. Examples are the diatom *Chaetoceros concavicornis* and perhaps the silicoflagellate *Dictyocha speculum.*

b) produce harmful chemicals, neurotoxins, hemolytic or blood agglutinating substances that cause physiological damage to gills, major organs, intestine, circulatory or respiratory systems or interfere with osmoregulatory processes. Several example species are listed in Table 23.1.

It may be argued that both of the above categories result in the same ultimate cause of fish death, i.e., malfunction of major organs such as the brain and heart due to blood hypoxia. However, in several cases there is large uncertainty regarding the precise kinds of chemicals involved and initial mechanism leading to blood-hypoxia and fish death (Table 23.1).

2) HABs are a major impediment to marine finfish aquaculture by causing biotoxin accumulation or fish death. Repeated HAB occurrences have completely extirpated commercial finfish aquaculture from some coastal regions such as the Sunshine Coast, north of Vancouver, British Columbia due to *Heterosigma carterae* and harmful *Chaetoceros* species. In many other coastal regions of the world where aquaculture remains active, HAB-caused fish kills have resulted in substantial monetary losses, and in some cases, loss of individual companies.

3) The occurrence of HABs is largely unpredictable, but the effects on fish farms may be managed or avoided through careful siting and certain mitigation techniques. Conceptual or

Table 23.1. Harmful phytoplankton species known or suspected of causing fish losses in aquaculture, concentration suspected to be harmful to fish, etiology and a few references.

CATEGORY AND SPECIES	HARMFUL CONCENTRATIONS & ETIOLOGY	REFERENCES
Diatoms[1]		
Chaetoceros concavicornis and *Ch. convolutus* and probably others of subgenus Phaeoceros	> 2-5 cells/ml for salmonids, depends on chain length. Cells lodge between gill lamellae causing mucus production, irritation & leads to blood-hypoxia/anoxia	Bell, 1961; Rensel, 1993; Taylor, 1993
Dinoflagellates		
Alexandrium tamarense possibly *A. catenella*	unknown; acute mortality to farmed fish not well documented; potential food web toxin accumulation could affect wild fish	White, 1980; Mortenson, 1985; Erickson, 1988
Ceratium fusus	unknown; gill irritation, poorly understood causes oyster larvae and shrimp loss also	Rensel and Prentice 1979
Cochlodinium spp.	unknown; some doubt about toxicity	Yuki and Yoshimatsu, 1989; Hallegraeff, 1991
Gymnodinium mikimotoi (= *G. nagasakiense*) may be identical to *G. aureolum*	unknown and possibly variable; causes damage to epithelial surfaces of gills and digestive system, poorly understood.	Takayama and Adachi, 1984; Okaichi, 1989; Tangen, 1977
Noctiluca miliaris (= *N. scintillans*)	variable; unionized ammonia causes gill damage and other problems for fish	Okaichi and Nishio, 1976
Prymnesiophyte Flagellates		
Chrysochromulina polylepis, *C. leadbeateri*	unknown; causes gill damage and osmoregulatory problems	Estep and MacIntyre, 1989 Skreslet *et al.*, 1993
Phaeocystis pouchetii	unknown; irritant substances and the alga's mucus can clog gills	Lancelot *et al.*, 1987; Gaines and Taylor, 1986
Prymnesium parvum	unknown; toxins cause tissue, blood-cell and neurological damage	Shilo, 1982
Raphidophyte Flagellates		
Chattonella antiqua (also *C. marina*)	500 c/l @ 23 °C; 110 c/l @ 29°C yellow-tail tuna; O_2 radical and hydrogen peroxide strips gills of mucus, leads to osmoregulatory problems and death.	Okaichi *et al.*, 1989; Onoue *et al.*, 1990; Tanaka *et al.*, 1994
Heterosigma carterae (formerly *H. akashiwo*)	probably variable, in most cases >750 to 1,000 cells/ml; cause of fish death unknown, may be similar to *Chattonella*	Chang *et al.*, 1990; Black *et al.*, 1991; Taylor and Haigh, 1993
Silicoflagellates		
Dictyocha speculum	unknown; siliceous skeleton may irritate gills, possible toxin action too.	Thomsen and Moestrup, 1985

1/ Blooms of the diatom *Chaetoceros* of the subgenus Hyalochaete that lack long, partly hollow setae have been implicated in a few kills of small Atlantic salmon soon after seawater entry in the Pacific Northwest (J. Rensel, R. Elston; unpublished data). Similarly the problem has been seen with the same species in Scotland (Bruno et al. 1989). Other spiny diatom species such as *Corethron* sp. (Speare *et al.*, 1989) may cause problems and even one without spines, *Leptocylindrus minimus*, has been implicated in recurring losses of net pen salmon in southern Chile but the mechanism is unknown (Clement and Lembeye, 1994).

mathematical models can be developed through hindcasting of empirical data and other means that may give indications of periods of increased HAB risk. Remote sensing from buoys, airplanes or satellites using sea surface temperature, chlorophyll *a* and other sensors can help track discrete parcels of water that contain HABs. This has been used successfully in some cases for monitoring the movement of blooms into or through coastal areas with aquaculture facilities, as discussed below.

METHODS OF AVOIDING AND DETECTING HARMFUL ALGAL BLOOMS

Site Selection

Judicious choice of aquaculture sites based on past or predicted HAB occurrence is an obvious but seldom exercised measure to reduce risk of fish kills. In the practical world of fish farming there are many important siting and permitting considerations that may take precedence over HAB concerns. Fish farms are sometimes located by trial and error with respect to HABs, but there have been exceptions where prior study has been effective in avoiding unsuitable locations. General aquaculture siting references include Edwards (1978); Sedgwick (1982); Beveridge (1987); and ICES (1992).

Basic hydrographic monitoring should be conducted in the general vicinity of a proposed finfish culture project to evaluate site suitability for the cultured fish species and to estimate HAB risks. Variables such as water temperature, salinity, vertical stratification profiles, dissolved oxygen saturation, water transparency and chlorophyll *a* can, in the hands of an experienced analyst, be surrogate indicators of the potential for certain types of harmful blooms to occur. Concurrently-collected phytoplankton composition data are very desirable to characterize a site. Sediment collection for examination of dinoflagellate or microflagellate cysts may be useful in some cases. However, strong tides necessary to disperse waste products may preclude the presence of a soft bottom substrate and cyst deposition. Hydrographic and phytoplankton data from prior studies by universities, government agencies or first-hand experience from others may be available at no expense to evaluate HAB risks in an area. If these are not available, field surveys should be conducted during the appropriate algal bloom seasons. Alternatively, only small scale or test facilities such as small net pens should initially be operated while hydrographic, phytoplankton, fish growth and survival data are gathered.

In general, adverse effects of HABs may be reduced or avoided by selecting marine aquaculture sites with moderate to strong vertical mixing and tidal current velocity. Dinoflagellate and microflagellate blooms are less likely to remain intact in such areas and turbulence may reduce cell growth rate (White, 1976). Additionally, strong currents will reduce or eliminate bottom sedimentation and adverse water column effects from aquaculture waste products. Strong vertical mixing may not be a deterrent to all harmful species, however, as the diatom *C. concavicornis* may be harmful at relatively low concentrations (Rensel, 1993) and be present throughout well mixed water columns (Rensel Associates and PTI Environmental Services, 1991). Even with extensive site surveys and location of facilities in strong current areas, there is still a risk of blooms being advected into a usually safe area from offshore or adjacent waters. Examples include *Gyrodinium aureolum* blooms along the coast of Norway (Dahl and Tangen, 1990), shellfish toxin-producing *Gymnodium catenatum* blooms on the northern Atlantic coast of Spain (Fraga *et al.*, 1988) and the fall 1994 *H. carterae* blooms in active tidal channels of the inland passage between Vancouver Island and the British Columbia mainland. In cases such as these, fish farmers must rely on mitigation techniques such as those discussed below.

HAB and Fish Health Monitoring by Fish Culturists

Routine monitoring of HABs is practiced at fish culture facilities in several countries. It varies from occasional, qualitative microscopic examination of plankton net tow contents to detailed, daily species counts from water bottle or composite depth samples. A few manuals for aquaculturists are available including Gaines and Taylor (1986), Hallegraeff (1991) and Horner (1992). General phytoplankton monitoring strategies are discussed by Venrick (1978), Margalef (1978) and Smayda (see chapter 21).

Very little summary information is available regarding the concentration of HAB cells that cause injury or death of various fish (Table 23.1), and it probably varies with species, stock, size and condition of fish, water temperature, exposure periods, etc. In some cases the mere presence of a few HAB cells in a non-concentrated water sample are reason for concern and justify an increase in monitoring efforts (e.g., harmful *Chaetoceros* spp.). At other times no fish mortality has been observed when concentrations of some HAB species (e.g., *H. carterae*) are relatively high, suggesting controls by environmental factors or genetic variation among blooms.

After training and practice, fish-farm technicians with an intellectual interest in the topic are able to accurately identify microalgal species that are harmful to finfish using a light microscope. Identification may focus on morphology, color, size, swimming behavior or appearance after fixation, but the use of taxonomic keys by technicians is uncommon. Permanent microscope slides or photographs of key species found in manuals are useful to guide the uninitiated. Palmer-Maloney counting chambers are often used, although other means may be utilized such as filtration of larger volumes of water onto filter paper for detecting harmful diatoms. Fish culturists may establish routine HAB monitoring protocols including the daily collection and storage of preserved water samples. Daily archiving of samples may help in predicting future blooms if matching hydrographic data were collected. If not analyzed, samples should be kept for at least two weeks prior to sample disposal and reuse of the bottles.

If a region's aquaculture resources are adequately developed, it may be useful to form cooperative monitoring services or information sharing networks for aquaculturists that employ rapid telephone or facsimile notification. For large scale bloom monitoring, sea surface temperature and other parameter monitoring by satellite (NOAA coastwatch in U.S.) or real-time data transmitting ocean buoys (e.g., MARINET in Norway and government ocean buoys in Canada) provide the ability to track discrete water masses that may contain HABs.

It may be possible to detect the onset of HABs by monitoring behaviour of cultured fish. In some cases fish will reduce or stop feeding, may orient themselves unusually in the water, may lose self-righting ability or may concentrate in the up or downcurrent ends of the pens or tanks. Different sized fish or species of fish may react with differing behavior patterns and physiological responses, and some related fish species are known to be more sensitive to HAB than others.

MITIGATIVE PRACTICES

A number of practical measures can minimize the effects of harmful algal blooms on finfish aquaculture. Usually more than one of the following methods will be employed, depending on the type of HAB, the fish species and the site location characteristics, etc.

Feeding and Handling Practices

One of the most effective and least costly mitigation practices for finfish aquaculture is to withhold feed immediately prior to, if possible, and during minor HAB episodes. This reduces the digestive demand for oxygen, which is still required for other basal metabolism. Over a

period of several weeks, however, this practice causes increased stress due to low glycogen stores, catabolism of tissues, and susceptibility to chronic diseases such as bacterial kidney disease. Cessation of all fish handling and restriction of activity nearby the cages acts to reduce stress on the fish, decreasing the demand for oxygen. However, both of these strategies are usually not sufficient to fully mitigate the effects of major HABs. Other forms of mitigation that may be utilized during severe HAB events are discussed below.

Increasing the amount of oxygen-rich carbohydrate in the diet while reducing the oxygen-poor fat content could reduce the metabolic demand for oxygen by the fish. Unfortunately, most carbohydrates are less digestible for salmonids than other dietary components. This is an area of further research that some day may yield useful mitigation strategies.

Oxygenation and Aeration

Injection of oxygen into fish culture water to create supersaturated conditions may be technically feasible for mitigating the effects of HABs that cause gill clogging or mucus production and gill tissue damage. However, the method has not been used on a commercial scale, but could be used in net pens equiped with perimeter skirts to retain treated water. Moderate levels of oxygen supersaturation may be beneficial to salmonids and yellowtail subjected to compromised water quality conditions (Boyd and Watten, 1989; Okaichi *et al.*, 1989). For 190 g rainbow trout "moderate" means <300% of the air saturation concentration of oxygen (approximately 477 mm Hg), without supersaturated nitrogen gas (Boyd and Watten, 1989). Colt *et al.*, (1991) suggest that high density salmonid culture requires conditions with up to 400 mm Hg oxygen pressure. Supersaturated oxygen has been used with some success in freshwater fish hatcheries and is often referred to as "oxygen supplementation". It is one of the key factors in design of on-shore marine salmon farms that must rear larger fish at very high densities to be economically feasible.

Although oxygen supersaturation has proven effective in the laboratory in reducing fish losses due to at least one harmful alga (*C. concavicornis*), the economics of supplying an entire net-pen facility with large volumes of oxygen are relatively poor (Rensel, 1992). Other measures to mitigate the effects of HABs on finfish reared in net pens should be considered first, unless the fish are very valuable such as brood stock of endangered species.

Aeration of fish culture water is not recommended to sustain marine fish exposed to HABs because it is often only marginally effective in increasing the ambient concentration of dissolved oxygen during algal blooms. This is because the transfer rate of dissolved oxygen to the water is proportional to the difference between ambient and desired concentrations. The exception would be during the senescent phase of very high density blooms that in some cases may result in severe reductions of dissolved oxygen due to algal respiration and cell decay.

Aeration in net pens serves only to create vertical convection cells in the water and does little to flush water or microalgal cells from the pens (Kils, 1979). Extremely vigorous aeration could help break the chains and setae of the harmful diatom *C. concavicornis* into smaller sections that have proven to be less harmful in the laboratory (Rensel, 1992). However, the amount of turbulence required would likely require prohibitively expensive equipment and could adversely affect the fish by causing stress and increased respiratory demand. Similarly, aeration-induced turbulence in net pens for reducing dinoflagellate growth (White, 1976) is unlikely to be practical.

Air-lift pumping of deep water into cages

This technique may be effective when a harmful bloom is restricted to the near-surface layers of the water column. Air injected into vertically-hung large pipes can be used to pump relatively cell-free water from depth. In some cases it has been very useful in mitigating the adverse effects of *H. carterae*. Air-lift pumping may be more efficacious if non-permeable skirts or

sheets of plastic material are vertically suspended around the perimeter of fish cages. The skirts can reduce the advection of HABs into the cages and reduce the dissipation of pumped water.

Air-lift pumps are a very cost effective means of moving water, but must be used with caution as gas supersaturation can result if the inflow of air is located too deep within the tube. Gas bubble trauma caused by excessive gas supersaturation may also be avoided by using large size bubble diffusers that act more to move the water than to increase dissolved gas concentration (Huguenin and Colt, 1989). Air-lift pump efficacy can be monitored by using a semi-conservative or conservative tracer such as temperature or salinity to evaluate the effectiveness of moving water from depth to the surface.

Moving net pens

Towing net pens from an area affected by HABs to a known refuge area is one of the most effective mitigative measures. The cost of towing net pens may be substantial, although a fish farm's insurance company may cover part of the expense. Towing involves the risks of structural damage to facilities or escape of the fish if not conducted properly. Furthermore, fish already affected by HAB will be particularly sensitive to the additional stress of towing. A towing contingency plan should be devised in advance that includes protocols for dealing with anchoring systems and timing of movement with regard to tides. Practice towing exercises may be warranted as well.

Towing of net-pens has been useful for preventing salmon net pen losses due to the harmful microflagellate *H. carterae* in Puget Sound, Washington and British Columbia. It has also been used with the harmful dinoflagellate *Gyrodinium aureolum* in Norway and *Chattonella antiqua* in the Seto Inland Sea of Japan. Aerial surveys by small airplane or visual surveys by boat can be useful in his regard, particularly when conducted in the morning before sea or land breezes have mixed cells away from the surface. Harmful *Chaetoceros* events are difficult for fish farms to manage by towing because the vertical and horizontal distribution of the cells in the water column is difficult to ascertain over large areas. Finding a true refuge area may therefore be difficult. The concentration of these diatoms required to harm fish is below the threshold of visible change to water transparency or effect on other easily measured hydrographic parameters such as dissolved oxygen or pH.

Submerging net pens

Submersion of cages to avoid HABs is generally not recommended as it is technically difficult and because net-pen cages are usually not designed to deal with the structural stresses of submerged nets. In some regions net pens are located in areas of relatively strong currents for environmental management reasons, which limits the ability to submerge cages. Additionally, physostomous fish such as salmonids must occasionally imbibe air for their float bladders to stay neutrally buoyant. This is not possible during continuous captive submersion. However, in cases where cages are located in physically quiescent areas, or in the case of specially designed pens with adequate site depth, the technique may have considerable merit.

Therapeutics

At present there are no readily-available or government-approved therapeutic drugs designed specifically to treat fish that have been affected by HABs, but this is a rapidly developing field. For example, Tanaka *et al.*, (1994) suggested that the production of superoxide anion radical (O_2^-) by *Chattonella antiqua* may strip mucus from fish gills and lead to osmoregulation problems that are thought to be the ultimate cause of fish death. The oxygen radical may even be reduced to the more harmful hydroxy radical in seawater. However, application of a combination of enzymes including superoxide dismutase, catalase, and glutathione peroxidase

may be useful in treating affected water (Colt *et al.,* 1991). It has been suggested (Yang *et al.*, 1994) that another raphidophyte, *H. carterae* may be treated by the same process, although laboratory results have not been published to date.

When hypersecretion of mucus from fish gills and blood hypoxia are suspected following exposure to certain species of harmful algae, chemicals that reduce mucus production could provide mitigative action. Mucolytic agents such as L-cysteine ethyl ester fed to fish have reportedly reduced mucus production and sustained fish during exposure to harmful *Chaetoceros* in the laboratory (Yang and Albright, 1993). This appears to be a useful approach to dealing with acute exposures, but there are potential problems with such an approach for longer exposures that may occur for a month or more. First, feeding of fish during HABs is not recommended because the high oxygen requirements needed for digestion competes with oxygen demand for basal metabolism. Secondly, a moderate amount of mucus production on the gills from chronic exposure to harmful *Chaetoceros* is likely beneficial to the fish. Mucus discharge along with coughing response provides a defense mechanism for removal of the HABs from the gills (Rensel, 1993). Without mucus, some species of spiny phytoplankton that lodge in the gills would likely be enveloped through lamellar fusion, as has been documented for *Corethron* sp. (Speare *et al.,* 1989). Mucus provides a protective barrier and lubricating ability for the gills, without which the gills are more susceptible to secondary infection from bacteria, viruses and parasites.

Other vasoactive drugs such as adrenaline and acetylcholine that, regulate the distribution of blood to the gill secondary lamellae (Part *et. al.*, 1982) could possibly be of some use in treating HAB -affected fish. The need to administer these orally and the potentially high costs, however, limits their usefulness.

Water Treatment

Treatment of fish-rearing water to destroy or precipitate HABs may be useful in on-shore tank farms or in areas of intense net pen use such as some of the Japanese prefectures. For most net-pen rearing conditions, however, it would not be economically feasible to treat the large volumes of water that surround and flow through the cages. One large scale treatment that may be possible is the proposed use of an submerged electrode set at a certain potential to neutralize harmful oxygen radicals thought to be produced by *Chattonella antiqua* (Tanaka *et al.,* (1994).

Acid-treated clay reportedly has been effective in disrupting and precipitating *Chattonella spp., Cochlodium '78* and other phytoplankton in laboratory and field trials in Japan (Maruyama *et al.*, 1987; Shirota, 1989). Copper sulfate has been widely used in the past for limiting freshwater blooms of algae, but it is presently not considered a viable option because of negative impacts on non-target organisms, contamination of sediments, temporary effects and high costs (Cooke *et al.,* 1993). Other chemicals such as formalin and hydrogen peroxide have similar problems. However, treatment with sodium per(oxo)carbonate eliminated 90% of *Chattonella* cells after only two hours both in onshore tanks and in the shallow surface layer of Shido Bay Japan in 1987 (Okaichi *et al.,* 1990). A concentration of 50 mg/L was required and did not significantly alter ambient pH.

A possible future treatment for aquaculture fish affected by *H. carterae* may be the surface addition of freshwater to skirt-surrounded net pens. Hershberger *et al.*, (in prep.) has reported rapid migration of the alga to the surface of columns in the laboratory when small amounts of distilled water are gently added to the surface. If a similar procedure is possible in commercial scale net pens, microalgal cells could be skimmed off the surface and replaced with cell-free water pumped from depth.

Other Techniques

Other mitigation practices for HAB-caused fish kills are possible. For nutrient-sensitive areas these include improvement of the environment by reduction in N and P discharge by

municipalities, industry, and agriculture. Aeration below the pycnocline to de-stratify the water column may be useful in some cases if the HAB is dependent on stratified conditions. Preemptive fish harvest just prior to a HAB is often practiced. Where fish already exposed to HAB are harvested for sale as food, care should be taken to ensure that fish tissue is free of toxins or harmful residues. Table 23.1 shows that some HAB species such as *H. carterae* leave no known toxin or harmful residue in the fish tissues.

At least a few species of fish may acclimate to blood hypoxia caused by gill problems. Anacrobic metabolism may be facilitated by increased perfused area of the gills and by elevated oxygen-carrying ability or numbers of erythrocytes in the blood (Shepard, 1955). However, acclimation to environmental or blood hypoxia for salmon may not be possible judging from the results with rainbow trout (Smith and Heath, 1980).

Although not yet attempted on a production scale, large volumes of freshwater could be used to lower the salinity to a level near fish blood content (approx. 10 ppt) during a HAB. This would reduce the energetic costs of osmoregulation and slightly increase the oxygen carrying capacity of water. Bath and Eddy (1979) found that dorsal aorta blood P_{O_2} of trout in freshwater was about 110 mm Hg. After seawater entry it dropped quickly to 80 mm Hg, then decreased after 28-h to about 60 mm Hg. This pattern was inversely mirrored by the oxygen consumption rate. The authors proposed that increased gill osmotic and ionic metabolic activity consumed a significant proportion of the oxygen before it reached the dorsal aorta of the seawater-held fish.

OVERVIEW OF FISH KILL SAMPLING PROCEDURES

Fish-kills in aquaculture may be caused by a wide variety of causes, including toxic chemical discharges, disease, environmental hypoxia or anoxia due to algal bloom decay, discharge of oxygen-demanding wastes, etc. In some cases it may be difficult to ascertain the cause of a specific fish kill. This necessitates an expeditious, broad-scale environmental and fish tissue sampling campaign if the cause is to be revealed. A contingency plan should be prepared by fish farmers to deal with HAB occurrence in a timely manner. A number of literature sources provide detailed information regarding fish kill investigations, including Svobodová *et al.* (1993), Gaines and Taylor (1986) for salmon net-pens, and Meyer and Barclay (1990) or AFS (1992) for wild fish kills. An overview of the approach and sampling protocols for HAB-caused fish kills in aquaculture is presented below.

Hydrographic and Phytoplankton Samples

Hydrographic and phytoplankton sampling procedures were previously discussed. Immediate phytoplankton sampling is perhaps the most important diagnostic tool if a HAB is suspected of causing a fish kill. It is typically more informative than normal techniques of fish tissue analysis practiced by pathologists. This is because many fish lesions and abnormalities including those of the gill are stereotypical of more than one cause (Mallatt 1985). For example, lifting or separation of gill epithelia is caused by a wide variety of toxicants and chemicals as well as exposure to several HAB species. Phytoplankton samples should be collected from several depths and over a broad enough geographic range to match the movement of tidal waters from the time a fish kill was first observed. If sampling is delayed too long and major weather changes occur or tidal transport is great, the causative HAB species may not be detected. With regard to hydrographic sampling, dissolved oxygen sampling throughout the day and especially just before sunrise should be conducted if microalgal bloom respiration or upwelling of low oxygen water is suspected as a cause of fish mortality (see chapters 2 and 21).

Gross Morphology of Affected Fish

External appearance of the fish should be carefully noted including the presence of excessive mucus on the gills or trailing from the opercular cavity, the color and shape of gills, condition of scales and fins, presence of lesions and other abnormalities. Because of the large volumes of water filtered through fish gills, scraping of the gill surface to prepare a microscope slide as a wet mount can be a simple but effective method to see if certain HAB species are prevalent. This is very diagnostic for larger-sized HAB species that cause gill mucus production and damage such as harmful *Chaetoceros.* It is much preferred to histological techniques that are time consuming, expensive and relatively inaccurate due to the extensive tissue preparation procedures that wash phytoplankton cells from the surface of samples.

Tissue for Histopathology, Bacteriology and Viral Analysis

Although the usefulness of fish tissue or blood analysis may be limited for diagnosing the causative HAB species, as discussed above, an experienced pathologist can provide important insight into fish condition and the possibility of other factors contributing to a fish kill such as chronic diseases. Sampling of moribund fish and apparently unaffected fish, rather than dead fish is highly recommended. Fish that have been dead for more than a few minutes may be useless in efforts to determine the cause of death using histopathology because tissues are rapidly affected by post-mortem changes (Speare and Ferguson, 1989). It may be useful to sample the gills, liver, kidney, gut, brain, heart and buccal cavity surfaces to look for aberrations. A variety of stains and counterstains may be used in histology, but alcian-blue stains are particularly useful for highlighting the prevalence of gill mucus. As a further precaution, samples for virus and bacterial analyses should be collected to detect the possibility that they contribute to a generalized stress response or to death of the fish (Wedemeyer, 1970).

Live Cage Bioassays

Fish farmers should consider placing small, portable fish cages to conduct *in situ* bioassays both at their farm sites and at remote locations where blooms may originate. Small cages have several advantages over large cages normally used in commercial aquaculture. Fish in small cages are more visible for behavior observation and easier to sample. They are also exposed to the shallowest waters during surface-oriented dinoflagellate or microflagellate blooms, allowing for a worst-case monitoring of effects. If a suspected HAB is being advected toward a fish farm site, it may be useful to test the virulence of the harmful species by transporting previously unexposed fish to the bloom and the area of highest HAB concentration. This method has been practiced by both researchers and fish farmers for blooms of *H. carterae* in the Pacific Northwest of U.S. and Canada.

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24. Epidemiology & Public Health

L.E. Fleming, J.A. Bean, D.G. Baden

Epidemiology is the study of the distribution and determinants of disease frequencies in human populations. The epidemiology of the human diseases caused by harmful marine phytoplankton is still in its infancy. This is true even for diseases such as ciguatera, Paralytic Shellfish Poisoning (PSP) and Pufferfish Poisoning (Fugu) which have been reported in the medical and lay literature for hundreds of years (Halstead, 1988; ILO, 1984). In general, the epidemiology of these diseases has consisted of case reports of acute illness, sometimes as epidemic outbreaks, associated with the ingestion of suspicious seafood (Anderson *et al.*, 1983; Bagnis *et al.*, 1979; Halstead, 1988; ILO, 1984; Falconer, 1993; Lange, 1987; Miller, 1991; Rodrigue *et al.*, 1990; Saldate Castaneda *et al.*, 1991; Sims, 1987; Shelley, 1991; Viviani, 1992; Yasumoto and Murata, 1993; Carmichael *et al.*, 1986; Fuhrman, 1967; Baden *et al.*, 1995).

The lack of progress in phycotoxin disease epidemiology is due to the lack of disease and exposure biomarkers in humans. The only way to study these diseases epidemiologically has been identification through their clinical presentation, and more recently, by applying the appropriate laboratory testing to the ingested seafood. Because diagnosis could not be made accurately for either the clinical diseases or the asymptomatic cases associated with these phycotoxin exposures, it has not been possible to investigate their true incidence in human populations. Nor has it been possible, without human biomarkers, to accurately evaluate the true clinical course, treatment and prognosis of the marine toxin diseases.

Therefore, this chapter will discuss general principles of epidemiology with respect to biomarkers and the phycotoxins. It will also discuss the issue of disease and exposure surveillance as essential components in the epidemiological study and public health control of phycotoxin disease in human populations. A brief summary of the known epidemiology of the phycotoxin diseases will be presented. Recommendations will be made for the future epidemiological study and public health control of the phycotoxin diseases in human populations. Finally, a brief set of guidelines for the epidemiological investigation of an acute outbreak of possible phycotoxin disease is presented in an Appendix.

EPIDEMIOLOGICAL ISSUES

Epidemiological research relies on observational rather than experimentally derived data. This is because, unlike the laboratory sciences and except for clinical trials, controlled experiments are difficult to perform in human populations. Using this type of data, epidemiologists study the distribution of disease across populations, geographic locations and across time. This information can be used to understand the clinical course and risk factors of diseases.

Epidemiology is an unique discipline which utilizes the tools of other disciplines in determining causation. As such, the following elements should be present to prove epidemiological causation: an appropriate time sequence (i.e. the disease follows the exposure), evidence of a dose response relationship (i.e. with increasing exposure dose, there is increased severity of disease), the strength of the association (the frequency of the disease increases as the exposure increases), consistency with other evidence such as biochemical and pharmacological, and additional epidemiological and other types of studies (such as toxicological) which support the causal relationship (MacMahon and Pugh, 1970; Last, 1980; Last, 1988; Hulka *et al.*, 1990; Schulte, 1987; Kelsey *et al.*, 1986; Monson, 1992; WHO, 1983; Beaglehole *et al.*, 1993; CDC, 1990; Fleming *et al.*, 1992).

Surveillance, a tool of both epidemiology and public health, is the ongoing and systematic collection, analysis and interpretation of data related to health. This information is used to plan, implement and evaluate public health interventions (Last, 1980; Last, 1988; MacMahon and Pugh, 1970; Baker, 1989; WHO, 1983; Beaglehole *et al.*, 1993; CDC, 1990). Thus, epidemiological research and public health are integrally intertwined.

In epidemiological research and for surveillance, it is necessary to identify the target population, formulate a case definition and measure the exposures.

Target populations are those which are believed to be at greatest risk for exposure and disease, and thus have the highest incidence and prevalence of disease. In the case of the phycotoxins, a target population would consume seafood with possible marine toxin contamination.

The other essential component in epidemiology is the measurement of exposure and their surrogates, physiological effects. These measurements are known as biomarkers (Hulka *et al.*, 1990; Schulte, 1987; Lioy, 1990; NRC, 1987).

Biomarkers have been divided into markers of exposure and markers of effect. Traditionally, biomarkers of exposure have been the toxicological analyses of the actual levels of the toxins or their metabolites in bodily fluids; an example would be a lead level in the blood of a lead smelter worker. Markers of effect tend to be more clinically based and are markers of subclinical physiological change, such as nerve conduction changes noted in the peripheral nerves of persons suffering with ciguatera (Ayyar and Mullaly, 1977; Sozzi *et al.*, 1988).

In order for any biomarker to be useful, it must be measured in easily accessible and acceptable human fluids, such as blood or urine. In addition, the biomarker must be both quantitative and qualitative. Biomarkers should be qualitative in order to make a diagnosis of exposure and/or effect. They should be quantitative in order to evaluate the amount of exposure and/or effect for treatment and prognostic prediction purposes. A quantitative biomarker of exposure and/or effect should be able to evaluate the extent and severity of both acute and chronic disease over time. Finally, biomarkers may identify persons who have been exposed but do not show signs and symptoms of disease, so called subclinical cases.

Other biomarker considerations are the rapidity of the test, ease of application, and the price. Needless to say, issues with regards to the sensitivity and specificity of the actual biomarker tests compared to some "gold standard" must also be worked out.

In order to measure the amount of disease caused by a specific exposure in the target population, it is necessary to make a case definition of this disease. The case definition is usually derived by historical consensus or developed for the purposes of a particular investigation. For example, a classic clinical definition of ciguatera would be the acute onset of gastrointestinal symptoms followed by neurological symptoms within hours of consuming contaminated reef fish. However, the case definition should incorporate, to the extent possible, some objective measurements to eliminate misdiagnosis and bias. An objective measurement of disease could be a biomarker of physiological effect, as well as a biomarker of exposure.

Usually, the case definition is fairly narrow so as to include only those cases which truly have the disease (ie. increased sensitivity). The case definition must be standardized such that it can be used in multiple surveillance programs and epidemiological studies to allow for pooling of data. The case definition must also have clinical relevance so that it can be used in the diagnosis and reporting of disease.

Once biomarkers of exposure and disease have been established and validated, as well as a case definition of disease, the epidemiological research can be performed in the target population. The target population, or some subgroup sampled in a systematic predetermined fashion, is evaluated for the incidence of disease, applying the case definition and collecting appropriate fluid samples for objective diagnosis. If qualitative and quantitative biomarkers of exposure and/or effect are available, then the incidence of both acute and chronic disease, as well as subclinical disease (ie. exposed but never clinically ill), can be determined.

Surveillance

When measures of exposure and/or disease can be performed, the surveillance mechanisms can be established. Surveillance involves the reporting and tracking of disease and exposure in the target population. Surveillance is essential for both epidemiological study and for public health intervention.

There are existing surveillance programs for marine toxin exposure, such as monitoring programs of shellfish beds for PSP and the other shellfish toxins (ILO, 1984; Falconer, 1993; Baden, 1983). There is also surveillance of disease, such as reports of cases of phycotoxin related diseases to health departments (Lawrence *et al.*, 1980). With these case reports, the incidence (i.e. the number of new cases of disease in a given population during a set time period) of clinical disease can be monitored to evaluate trends and changes. In particular, disease clusters, which would manifest as increases in the incidence of disease compared to historical baseline data, can be studied to evaluate new manifestations of disease and/or breakdown in the exposure monitoring system. A brief set of guidelines for the epidemiological investigation of an acute outbreak of possible phycotoxin disease is presented in an Appendix.

Public Health Intervention

The ideal public health intervention is primary prevention such that human exposure and subsequent disease never occur; primary prevention seeks to decrease the incidence of disease (Last, 1988; Last, 1980; Baker, 1989; CDC, 1990; WHO, 1983; Beaglehole *et al.*, 1993). In the case of the phycotoxins, this situation is realized in the exposure monitoring of shellfish beds. Secondary prevention attempts to decrease the prevalence of disease by reducing the duration of clinical illness and by instituting early detection. Secondary prevention of marine toxin diseases would involve the surveillance of biomarkers of subclinical effect and exposure in target populations for the presence of phycotoxin exposure, and then the development of educational and monitoring systems for future primary prevention. The aim of tertiary prevention is to reduce the complications resulting from actual phycotoxin disease; this would involve the early treatment of clinical disease, such as the use of mannitol in the treatment of acute ciguatera, to prevent chronic sequelae (Palafax *et al.*, 1988; Pearn *et al.*, 1989). Obviously, primary prevention is the most important form of prevention from the point of view of public health, although it is not always logistically possible.

EPIDEMIOLOGICAL ISSUES & HARMFUL MARINE PHYTOPLANKTON

As stated above, the epidemiology of the phycotoxins is in its infancy when compared with the epidemiology of the infectious diseases (e.g. tuberculosis), the chronic diseases (*eg.* cancer and cardiovascular disease) and even occupational diseases (e.g. asbestosis) (Last, 1980). This is primarily due to the lack of appropriate biomarkers in humans for the marine toxin illnesses.

Phycotoxin Epidemiology: Known

(Anderson *et al.*, 1983; Bagnis *et al.*, 1979; Halstead, 1988; ILO, 1984; Falconer, 1993; Lange, 1987; Miller, 1991; Rodrigue *et al.*, 1990; Saldate Castaneda *et al.*, 1991; Sims, 1987; Viviani, 1992; Yasumoto, 1993; Carmichael *et al.*, 1986; Ayres and Cullum, 1978; Baden, 1983; Fuhrman, 1967; Perl *et al.*, 1990; Teitelbaum *et al.*, 1990; Baden *et al.*, 1995).

What has been determined epidemiologically for the phycotoxin diseases is summarized in Table 24.1 (Sims, 1987; Sakamoto *et al.*, 1987; Baden *et al.*, 1995). This is based on case

Table 24.1. Intoxication Syndromes Caused by Phycotoxins Consumed in Seafood.

Disease	PSP	NSP	ASP	DSP	Ciguatera	Puffer Fish
Causative Organism	Pelagic[1] Dinoflagellate	Pelagic Dinoflagellate	Pelagic Diatom	Pelagic or Benthic Dinoflagellate	Epibenthic[2] Dinoflagellate	Bacteria?
Major Transvector	Shellfish	Shellfish	Shellfish	Shellfish	Fish	Fish
Geographic Distribution	Temperate to Tropical World-wide	Gulf of Mexico Japan, New Zealand	Canada, NW U.S.A.	Temperate World-wide	Sub-Tropical to Tropical World-wide	Japan, World-wide
Major Toxin (Number)	Saxitoxin (18+)	Brevetoxin (10+)	Domoic Acid (3)	Okadaic Acid (4)	Ciguatoxin (8+) Scaritoxin, Maitotoxin	Tetrodotoxin (3+)
Neuro-Mechanism	Na+ Channel Blocker	Na+ Channel Activator	Glutamate Receptor Agonist	Phosphorylase Phosphatase Inhibitor	Na+, Ca2+, Channel Activators	Na+ Channel Blocker
Incubation Time	5-30 min	30 min to 3 hr	hours	hours	hours	5-30 min
Duration	days	2 days	years	days	years	days
Acute Symptoms	n,v,d **p,r**	n,v,d, **b, t**, p	n,v,d,**a**, p,r	**d**, n,v	n,v,d, **t**, p	n,v,d,**p,r,↓b p**
Chronic Symptoms	none	none	amnesia	none	paraesthesias	none
Fatality Rate	1-14%	0%	3%	0%	<1% (0.1-12%)	60%
Diagnosis	clinical, mouse bioassay of food, HPLC	clinical, mouse bioassay of food, ELISA	clinical, mouse bioassay of food, HPLC	clinical, mouse bioassay, HPLC, ELISA	clinical, mouse bioassay, immunoassay	clinical, mouse bioassay, Fluorescence
Therapy	Supportative (respiratory)	Supportive	Supportive (respiratory)	Supportive	mannitol? TCA? Supportive	Supportive (respiratory)
Prevention	red tide and seafood surveillance, report cases	red tide, then seafood surveillance, report cases	seafood surveillance, report cases	seafood surveillance, some red tide, report cases	seafood surveillance, report cases (clusters)	regulated food preparation, report cases

[1]pelagic water column blooms of motile single celled microalgae . [2]epibenthic forms live on solid surfaces or macroalgae and are inadvertently consumed during fish grazing activities. Taken together, it is readily evident that the toxins of bloom organisms accumulate in filter-feeders, while toxins of epibenthic forms accumulate in fish. n=nausea, v=vomiting, d=diarrhea, p= paraesthesias, r= respiratory despression, b= bronchoconstriction, t= reversal of temperature sensation,a= amnesia,↓bp= decreased blood pressure. Letters in **bold** indicate pathognomonic symptoms (adapted from Sakamoto, 1987; Sims, 1987; and Baden *et al*, 1994.

reports, case series and reports of disease clusters, without any real knowledge of the true incidence of these diseases due to the lack of definitive diagnostic tests.

In general, the phycotoxin diseases can be divided into those associated with the ingestion of contaminated shellfish (i.e. Paralytic Shellfish Poisoning, Neurotoxic Shellfish Poisoning (NSP), Amnesic Shellfish Poisoning (ASP) and Diarrhetic Shellfish Poisoning (DSP)) and those associated with contaminated fish ingestion (i.e. ciguatera and Fugu). An exception to this has been recent reports of human poisonings from the ingestion of fish and wildlife contaminated with shellfish poisons (MacLean and White, 1989; Viviani, 1992).

In the past, phycotoxin disease in humans have been highly localized to coastal, often island, communities of indigenous peoples. There have been no biomarkers of exposure and effect in humans for these phycotoxin diseases, so that the true incidence (including the asymptomatic cases) are unknown in these highly exposed communities, although there are estimates of 70% lifetime occurrence in some Polynesian islands of ciguatera by clinical case report, for example (Bagnis *et al.*, 1979; Lewis, 1986). To the extent that cases or disease clusters have been reported in the past, the distributions of the phycotoxin diseases have been localized to the geographic distribution of the particular dinoflagellate; for example, PSP was reported in temperate coastal areas and ciguatera in tropical island communities. However, with fairly recent increases in seafood trade, increased worldwide seafood consumption, and international tourism, the target populations have become international (Schatz, 1989; Lange *et al.*, 1992; Pocchiari, 1977; NRC, 1989).

Apparently, anyone who ingests enough of the contaminated seafood can become ill, although the true attack rates (i.e. percentage of people who become ill from the truly exposed population) are unknown. Persons at the extremes of age, and possibly those with existing chronic diseases, may be more susceptible (e.g. ASP and ciguatera). The illnesses associated with the marine toxins appear to be acute in onset (i.e. within hours of ingestion), although the incidence of asymptomatic, subclinical and/or chronic disease are unknown.

The phycotoxins are predominantly neurotoxins, both central and peripheral; this results in a myriad of symptoms. Acutely, persons present with gastro-intestinal symptoms, and often, with respiratory compromise; the latter can be fatal in all except DSP and NSP. Further neurological manifestations, ranging from memory loss (i.e. ASP) to paresthesias (i.e. ciguatera), can present acutely and continue apparently for weeks to even years. Again, the true incidence of chronic disease is unknown. The treatment of these diseases is primarily supportive and symptomatic; for example, fluid and electrolyte replacement have been the classic therapy for the vomiting and diarrhea of acute ciguatera (Lange, 1987). Above all, for all the phycotoxin diseases primary prevention is the key.

Phycotoxin Epidemiology: Unknown

The phycotoxins have been studied intensively over the last 30 years, with remarkable progress in the areas of defining the etiological organisms, toxin biochemical characterization, neurophysiological toxin research and toxin biomarkers in the contaminated seafood and shellfish (Falconer, 1993; Viviani, 1992; Narahashi, 1974; Baden, 1983; Scheuer *et al.*, 1967; Hokama, 1985; ILO, 1984; Baden *et al.*, 1995). However, there has been very limited research and progress in the area of human diagnostics and biomarkers.

The only diagnostic methodologies available for epidemiological and clinical study of the marine toxin diseases in humans have been the clinical history and, more recently, testing on the allegedly contaminated seafood. There have been no exposure biomarker tests available to study these toxin diseases in human populations. Even physiological effect biomarkers, such as nerve conduction studies in ciguatera, have been explored minimally to aid in the diagnosis, prognosis and follow-up of disease due to this diagnostic uncertainty (Cameron *et al.*, 1991a; Cameron *et al.*, 1991b; Ayyar and Mullaly, 1977; Sozzi *et al.*, 1988; Teitelbaum *et al.*, 1990).

Furthermore, there has been very little research performed in animals or other *in vivo* systems to study these diseases with human applicability as the goal. Thus, relatively little is known about the chronic course of these disease; for example, ciguatera is reported to continue

as a neurological syndrome for months to years (Halstead, 1988; Lawrence *et al.*, 1980; Bagnis *et al.*, 1979), however this cannot be confirmed without accurate diagnosis. Nor can appropriate comparisons be made between geographical areas for the same marine toxin disease without accurate biomarker diagnosis (for example, there is an apparent clinical difference between ciguatera in the Caribbean and in the Pacific) (Lange, 1987; Halstead, 1988; Bagnis *et al.*, 1979; Lawrence *et al.*, 1980).

Treatment studies of acute and chronic disease are difficult, if not impossible to perform. As mentioned, the existing treatments for the majority of the phycotoxin diseases are only supportive and symptomatic, even though use of specific anti-toxins and other modalities are at least theoretically possible (Kaufman *et al.*, 1991; Viviani, 1992).

The new international distribution of phycotoxin contaminated seafood has epidemiological and public health implications. Epidemiologically, it is much more difficult to study human disease when the target populations become diffuse, and from the public health point of view, control of disease is also more difficult in a diffuse target population. Therefore, it is necessary for epidemiological research purposes to identify those persons at greatest risk for a high incidence of disease due to greatest exposure as the target population. It would be easiest to study both the fish and shellfish-borne diseases in coastal communities with nutritional dependence on contaminated seafood.

In the case of shellfish, as noted above, the development of shellfish organism and toxin measurement methodologies has lead to excellent primary prevention programs by shellfish bed monitoring (ILO, 1984; Baden, 1983; Viviani, 1992). However, in the case of ciguatera which is fish-borne, there are no primary prevention programs, except fish avoidance; for example, there is an official ban of commercial barracuda in South Florida, and in endemic areas of the Caribbean and South Pacific, there is reportedly voluntary avoidance of all local fish due to the high incidence of ciguatera (Lewis, 1986; Morris *et al.*, 1982; Lawrence *et al.*, 1980). In fact, as opposed to sedentary shellfish, it may not be feasible to practice primary preventive measures with respect to the fishborn phycotoxin diseases unless mandatory testing of all high risk large reef species is performed.

PREVENTION OF DISEASE IN HUMAN POPULATIONS FROM HARMFUL MARINE PHYTOPLANKTON

In order to prevent the occurrence of these diseases in human populations, there must be a concerted effort to develop human biomarkers of exposure and effect to study these illnesses both epidemiologically and clinically. If the biomarkers are both qualitative and quantitative, then not only can the appropriate diagnosis be made, but also prognosis for both acute and chronic diseases can be determined. Furthermore, appropriate treatments for acute and chronic disease, tested in human populations, can be developed. With appropriate epidemiological study of these diseases, risk factors for exposure and disease, including clinical severity, can be determined. Finally, disease surveillance in at risk target populations will be able to monitor these diseases for any changes, including breakthroughs in the public health primary prevention programs. Research has begun in this area with the development of an exposure biomarker for ciguatera and brevitoxin which is applicable for both seafood and human testing (Trainer and Baden, 1990; Trainer and Baden, 1991).

At the same time, on-going efforts in primary prevention must continue. These efforts must be different for the different seafood vectors. As noted above, for the shellfish borne diseases, seafood bed monitoring is appropriate; this includes monitoring for the organisms and toxins associated with PSP, NSP, ASP and DSP. This intervention works because the shellfish are sedentary and evaluation of the shellfish bed reflects the state of the individual shellfish. However, for the fish borne diseases, especially ciguatera, but also puffer fish related diseases, the mobility of the seafood vector makes the exposure individualized to the particular fish. As mentioned, complete banning, both voluntarily and mandatory, of at risk fish species can be

instituted. However, this will result in possible economic and nutritional hardship, as well as being very difficult to enforce (Lewis, 1986). Therefore, probably mandatory catch testing will be important for the primary prevention of phycotoxin exposure and disease, although it is relatively expensive and labor intensive.

Catch testing requires the use of an exposure biomarker which is rapid, inexpensive and can be used in the field. Then, using both risk exclusion and sampling techniques, the catch to be tested can be isolated from the majority of the catch. Thus, in the case of ciguatera catch screening, non-reef fish can be excluded while large reef fish with a high incidence of ciguatera such as barracuda would be selected for individual or subsample lot testing. In order to perform subsample testing of high risk catch groups and avoid individualized testing of the entire high risk lot, prior research would be necessary to determine the probabilities of positive proportions of sampled fish. Even in the case of red tides, catch testing techniques could also be applied to fish caught near known red tide areas, since poisonings from ingestion of seafood contaminated by shellfish toxins have been reported in humans (MacLean and White, 1989; Viviani, 1992; ILO, 1984).

Surveillance of both phycotoxin exposure and diseases in target human populations will still be necessary. This could be used in conjunction with seafood exposure monitoring to evaluate for changes in disease patterns. However, disease surveillance would be especially important for countries unable to afford expensive catch monitoring, or even continuous shellfish bed monitoring, as an alternative method of secondary and primary prevention. In this type of surveillance, the at risk population is monitored for sentinel cases (i.e. the first cases which present to medical and public health attention); this sentinel surveillance can prevent further disease if followed up appropriately. If cases are reported associated with the ingestion of seafood from a particular location, then commercial catch or shellfish from these areas can be banned and exposure monitoring instituted at least temporarily. A brief set of guidelines for the epidemiologic investigation of an acute outbreak of possible phycotoxin disease is presented in an Appendix.

Surveillance requires the existence of knowledgeable public health personnel who are able to work in the field collecting information and exposure samples. Therefore, a public health infra-structure is necessary, not only for surveillance, but for disease prevention (Baker, 1989; Last, 1980; WHO, 1983; Beaglehole *et al.*, 1993; CDC, 1990).

Laboratories with appropriately trained personnel and with the capability for biomarker testing in seafood and humans are essential for both surveillance and the primary prevention interventions described above. Inter-lab standardization, as well as issues of sensitivity and specificity for the individual biomarker methods, is also essential (Hulka *et al.*, 1990; Schulte, 1987; Lioy, 1990; NRC, 1987; Baker, 1989; WHO, 1983; Beaglehole *et al.*, 1993).

Obviously education of healthcare and public health personnel concerning the diagnosis, treatment and reporting of these sentinel cases would be extremely important to a successful surveillance program. Education of at risk populations about possible preventive measures (such as not consuming shellfish during times of red tides and/or fish kills) and diagnosis recognition for early treatment interventions would be essential in this type of surveillance. Finally, education and cooperation of the seafood industry concerning the risks of the marine toxin diseases as well as primary and secondary prevention programs are necessary for these programs to function effectively (Baker, 1989; Last, 1980; WHO, 1983; Beaglehole *et al.*, 1993; CDC, 1990).

APPENDIX

Epidemiological Investigation of an Acute Outbreak of Possible Phycotoxin Disease

The following is a outline of guidelines for the epidemiological investigation of an acute outbreak of possible phycotoxin disease. In addition, the reader is referred to a number of

epidemiological texts, including those from the World Health Organization, concerning the evaluation of a disease outbreak and related topics. (WHO, 1983; Beaglehole *et al.*, 1993; CDC, 1990; MacMahon and Pugh, 1970; Last, 1980; Last, 1988; Hulka *et al.*, 1990; Schulte, 1987; Kelsey *et al.*, 1986; Monson, 1992; Fleming *et al.*, 1992; Checkoway *et al.*, 1989; Hennekens *et al.*, 1987; Lilienfeld and Stolley, 1994).

These guidelines assume that there are existing mechanisms for human disease case surveillance and reporting, as well as the resources for Environmental Monitoring and Public Health Education. As discussed in the text, the tools for human monitoring with marine toxin diseases (i.e. biomarkers) are not yet in widespread use; in the future, biomarkers should play an integral part of any epidemic investigation. In addition, because definitive epidemiological information for many of the phycotoxin diseases with respect to the true attack rates, response to treatment, the existence of chronic disease and duration of disease are lacking, uncertainty of information is indicated in the guidelines outline below.

Guidelines Outline

***Stage I.* Initial Contact and Response**

1. Report of **Index Cases**

***Stage II.* Assessment**

1. Create a **Case Definition**

   a. Case Definition of Acute Phycotoxin Disease

      1. Subjective **Clinical Definition**
         - Disease Cluster
         - Appropriate Seafood Ingestion
         - Short Incubation Time (minutes-hours)
         - Gastrointestinal and/or Neurologic Symptoms
         - ?High Attack Rate
         - ?Fatality
         - ?Response to Treatment
         - ?Duration
         - ?Chronic Symptoms

      2. Objective **Laboratory Definition**
         a. Seafood Testing
            - Shellfish: Red Tide
            - Fish: Large Reef or Pufferfish
         b. ?Human Testing
            - ?Serum, ?Other
            - ?Neurophysiologic Testing

   b. Exclude other possible etiologies

2. Index **Case Verification**

   a. Exclude other etiologies

3. Establish **Environmental Exposures**

   a. Shellfish: Red Tide

b. Fish: Large Reef or Pufferfish

Stage III. **Further Study**

1. Search for **Other Cases**

   a. Disease Clusters
   b. Apply Case Definition
   c. Exclude other Etiologies

2. **Seafood Monitoring**

   a. Shellfish: Red Tide
   b. Fish: Large Reef or Pufferfish

3. ?**Human Testing**
   a. ?Serum, ?Other
   b. ?Neurophysiological Testing

Stage IV. **Etiologic Investigation**

1. Confirm Epidemic & **Risk Communication**

2. Institute **Source Control Measures**

   a. Shellfish: Shellfish Bed Ban & Monitoring
   b. Fish: Sampling, Distributor Ban
   c. Continued Disease Surveillance

3. **Public Health Education**
   - Fishing Industry
   - Food Industry
   - Health Professionals
   - General Public

DISCUSSION

These guidelines assume that there is continual communication concerning the progress of the investigation with those persons directly involved, as well as the health and seafood communities, the media and the general public. This communication is essential for the successful investigation and control of an outbreak of phycotoxin disease.

In Stage I, index or initial cases which suggest the possibility of phycotoxin disease are reported to health authorities. Again, this assumes that the health care providers are able to both recognize and report phycotoxin disease, and that the public health authorities are able to collect and follow-up on such reports.

In Stage II, the health authorities need to develop a Case Definition of phycotoxin disease. This definition should include both subjective and objective criteria, as detailed in the guidelines outline above. In addition, the definition should be able to distinguish and exclude competing illnesses; for the phycotoxin diseases, the differential diagnosis should minimally include acute infectious foodborne illnesses (both bacterial and viral) and organophosphate pesticide poisoning. All the index cases should be verified using the Case Definition. At the

same time, environmental testing should be performed for both the implicated food source and in the case of the shellfish associated diseases, at the geographical source.

Assuming that the index cases are verified, other etiologies excluded and environmental monitoring consistent with a phycotoxin source, the investigation should widen in Stage III. This is especially true for the case of the shellfish associated diseases because they are often associated with red tides and a large geographic area; the fish associated diseases, in the case of ciguatera can be associated with fish from a particular reef but not with the same intensity as with red tides, and in the case of pufferfish, can be associated with a particular species. New cases of the original disease cluster should be investigated by tracking the human associations (ie. sharing of seafood) as well as the food source associations (ie. where seafood obtained). If warranted, further testing of the seafood and environmental monitoring should be initiated to evaluate the geographical extent of the contamination. If human biomarkers are available, then collection of fluids from geographically and seafood-exposed persons should begin, to determine the extent of the epidemic in the human population.

Once the extent of the epidemic, both in human and environmental terms, has been determined, it is important to communicate the results and risk to the public and other interested parties. Appropriate source control, as described in the text, should be instituted to prevent further spread of disease. Finally, education of the seafood and food industries and seafood consumer, as well as health professionals and the media, is essential for further prevention and future surveillance activities.

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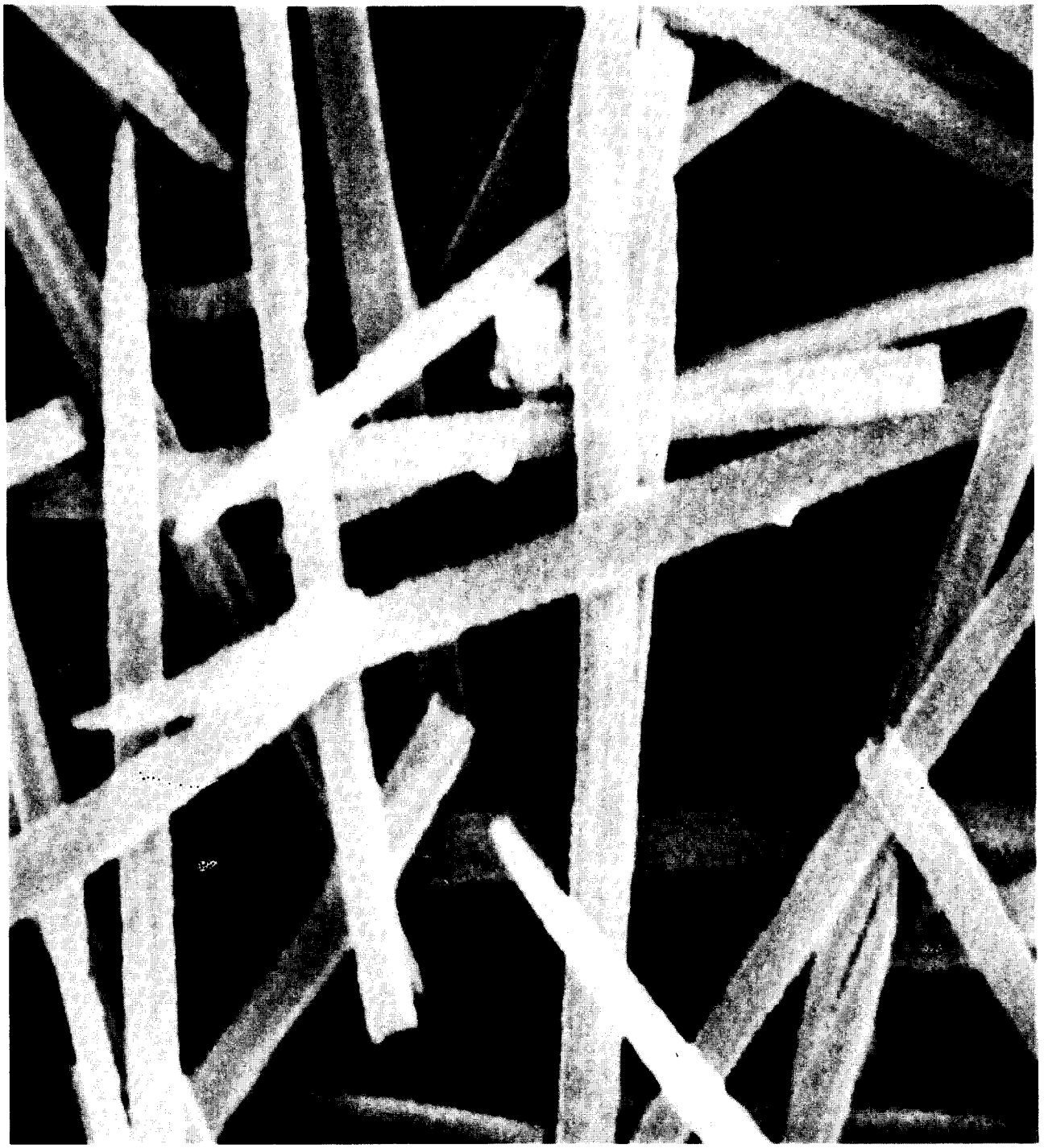
Part IV

Resources



25. Algal Culture Collections and Toxic Algal Strains

R.A. Andersen, S.I. Blackburn, F.J.R. Taylor and C.R. Tomas

The purpose of this chapter is to provide a world list of algal culture collections and a list of toxic, or possibly harmful, marine phytoplankton strains. We have also included a list of toxic or possibly harmful freshwater blue-green algal strains. The world list of culture collections includes all the algal culture collections known to us, both marine and freshwater (Table 25.1). Most public collections provide cultures to scientists, however, private collections may only provide cultures on a selective basis. Each collection, public or private, may have restrictions. We have not listed these restrictions, and users should contact collections directly to see if restrictions apply. Most public collections sell cultures and users should contact the collections for specific prices (cost of cultures, shipping and handling fees). If users are obtaining cultures from a collection located in another country then careful attention should be paid to restrictions for their home country. Customs documentation is often required, and improper customs documentation may lead to delays, possibly resulting in the death of the organisms. In general, "overnight" courier services (e.g., Federal Express, DHL, TNT Express) are recommended. These couriers provide rapid service and the courier companies can quickly locate delayed or lost shipments via tracking numbers. Good communications between the user and the collection are recommended so that cultures do not arrive on holidays or when users are away from their institution.

Many toxic strains are difficult to transport and successful shipment of these strains may not always be possible. Dinoflagellates in particular are difficult to ship because their respiration rates are high relative to their stored foods (i.e., they "starve" when kept in the dark for more than 1-2 days where they are unable to photosynthesize) and because they may be sensitive to dramatic photoperiod changes.

Users should be prepared to receive the cultures when they arrive. The proper culture medium and appropriate culture conditions (temperature, light, etc.) should be prepared in advance. Most collections provide instructions on how to handle cultures once they arrive. Users should immediately examine the cultures for viability, remembering that cells which are normally swimming in the culture vessel may be settled to the bottom as result of the shipping event. Finally, there are many factors which can affect the growth and viability of marine phytoplankton. Good quality seawater or artificial seawater is important. Also, some laboratory vessels release substances that are toxic to sensitive strains (e.g., some types of glass, some types of test tube caps, etc.). For culturing details, see Chapter 3.

Users should be very careful about disposing cultures of toxic phytoplankton. It is often illegal to dispose of toxic phytoplankton cultures into natural waters. In cases where such releases are not illegal, it is generally considered undesirable to release toxic phytoplankton into the local environment. Besides avoiding the release of toxic phytoplankton into native waters, users should be careful about releasing toxins from phytoplankton cultures into the environment.

The list of toxic, or possibly harmful, strains is limited to only marine phytoplankton strains plus toxic or possibly harmful freshwater blue-green algae (Table 25.2). The first level of organization is by algal class, the second is by generic name, the third by specific epithet, the fourth by culture collection acronym, and the fifth is by strain number. The culture collection acronym in Table 25.2 is defined in Table 25.3, and the details for each collection can be found in Table 25.1. The list of toxic strains (Table 25.2) is almost certainly incomplete, and it is very likely that the contents of Table 25.2 will change over time as additional toxic strains are isolated into culture and existing strains are deleted or lost in collections.

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Table 25.1. A world list of algal culture collections arranged alphabetically by country.

AUSTRALIA

The Australian Collection of Marine Microorganisms
Department of Biomedical & Tropical Veterinary Science
James Cook University
Townsville, Queensland, 4811 Australia
Collection Abbreviation: ACCM
Curator: Dr. Paul Muir
Dr. Warren Shipman
Telephone: (61)(77) 814-278
Fax Number: (61)(77) 791-526
Catalog

Australian Collection of Micro-organisms
Centre for Bacterial Diversity and Indentification
Department of Microbiology, University of Queensland
Brisbane, Queensland, 4072 Australia
Collection Abbreviation: ACM
Director: Dr. L.I. Sly
Telephone: (61)(7) 365-4617
Fax Number: (61)(7) 365-4620
Catalog

Australian Water Quality Centre
Private Mail Bag 3
Salisbury, SA, 5108 Australia
Collection Abbreviation: AWQC
Curator: Mr. Peter Baker
Telephone: (61)(8) 259-0338
Fax Number: (61)(8) 259-0228
email: peter.baker@sawater.sa.gov.au

Commonwealth Scientific and Industrial Research Organization
CSIRO Division of Fisheries Research
GPO Box 1538
Hobart, Tasmania, 7001 Australia
Collection Abbreviation: CSIRO
Director: Dr. Susan Blackburn
Curator: Jeannie-Marie LeRoi
Telephone: (61)(02) 32-5222
Fax Number: (61)(02) 32-5000
email: Sue.Blackburn@ML.CSIRO.au
Catalog

Melbourne University Culture Collection
School of Botany, University of Melbourne
Parkville, Victoria, 3052 Australia
Collection Abbreviation: MUCC
Curator: Dr. David Hill
Telephone: (61)(3) 344-5131
Fax Number: (61)(3) 347-5460
email: u6067930@hermes.unimelb.edu.au

Murdoch University Algal Culture Collection
School of Environmental and Life Sciences
Murdoch, Western Australia, 6150 Australia
Collection Abbreviation: MUR
Director: Dr. Michael A. Borowitzka
Telephone: (61)(9) 360-2333
Fax Number: (61)(9) 310-3505
email: borowitz@possum.murdoch.edu.au

BULGARIA

Plovdiv Algal Culture Collection
Department of Botany
"Paissi Hilendarsky" University of Plovdiv
Todor Samodumov Street Nr. 2
Plovdiv, 4000 Bulgaria
Collection Abbreviation: PACC
Director: Prof. Dimitar Vodenicharov
Curator: D. Belkinova
Fax Number: 32-238607
Catalog

CANADA

North East Pacific Culture Collection
Department of Oceanography
University of British Columbia
6270 University Boulevard
Vancouver, BC, V6T 1Z4 Canada
Collection Abbreviation: NEPCC
Director: Dr. F.J.R. Taylor
Curator: Elaine P. Simons
Telephone: 604-822-4378
Fax Number: 604-822-6091
email: nepcc@unixg.ubc.ca
Catalog

University of Toronto Culture Collection
Department of Botany, University of Toronto
Toronto, Ontario, M5S 3B2 Canada
Collection Abbreviation: UTCC
Director: Dr. C. Nalewajko
Curator: J.C. Acreman
Telephone: 416-978-3641
Fax Number: 416-978-5878
email: jacreman@botany.utoronto.ca
Catalog

CZECH REPUBLIC

Culture Collection of Autotrophic Organisms
Institute of Botany
Czech Academy of Sciences
Dukelska 145, Trebon, CZ-379 82 Czech Republic
Collection Abbreviation: CCALA
Curator: Dr. Lukavsky
Telephone: 42-333-2522
Fax Number: 42-333-2391
email: hauser@sigma.jh.jcu.cz
Catalog

DENMARK

Scandinavian Culture Centre for Algae and Protozoa
Botanical Institute
Øster Farimagsgade 2D, DK-1353 Copenhagen K, Denmark
Collection Abbreviation: SCCAP
Curator: Niels Henry Larsen
Telephone: 35-32-23-03
Fax Number: 35 32 23 21
email: sccap@bot.ku.dk
Restrictions: closed during August
Catalog

FINLAND

University of Helsinki Algal Culture Collection
Department of Applied Chemistry and Microbiology
University of Helsinki
P.O. Box 27, Helsinki, FIN-0014 Finland
Collection Abbreviation: UHACC
Curator: Dr. Kaarina Sivonen
Telephone: 358-0-708-5288
Fax Number: 358-0-708-5212
email: kaarina.sivonen@helsinki.fi

FRANCE

Caen Algal Culture Collection
UniversitÈ de Caen
Laboratoire de Biologie et Biotechnologies Marines
(Equipe Phycologie)
Esplanade de la Paix
Caen, 14032 France
Collection Abbreviation: CAEN
Director: Dr. Chantal Billard
Telephone: (33) 31-45-58-85
Fax Number: (33) 31-45-56-00

Centre d'Oceanologie de Marseille
CNRS URA n41
Station Marine d'Endoume
Rue de la Batterie des lions
Marseille, 13007 France
Collection Abbreviation: COM
Director: Dr. Brigitte Berland
Telephone: (33) 91 04 16 38
Fax Number: (33) 91 04 16 35
email: berland@com.univ-mrs.fr

Centre de Nants
Laboratoire Phycotoxincs et Nuisances
BP 1105
44311 Nantes, Cedex 03, France
Collection Abbreviation: CN
Director: Dr. Patrick Lassus
Telephone: (33) 40 37 41 30
Fax Number: (33) 40 37 40 73
email: plassus@ifremer.fr

Pasteur Culture Collection of Cyanobacterial Stains in Axenic Culture
Unite' de Physiologie Microbienne
Institut Pasteur
28 rue du Docteur Roux
Paris 75724, Cedex 15, 75724 France
Collection Abbreviation: PCC
Director: Dr. Rosmarie Rippka
Curator: Dr. Michael Herdman
Telephone: (33-1)4568-8416
Fax Number: (33-1)4061-3042
email: cyano@pasteur.fr
Catalog

GERMANY

Sammlung von Algenkulturen
Pflanzenphsiologisches Institut der Universit‰t Gottingen
Nikolausberger Weg 18
Gottingen, D-37073 Germany
Collection Abbreviation: SAG
Director: Prof. Dr. U.G. Schl^sser
Fax Number: 0551-397871
Catalog

Sammlung von Conjugaten-Kulturen
Institut f¸r Allgemeine Botanik der Universit‰t Hamburg
Ohnorststrasse 18
D-22609, Hamburg, Germany
Collection Abbreviation: SVCK
Director: Dr. Monika Engels
Fax Number: 822-82-254
Catalog

GREECE

National Centre for Scientific Research
153 10 AG. Paraskevi Attikis
POB 60228, Greece
Curator: Dr. Lydia Ignatiades
Collection Abbreviation: NCSR
Telephone: 651-311-19
email: igna@cyclades.nrcps.ariadne-t.gr

University of the Aegean Culture Collection
Department of Environmental Studies
Kavetsou 12-14, Mytilini, 81100 Greece
Collection Abbreviation: UACC
Director: Prof. M. Karydis

INDIA

National Collection of Industrial Microorganisms
Biochemistry Division
National Chemical Laboratory, CSIR
Poona, Mahasahtra, 411 008 India
Collection Abbreviation: NCIM
Director: Mr. S.R. Modak

ISRAEL

Israel Oceanographic and Limnological Research LTD
P.O. Box 8030, Haifa, 31080 Israel
Collection Abbreviation: IOLR
Curator: Prof. Ami Ben-Amotz
Telephone: 04-515202
Fax Number: 04-511911

JAPAN

Faculty of Agriculture
University of Tokyo
Yayoi, Bunkyo-ku
Tokyo 113, Japan
Collection Abbreviation: FAUT
Curator: Prof. Yasuwo Fukuyo

Institute of Molecular and Cellular Biosciences
The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku
Tokyo, 113 Japan
Collection Abbreviation: IAM
Director: Prof. Junta Sugiyama
Telephone: 81-3-3812-2111
Fax Number: 81-3-3818-0444
Catalog

Akashiwo Research Institute of Kagawa Prefacture
Yashima-Higashi-machi, Takamatsu
761-01, Japan
Collection Abbreviation: KAGAWA
Director: Dr. Chitari Ono
Curator: Dr. Sadaaki Yoshimatsu
Telephone: (0878)-43-6511
Fax Number: (0878)-41-8133

Kyoto University Culture Collection
Laboratory of Microbiology
Department of Fisheries
Faculty of Agriculture
Kyoto, 606 Japan
Collection Abbreviation: KUCC
Director: Prof. Yuzaburo Ishida
Telephone: (075) 763-6217
email: h51405@sakura.kudpc.kyoto-u.ac.jp

Marine Biotechnology Institute
Kamaishi Laboratories
3-75-1 Heita
Kamaishi-shi, Iwate, 026 Japan
Collection Abbreviation: MBI
Director: Dr. Shigetoh Miyachi
Curator: Dr. Hisato Ikemoto
Telephone: 81-193-266538
Fax Number: 81-193-266584

Department of Marine Science
School of Marine Science and Technology
Tokai University
3-20-1 Orido, Shimizu, Shizuoka 424, Japan
Collection Abbreviation: MSTU
Director: Prof. Kaori Ohki
Fax Number: 543-34-9834

National Institute for Environmental Studies
Microbial Culture Collection
16-2 Onagawa, Tsukuba
Ibaraki, 305 Japan
Collection Abbreviation: NIES
Director: Dr. Makoto M. Watanabe
Curator: Dr. Nozaki
Telephone: 81-298-51-6111
Fax Number: 81-298-51-4732
Catalog

MEXICO

La Coleccion de Microalgas del CICESE
Centro de Investigacion Cientifica Y de Educacion
Superior de Ensenada
Km. 107 Carretera
Tijuana, Ensenada, Mexico
Collection Abbreviation: CICESE
Curator: M.L. Trujillo Valle
Telephone: 91-617-448-80
Fax Number: 91-617-448-80
Catalog

NEW ZEALAND

The Cawthron Microalgae Culture Collection
Cawthron Institute, Private Bag 2
Nelson, New Zealand
Collection Abbreviation: CAW
Curator: Ms Maggie Atkinson
Telephone: (64)(3) 548-2319
Fax Number: (64)(3) 546-9464
email: maggie@environment.cawthorn.org.nz
Catalog

New Zealand Oceanographic Institute Phytoplankton Culture Collection
National Inst. Water and Atmospheric Research
P.O. Box 14-901
Kilbirnie, Wellington, New Zealand
Collection Abbreviation: NZOI
Curator: Dr. F. Hoe Chang
Telephone: 64-4-38 60300
Fax Number: 64-4-38 62153
email: Chang@greta.niwa.cri.nz

NORWAY

Culture Collection of Algae
Norwegian Institute for Water Research
P.O. Box 173 KjelsÂs
N-0411, Oslo, Norway
Collection Abbreviation: NIVA
Curator: Dr. Olav M. Skulberg
Telephone: 47-22-185266
Fax Number: 47-2235280
Catalog

University of Oslo
Department of Biology, Marine Botany
P.O. Box 1069
Blindern N-0316, Oslo, Norway
Collection Abbreviation: UIO
Curator: Dr. Jahn Throndsen
Telephone: 47-22854526
Fax Number: 47-22854438
email: jahn.throndsen@bio.uio.no

PEOPLE'S REPUBLIC OF CHINA

Collection of Asian Phytoplankton
Institute of Oceanology Academia Sinica
7 Nanhai Road
Qingdao, People's Republic of China
Collection Abbreviation: CAP
Curator: Dr. C.Y. Wu

Freshwater Algae Collection
Institute of Hydrobiology
The Chinese Academy of Sciences
Wuhan, People's Republic of China
Collection Abbreviation: FACHB
Curator: Dr. Li-Rong Song
Fax Number: 27-7875132

Institute of Hydrobiology Algal Collection
Jinan University
Guangzhou, People's Republic of China
Collection Abbreviation: JINAN
Curator: Prof. Yu-zao Qi

Marine Algal Culture Collection
Ocean University
Aquaculture Food Organism Research Laboratory
Qingdao, People's Republic of China
Collection Abbreviation: MACC
Director: Prof. Wang Yi-yi

THE PHILIPPINES

Blue-green Algal Collection at IRRI
International Rice Research Institute
Soil Microbiology Division
P.O. Box 933
Manila, Philippines
Collection Abbreviation: IRRI
Curator: Dr. J.K. Ladha
Telephone: 818-1926 loc 737
Fax Number: (63-2) 817-8470
email: j.k.ladha@cgnet.com

Marine Science Institute
University of the Philippines
U.P.P.O. Box 1, Diliman
Quezon City, 1101 The Philippines
Collection Abbreviation: MSIUP
Director: Dr. Edgardo D. Gomez
Curator: Dr. Rhodora Corrales
Fax Number: 632-921-5967
email: rhod@msi.upd.edu.ph

Algal Culture Collection of University of Philippines - Los Banos
Museum of Natural History, Institute of Biological Sciences
P.O. Box 169
University of Philippines at Los Banos
Laguna, 4031, The Philippines
Collection Abbreviation: UPLB
Curator: Dr. Milagrosa R. Martinez-Goss
Telephone: (63-94) 2570
Fax Number: (63-94) 3249
email: mrmg@mudspring.uplb.edu.ph

PORTUGAL

Instituto Português de Investigação Maritma
Av. Brasilia
1400 Lisboa, Portugal
Collection Abbreviation: IPIMAR
Curator: Manuela Morais
Director: Dr. Maria Antónia de M. Sampayo
Telephone: 351-1-3016361
Fax Number: 351-1-3015948

Estela S. Silva LME Algal Collection
Instituto Nacional da Saúde
Laboratory of Experimental Biology
National Institute of Health
Av. Padre Cruz
1699 Lisboa Codex, Portugal
Collection Abbreviation: LME
Curator: Dr. Susana Franca
Telephone: 351-1-7577070
Fax Number: 351-1-7590441

RUSSIA

Biological Institute of St. Petersburg State University
Oranienbaumskoye sch. 2
Stary Peterhof
St. Petersburg, 198904 Russia
Collection Abbreviation: BISPSU
Curator: Dr. Serguei Karpov
Telephone: (812)427-9669
Fax Number: (812)428-6649 & 218-1346
email: igor@hg.bio.lgu.spb.su

Algological Collection
Institute of Plant Physiology RAS
ul. Botanicheskaya
35 Moscow, 127276 Russia
Collection Abbreviation: IPPAS
Curator: Dr. E.S. Kuptsova
Telephone: 095-482-4491
095-482-5447
Fax Number: 095-482-1685
Catalog

Collection of Green Algal Cultures
Laboratory of Algology
Botanical Institute by V.L. Komarov of RAS
ul. Prof. Popov 2
St. Petersburg, 197376 Russia
Collection Abbreviation: LABIK
Curator: Dr. V.M. Andreyeva & A.F. Luknitskaya
Fax Number: (812)234-45-12
email: binran@glas.apc.org.
Catalog

SAUDI ARABIA

King Fahd University of Petroleum & Minerals
Research Institute
Marine Group, Algal Collection
Dhahran, 31261 Saudi Arabia
Collection Abbreviation: KFUPM
Curator: Dr. Assad A. Al-Thukair
Telephone: (966)-3-8604180
Fax Number: (966)-3-8604180

SOUTH AFRICA

University of Witwatersrand Culture Collection
Department of Botany
University of Witwatersrand
Private Bag 3 WITS
Johannesburg, 2050 South Africa
Collection Abbreviation: WITS
Curator: Prof. Richard N. Pienaar
Telephone: (27)(11)-716-2251
Fax Number: (27)(11)-403-1429
email: richard@gecko.biol.wits.ac.za

SPAIN

Instituto Espanol de Oceanografia
CaboEstay-Canido Ap. de Correos 1552
36280 Vigo, Spain
Collection Abbreviation: VIGO
Director: Dr. I. Bravo
Curator: I. Ramilo
Telephone: 34-86-49-21-11
Fax Number: 34-86-49-23-51
email: insovigo@cesga.es

SWEDEN

AVD for Marine Ekologi
Department of Marine Ecology
Lund University
Lund, Sweden
Collection Abbreviation: AVD
Director: Dr. Lars Gisselson
Telephone: 46-46-108366
Fax Number: 46-46-104003

TAIWAN

Tungkang Marine Laboratory
Taiwan Fisheries Research Institute
Tungkang, Pingtung, 928
Taiwan R.O.C.
Collection Abbreviation: TML
Director: Dr. Mao-Sen Su
Curator: Dr. Huei-Meei Su
Telephone: 886-8-8324121
Fax Number: 886-8-8320234

THAILAND

Thailand Institute of Scientific and Technological Research
196 Phahonyothin Road
Bangkok, 10900 Thailand
Collection Abbreviation: TISTR
Curator: Pongtep Antarikanonda

UKRAINA

Collection of Algal Strains
Division of Spore Plantae
Institute of Botany by Kholodny of UkrAS
ul. Tereschenkovskaya, 2
Kiev, 252601 Ukraine
Collection Abbreviation: IBASU-A
Director: Dr. K.M. Sitnick
Curator: Dr. V.P. Yunger
Telephone: 224-51-57
Fax Number: 224-10-64
Catalog

UNITED KINGDOM

Culture Collection of Algae and Protozoa
Dunstaffnage Marine Research Laboratory
P.O. Box 3
Oban, Argyll, PA34 4AD Scotland
Collection Abbreviation: CCAP at DML
Curator: Michael F. Turner
Telephone: 1631-562244
Fax Number: 1631-565518
email: CCAPN@DML.AC.UK
Catalog

Culture Collection of Algae and Protozoa
Institute of Freshwater Ecology
The Ferry House, Ambleside
Cumbria, LA22 0LP England
Collection Abbreviation: CCAP at IFE
Curator: Dr. J.G. Day
Telephone: 53-94 42468
Fax Number: 153-94 46914
email: CCAP@IFE.AC.UK
home page: http://wiua.nwi.ac.uk/
ccap/ccaphome.html
Catalog

Plymouth Culture Collection
Plymouth Marine Laboratory
Citadel Hill
Plymouth, Devon, LP1 2PB UK
Collection Abbreviation: PLY
Director: Dr. J. Green
Telephone: (44) 752-222772
Fax Number: (44) 752-226865
j.green@pml.ac.uk

Swansea Algal Research Unit
University of Wales Swansea
Singleton Park
Swansea, SA2 8PP Wales, U.K.
Collection Abbreviation: SARU
Director: Dr. Kevin Flynn
Telephone: (44) 792-295726
Fax Number: (44) 792-295447
email: K.J.Flynn@swansea.ac.uk

University of Westminster Algal Collection
115 New Cavendish St.
London WIM SJS UK
Collection Abbreviation: UW
Curator: Dr. J. Lewis
Telephone: 71-911-5000
Fax: 71-911-5087

UNITED STATES OF AMERICA

American Type Culture Collection
Protistology Department
12301 Parklawn Drive
Rockville, Maryland, 20852 USA
Collection Abbreviation: ATCC
Curator: Dr. Thomas Nerad
Telephone: 800-638-6597
Fax Number: 301-816-4361
email: sales@atcc.org
home page: http://www.atcc.org/
Catalog

American Type Culture Collection
Bacteriology Department
12301 Parklawn Drive
Rockville, Maryland, 20852 USA
Collection Abbreviation: ATTC
Curator: Dr. Robert Gherna
Telephone: 800-638-6597
Fax Number: 301-816-4361
email: sales@atcc.org
home page: http://www.atcc.org/
Catalog

Carolina Biological Supply Company
Algae Department
2700 York Road
Burlington, North Carolina, 27215 USA
Collection Abbreviation: CBSC
Curator: Daniel James
Telephone: 910-584-0381
Fax Number: 910-584-3399
Catalog

Provasoli-Guillard National Center for Culture of Marine Phytoplankton
Bigelow Laboratory for Ocean Sciences
McKown Point
West Boothbay Harbor, Maine, 04575 USA
Collection Abbreviation: CCMP
Director: Dr. Robert A. Andersen
Curator: Dr. Steven Brett
Telephone: 207-633-9630
Fax Number: 207-633-9641
email: ccmp@ccmp.bigelow.org
home page: http://ccmp.bigelow.org
Catalog

Chlamydomonas Genetics Center
Department of Botany, Duke University
DCMB Box 91000
Durham, North Carolina, 27708-1000 USA
Collection Abbreviation: CGC
Director: Dr. Elizabeth H. Harris
Telephone: 919-613-8164
Fax Number: 919-613-8177
email: chlamy@acpub.duke.edu
home page: http://probe.nalusda.gov:8000/
plant/aboutchlamydb.html
Catalog

Freshwater Diatom Culture Collection
Department of Biology, Loras College
Dubuque, Iowa, 52001-0178 USA
Collection Abbreviation: FDCC
Curator: Dr. David B. Czarnecki
Telephone: 319-588-7231
Fax Number: 319-588-7964
email: czdiatom@LCAC1.LORAS.edu
Catalog

Marine Microalgae Research Culture Collection
Florida Department of Environmental Protection
Marine Research Institute
100 Eighth Ave. S.E.
St. Petersburg, Florida, 33701 USA
Collection Abbreviation: FLORIDA
Curators: Dr. Karen Steidinger & Dr. Carmelo Tomas
Telephone: 813-896-8626
Fax Number: 813-823-0166
email: steidinger_k@sellers.dep.state.fl.us

Algal Collection at Monterey Bay Aquarium Research Institute
Monterey Bay Aquarium Research Institute
Pacific Grove, CA, 93950 USA
Collection Abbreviation: MBARI
Curator: Dr. Chris Scholin
Fax Number: 408-647-3779
email: scholin@mbari.org

University of Miami Algal Culture Collection
School of Oceanography, University of Miami
Miami, Florida 33149 USA
Collection Abbreviation: MIAMI
Curator: Dr. Larry Brand
Telephone: 305-361-4138

Milford Laboratory Culture Collection
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Milford, Connecticut, 06460-6499 USA
Collection Abbreviation: MIL
Director: Gary H. Wikfors
Curator: Jennifer H. Alix
Telephone: 203-783-4225
Fax Number: 203-783-4217

University of California-Santa Barbara Culture Collection
Department of Biological Sciences
University of California-Santa Barbara
Santa Barbara, California, 93105 USA
Collection Abbreviation: UCSB
Curator: Dr. Robert K. Trench
Fax Number: 805-893-4724

University of Rhode Island
Department of Pharmacognosy & Environmental Health Sciences
Kingston, Rhode Island, 02881 USA
Collection Abbreviation: URI-1
Director: Dr. Yuzuru Shimizu
Telephone: 401-792-2751
Fax Number: 401-792-2181

University of Rhode Island Culture Collection
Graduate School of Oceanography
University of Rhode Island
Narragansett, Rhode Island, 02882-1197 USA
Collection Abbreviation: URI-2
Curator: Dr. Paul Hargraves
Telephone: 401-792-6241
Fax Number: 401-792-6240
email: pharg@gsosun1.gso.uri.edu

Culture Collection of Algae at University of Texas
Department of Botany
University of Texas at Austin
Austin, TX, 78713-7640 USA
Collection Abbreviation: UTEX
Director: Dr. Richard Starr
Curator: Dr. Jeff Zeikus
Telephone: 512-471-4019
Fax Number: 512-471-3878
email: jeff_n_judy@mail.utexas.edu
home page: http://www.botany.utexas.edu/
infores/utex/
Catalog

Woods Hole Oceanographic Institution
Biology Department, Redfield 3-32
Woods Hole, Massachusetts, 02543 USA
Collection Abbreviation: WHOI-1
Director: Dr. Donald Anderson
Curator: David Kulis
Telephone: 508-457-2000 ext. 2351
Fax Number: 508-457-2169
email: danderson@whoi.edu

Woods Hole Oceanographic Institution
Woods Hole, Massachusetts 02543 USA
Collection Abbreviation: WHOI-2
Curator: Dr. John Waterbury
Telephone: 508-457-2000 ext. 2742
Fax Number: 508-457-2169
email: jwaterbury@whoi.edu
Restrictions: cultures sent by overnight courier only

VIETNAM

National Scientific Research Centre of Vietnam
Institute of Biology
Tu Liem, Ha Noi, Vietnam
Collection Abbreviation: Vietnam
Curator: Prof. Nguen Huu Thuoc

Table 25.2. List of toxic, harmful, or possibly harmful strains of marine phytoplankton and freshwater blue-green algae. Organisms are classified by taxonomic class and arranged alphabetically. The culture collection abbreviations are defined in Table 25.3 and details for each culture collection can be found in Table 25.1. When several strains of a specific species were isolated from the same collection site and have succeeding strain numbers then they are shown in only one line and the strain numbers are shown collectively separated by a hyphen (e.g., Pseudo-nitzschia pseudodelicatissima strains CCMP1562, CCMP1563, CCMP1564 and CCMP1565 are shown as CCMP1562-5). When strain numbers or collection sites are unknown then the field is blank. The listing is extensive, being compiled with the help of curators from around the world, however, it is probable that some strains have been inadvertently missed. The strains listed below are not necessarily available to the public; please contact the collection for strain availablity.

ORGANISM	COLLECTION	STRAIN	COLLECTION SITE
BACILLARIOPHYCEAE			
Cerataulina pelagica	NEPCC	NEPCC359	unknown
Chaetoceros concavicornis	CCMP	CCMP170	42°40.5'N 69°36W
C. convolutus	CCMP	CCMP169	Puget Sound, WA, USA
C. convolutus	NEPCC	NEPCC700	Jericho Beach, Vancouver, British Columbia, Canada
C. criophilus	(no cultures?)		
C. danicum	CCMP	CCMP171	Bay of Marseilles, France
C. dubile	CCMP	CCMP172	Friday Harbor, WA, USA
C. dubile	CCMP	CCMP1578	Narragansett Bay, RI, USA
C. dubile	NIES	NIES270	Hachinohe Harbor, Aomori, Japan
C. socialis	CCMP	CCMP201	Black Sea, Russia
C. socialis	CCMP	CCMP202	(unknown)
C. socialis	CCMP	CCMP203	Gulf of Mexico, 29°27'N 85°37.2'W
C. socialis	CCMP	CCMP204	Bay of Marseilles, France
C. socialis	CCMP	CCMP205	North Atlantic Ocean
C. socialis	CCMP	CCMP1579	Renesee, The Netherlands
C. socialis	NEPCC	NEPCC278	unknown
C. socialis	NEPCC	NEPCC653	Jericho Beach, Vancouver, British Columbia, Canada
C. sp.	JINAN	KD(J)900221	(Inst. Oceanology, Academia Sinica)
Coscinodiscus centralis	(no cultures?)		
C. concinnus	(no cultures?)		
C. wailesii	(no cultures?)		
Leptocylindrus minimus	NEPCC	NEPCC431	Jericho Beach, Vancouver, British Columbia, Canada
Pseudo-nitzschia australis	CAW	CAWB02	Marsden Point, New Zealand
P. australis	CCMP	CCMP1709-10	Santa Cruz, CA, USA
P. australis	VIGO	PS2V	Ria de Muros, Galicia, Spain
P. australis	VIGO	PS3V	Ria de Muros, Galicia, Spain

Pseudo-nitzschia australis	VIGO	PS4V	Alboran Sea, Spain
P. australis	VIGO	PS6V	Ria de Pontevedia, Galicia, Spain
P. delicatissima	(no cultures?)		
P. multiseries	MBARI	CV19	Monterey Bay, CA, USA
P. multiseries	MBARI	CV22	Monterey Bay, CA, USA
P. multiseries	MBARI	CV26	Monterey Bay, CA, USA
P. multiseries	CCMP	CCMP1573-4	Narragansett Bay, RI, USA
P. multiseries	CCMP	CCMP1659-60	Prince Edward Island, Canada
P. multiseries	CCMP	CCMP1711-2	Santa Cruz, CA, USA
P. pseudodelicatissima	CCMP	CCMP1562-5	Bass River, Cape Cod, MA, USA
P. seriata	CCMP	CCMP1309	Resolute Passage, Barrow Strait, NWT, Canada
P. turgidula	CAW	CAWB05	Tapeka Point, New Zealand
P. turgidula	CAW	CAWB06	Tauranga, New Zealand
P. sp.	JINAN	KD(J)930501	Dapeng Bay, South China Sea, People's Republic of China
Rhizosolenia cf. chunii	(no cultures?)		
Thalassiosira curviseriata	(no cultures?)		
T. delicatula	(no cultures?)		
T. diporocyclus	(no cultures?)		
T. fragilis	(no cultures?)		
T. gravida	CCMP	CCMP968	Tromso, Norway
T. gravida	CCMP	CCMP987	Tromso, Norway
T. gravida	CCMP	CCMP1442	McMurdo Sound, Antarctica
T. gravida	CCMP	CCMP1457-64	McMurdo Sound, Antarctica
T. gravida	CCMP	CCMP1546-7	McMurdo Sound, Antarctica
T. gravida	CCMP	CCMP1552	McMurdo Sound, Antarctica
T. cf. gravida	CCMP	CCMP1543	Boothbay Harbor, ME, USA
T. mala	(no cultures?)		
T. mediterranea (=stellaris)	CSIRO	CS16	Port Phillip Bay, Vic., Australia
T. minuscula	(no cultures?)		
T. partheneia	CCMP	CCMP1093	Scripps Institution, LaJolla, CA, USA
T. partheneia	CCMP	CCMP1113	
T. proschkinae	(no cultures?)		
T. subtilis	CCMP	CCMP1586	Jakarta Harbor, Indonesia
T. tubifera	(no cultures?)		
CHLOROPHYCEAE			
Oltmannsiella lineata	FLORIDA	000LTN	Gulf of Naples, Italy
O. virida	VIGO	OV1V	Ria de Vigo, Galicia, Spain

CYANOPHYCEAE

Anabaena affinis	NIES	NIES40	Lake Ksumigaura, Ibaraki, Japan
A. arnoldii	FACHB		Wudalianchi, Helongjiang, People's Republic of China
A. circinalis	CSIRO	ACBE03-5	Bernard River, Woogara, Australia
A. circinalis	CSIRO	ACBR01-2	Blackwood River, Western Australia
A. circinalis	CSIRO	ACBU01-2	Burrinjuck Dam, NSW, Australia
A. circinalis	CSIRO	ACHV01	Hope Valley Reservoir, South Australia
A. circinalis	CSIRO	ACCR02-3,8-13	Canning River, Western Australia
A. circinalis	CSIRO	ACFR02,5-6	Fitzroy River, Qld, Australia
A. circinalis	CSIRO	ACMB01-13	MtBold Reservoir, South Australia
A. circinalis	CSIRO	ACMR01-7	Murrumbidgee River, NSW, Australia
A. circinalis	CSIRO	ACPI01-5,7-9	Palm Island, Australia
A. circinalis	CSIRO	ACPJ05	Pijar Dam, NSW, Australia
A. circinalis	CSIRO	ACTA03-6	Tullaroop Reservoir, Vic.,Australia
A. circinalis	NIES	NIES41	Lake Kasumigaura, Ibaraki, Japan
Anabaena flos-aquae (freshwater)	CCAP	CCAP403/2S	Cumbria, England
A. flos-aquae	CCAP	CCAP1403/13A-13H,20-28	Various sources
A. flos-aquae	CCAP	CCAP1446/1C	Unknown
A. flos-aquae	NIES	NIES73-5	Lake Kasumigaura, Ibaraki, Japan
A. flos-aquae	NIVA	NIVACYA44	NERS, Corrallis, USA
A. flos-aquae	NIVA	NIVACYA83	Lake Edlandsvatn, Norway
A. flos-aquae	NIVA	NIVACYA138	Hastings Lake, Canada
A. flos-aquae	NIVA	NIVACYA139	Beaverhill Lake, Canada
A. flos-aquae	NIVA	NIVACYA223	Hungary
A. flos-aquae	UTEX	UTEX1444	MS. USA
A. flos-aquae	UTEX	UTEX2383	Burton Lake, Saskatchewan, Canada
A. flos-aquae	UTEX	UTEX2391	Beaverhill Lake, Alberta, Canada
A. flos-aquae	UTEX	UTEX2557-8	Hebgen Lake, MT, USA
A. hassallii (freshwater)	(no cultures?)		
A. lemmermanni (freshwater)	(no cultures?)		
A. spiroides (freshwater)	UTEX	UTEX1552	Fish pond, Israel
A. variabilis (freshwater)	CCAP	CCAP1403/12	Griefswald, Germany
A. variabilis	NIES	NIES 23	
A. variabilis	NIVA	NIVACYA19	Trondheim, Norway
A. variabilis	UTEX	UTEX377	(from CCAP1403/4)
Anabaenopsis circularis (freshwater)	NIES	NIES21	
A. milleri (freshwater)	(no cultures?)		
Aphanizomenon flos-aquae (freshwater)	NIES	NIES81	Lake Kasumigaura, Ibaraki, Japan
A. flos-aquae	NIVA	NIVACYA103	Lillehammer, Norway
A. flos-aquae	NIVA	NIVACYA141	Buffalo Pound Lake, Canada

Aphanizomenon flos-aquae	UTEX	UTEX 2384	Helbig's Pond, Warburg, Alberta, Canada (nontoxic?)
Coelosphaerium kuetzingianum	FACHB		Grand View Garden, Beijing, People's Republic of China
Lyngbya majuscula	CCAP	CCAP1446/2	Norfolk, England
Microcystis aeruginosa (freshwater)	CSIRO	MABA01	Barambola, NSW, Australia
M. aeruginosa	CSIRO	MABU01	Burrinjuck Dam, NSW, Australia
M. aeruginosa	CSIRO	MACR01-1	Canning River, Western Australia
M. aeruginosa	CSIRO	MAGL02	Gippsland Lakes, Vic.,Australia
M. aeruginosa	CSIRO	MAGR01	Griffith, NSW, Australia
M. aeruginosa	CSIRO	MALB01	Lake Burley Griffin, Australia
M. aeruginosa	CSIRO	MALD01	Lesley Dam, Qld, Australia
M. aeruginosa	CSIRO	MAMB01-3	Mt Bold Reservoir, South Australia
M. aeruginosa	CSIRO	MASY01	Sydney Water Board, Australia
M. aeruginosa	CSIRO	MAPI01-2	Palm Island, Qld, Australia
M. aeruginosa	CSIRO	MASD04-5	Sooley Dam, NSW, Australia
M. aeruginosa	CSIRO	MASH01	Shepparton, Vic.,Australia
M. aeruginosa	CSIRO	MASM01	St Marys, Tas., Australia
M. aeruginosa	CSIRO	MATA01	Taalarook, Vic.,Australia
M. aeruginosa	CSIRO	MATE01	Temora, NSW, Australia
M. aeruginosa	CSIRO	MAWB01	Werribee, Vic.,Australia
M. aeruginosa	CSIRO	CCAP1450/1,3,4,6,8,10-11	various sources
M. aeruginosa	FACHB	8641	East Lake, Wuhan, People's Republic of China
M. aeruginosa	FACHB	8696	Fish Pond, Wuhan, People's Republic of China
M. aeruginosa	IPIMAR	Lx81	Lisbon, Portugal
M. aeruginosa	NIES	NIES44,87	Lake Kasumigaura, Ibaraki, Japan
M. aeruginosa	NIES	NIES91,98	Lake Kasumigaura, Ibaraki, Japan
M. aeruginosa	NIES	NIES298,299,478	Lake Kasumigaura, Ibaraki, Japan
M. aeruginosa	NIES	NIES88-90	Lake Kawaguchi, Yamanashi, Japan
M. aeruginosa	NIES	NIES99-101	Lake Suwa, Nagano, Japan
M. aeruginosa	NIVA	NIVACYA22	Uppsala, Sweden
M. aeruginosa	NIVA	NIVACYA31	Little Rideau Lake, Canada
M. aeruginosa	NIVA	NIVACYA43	EPA,NERS, Corvallis, USA
M. aeruginosa	NIVA	NIVACYA57	Lake Froylandsvatn, Norway
M. aeruginosa	NIVA	NIVACYA118	Lake Gjersjoen, Norway
M. aeruginosa	NIVA	NIVACYA123	Lake Malaren, Uppland, Sweden
M. aeruginosa	NIVA	NIVACYA140	Bendig's Pond, Bruno, Canada
M. aeruginosa	NIVA	NIVACYA143,160	Lake Akersvatn, Norway
M. aeruginosa	NIVA	NIVACYA228	Lake Akersvatnet, Norway
M. aeruginosa	NIVA	NIVACYA144	Lake Borrevatn, Norway
M. aeruginosa	NIVA	NIVACYA162	Lake Arreso, Denmark
M. aeruginosa	NIVA	NIVACYA166	Lake Hellesjovatnet, Norway
M. aeruginosa	UPLB	Cy266	Laguna de Bay, The Philippines

Microcystis aeruginosa	UTCC	UTCC299	Pretzlaff Pond, Alberta, Canada
M. aeruginosa	UTCC	UTCC300	Pretzlaff Pond, Alberta, Canada
M. aeruginosa	UTEX	UTEX2061,3	Lake Mendota, Madison, WI, USA
M. aeruginosa	UTEX	UTEX2385-6	Little Rideau Lake, Ontario, Canada\
M. aeruginosa	UTEX	UTEX2387	Saskatchewan, Canada
M. aeruginosa	UTEX	UTEX2388	Bruno, Saskatchewan, Canada
M. viridis (freshwater)	NIES	NIES102-3	Lake Kasumigaura, Ibaraki, Japan
M. viridis	NIVA	NIVACYA122	Lake Finjasjon, Sweden
M. viridis	NIVA	NIVACYA169	Lake Arreso, Denmark
M. wesenbergii (freshwater)	NIES	NIES104	Chiyoda-ku, Tokyo, Japan
M. wesenbergii	NIES	NIES105-6,110-11, 604	Lake Kasumigaura, Ibaraki, Japan
M. wesenbergii	NIES	NIES108,112	Lake Suwa, Nagano, Japan
M. wesenbergii	NIES	NIES109	Lake Yogo,Shiga, Japan
Nodularia spumigena	UTEX	UTEX2091-2	soil, Spotted Lake, Osoyoos, BC,Canada
N. spumigena	CSIRO	NSBL03,5,6,8	Lake Bullenmeri, Vic., Australia
N. spumigena	CSIRO	NSBR01,3-5	Blackwood River, Western Australia
N. spumigena	CSIRO	NSGL01-2	Gippsland Lakes, Vic., Australia
N. spumigena	CSIRO	NSLA01-4	Lake Alexandrina, South Australia
N. spumigena	CSIRO	NSOR01-17	Orielton Lagoon, Tas., Australia
N. spumigena	CSIRO	NSPH01-6	Peel-Harvey Estuary, Western Australia
N. spumigena	UTEX	UTEX2091-2	Spotted Lake, Osoyoos, British Columbia, Canada
Oscillatoria acutissima (freshwater)	(no cultures?)		
O. agardhii (freshwater)	CCAP		
O. agardhii	CCMP	CCMP600-1	Lake Kolbotnvatn, Akershus, Norway
O. agardhii	NIES	NIES204-5	Lake Kasumigaura, Ibaraki, Japan
O. agardhii	NIVA		
O. agardhii	PCC		
O. formosa (freshwater)	NIVA		
O. nigro-viridis	CCAP	CCAP1459/9	Lowestoft, England
O. raciborskii	NIES	NIES207	Lake Kasumigaura, Ibaraki, Japan
O. rubescens (freshwater)	NIVA	NIVACYA97	Lake Steinsfjorden, Norway
Schizothrix calcicola	UTEX	UTEX1817	air, Austin, TX, USA
S. calcicola	UTEX	UTEX1934	La Jolla, CA, USA
S. calcicola	UTEX	UTEX1935	anaerobic bottle
S. calcicola	UTEX	UTEX1936	Salton Sea, CA, USA
S. calcicola	UTEX	UTEX1937	Mosquito Creek Reservior, Youngstown, OH, USA
Trichodesmium erythraeum	(no cultures?)		
T. hildebrandtii	(no cultures?)		
T. thiebautii	(no cultures?)		

DICTYOCHOPHYCEAE			
Dictyocha speculum	CCMP	CCMP1381	Musholm Bugt, Storebalt, Denmark
D. speculum	SCCAP	K-0031	Musholm Bugt, Storebalt, Denmark
D. speculum	SCCAP	K-0033	Musholm Bugt, Storebalt, Denmark
D. speculum	SCCAP	K-0035-6	Musholm Bugt, Storebalt, Denmark
D. speculum	SCCAP	K-0301	Southern Kattegat, Denmark
DINOPHYCEAE			
Alexandrium acatenella	(no cultures?)		
A. affine	CCAP	NEPPCC667	Ria de Vigo, Galicia, Spain
A. affine	CCMP	CCMP112	Ria de Vigo, Galicia, Spain
A. affine	CSIRO	AABB01/2	Bell Bay, Tasmania, Australia
A. affine	KAGAWA	KAGAWA37	Harima Nada, Seto Inland Sea, Japan
A. affine	KAGAWA	KAGAWA149	Gotou-rettou, Nagasaki, Japan
A. affine	NEPCC	NEPCC667R	Ria de Vigo, Galicia, Spain
A. affine	SARU	PA5V	Ria de Vigo, Galicia, Spain
A. affine	VIGO	PA2V-5V	Ria de Vigo, Galicia, Spain
A. affine	WHOI-1	PA5V	RÌa de Vigo, Galicia, Spain
A. affine	WHOI-1	PA71	RÌa de Vigo, Galicia, Spain
A. cf. affine	CSIRO	AABB1-3	Bell Bay, Tasmania, Australia
A. angustitabulatum	(no cultures?)		
A. andersonii	CCMP	CCMP1597	Town Cove, Eastham, MA, USA
A. andersoni	WHOI-1	GTM242	Town Cove, Eastham MA (41o45fN)
A. andersoni	WHOI-1	GTTC02	Town Cove, Eastham MA (41∞45fN)
A. catenella	CCMP	CCMP1493	Da Yia Bay, China
A. catenella	CCMP	CCMP1598	Da Yia Bay, China
A. catenella	CSIRO	ACJP01-3	ballast water (Kashima, Japan)
A. catenella	CSIRO	ACPP01-11	Port Philip Bay, Vic., Australia
A. catenella	CSIRO	CS319	Golden Crux, Singapore
A. catenella	KAGAWA	KAGAWA41	Owase Bay, Mie, Japan
A. catenella	KAGAWA	KAGAWA91	Tsuda Bay, Seto Inland Sea, Japan
A. catenella	KAGAWA	KAGAWA106	Tanabe Bay, Wakayama, Japan
A. catenella	KAGAWA	KAGAWA118	Yamakawa Bay, Kagoshima, Japan
A. catenella	KAGAWA	KAGAWA123	Uranouchi Bay, Kouchi, Japan
A. catenella	NEPCC	NEPCC254R	Hidden Basin
A. catenella	NEPCC	NEPCC743-4	Toquart Bay, Barkley Sound, British Columbia, Canada
A. catenella	NEPCC	NEPCC574R	Bamfield, British Columbia, Canada
A. catenella	NIES	NIES519	Owase Bay, Mie, Japan
A. catenella	NIES	NIES220	Tsuda Bay, Kagawa, Japan

Alexandrium catenella	NIES	NIES520	Hachinohe Harbor, Aomori, Japan
A. catenella	WHOI-1	ACQH01-2	Quarter Master Hbr., Vashon Is., Puget Sound, WA
A. catenella	WHOI-1	N520	Hachinohe Harbor, Aomori, Japan.
A. catenella	WHOI-1	OF041	Ofunato Bay, Iwate Prefecture, Japan
A. catenella	WHOI-1	OF051	Ofunato Bay, Iwate Prefecture, Japan
A. catenella	WHOI-1	OF101	Ofunato Bay, Iwate Prefecture, Japan
A. catenella	WHOI-1	OF878-C2	Ofunato Bay, Japan
A. catenella	WHOI-1	OF878-C5	Ofunato Bay, Japan
A. catenella	WHOI-1	TN9	Tanabe Bay, Wakayama Prefecture, Japan
A. catenella	WHOI-1	WKS-3	Tanabe Bay, Japan
A. catenella	WHOI-1	WKS-8	Tanabe Bay, Japan
A. cf. catenella	JINAN	KD-(J)910502	
A. cf. catenella	MBARI	A1-15	Santa Cruz, CA, USA
A. cf. catenella	WHOI-1	ACCA03,05,17	Santa Cruz Pier, Santa Cruz, CA
A. cohorticula	KAGAWA	KAGAWA139	Gulf of Thailand
A. cohorticula	WHOI-1	MMBS8811-3	Sagami Bay, Japan
A. cf. cohorticula	JINAN	KD(J)930205	Dapeng Bay, South China Sea, People's Republic of China
A. excavatum	VIGO	PE1V	Ria de Vigo, Galicia, Spain
A. excavatum	WHOI-1	PE1V	Ria de Vigo, Galicia, Spain
A. excavatum	WHOI-1	PE2V	Ria de Vigo, Galicia, Spain
A. fundyense	CN	GT7	Bay of Fundy, Canada
A. fundyense	WHOI-1	AFNFA3-4	Harbor Grace, NF, Canada (47°30'N, 53°10'W)
A. fundyense	WHOI-1	AFNS85	Nantucket shoals, MA, St. #8 (41°36'N, 69°45'W)
A. fundyense	WHOI-1	AFNS88	Nantucket shoals, MA, St. #8 (41°36'N, 69°45'W)
A. fundyense	WHOI-1	GT17xxx	Laboratory hybrids
A. fundyense	WHOI-1	GT2	Campobello Is., Bay of Fundy (44°46'N. 67°11'W)
A. fundyense	WHOI-1	GT429	Ipswich Bay, Gloucester, MA (42°40'N, 70°45'W)
A. fundyense	WHOI-1	GT7	Campobello Is., Bay of Fundy (44°46'N, 67°11'W)
A. fundyense	WHOI-1	GTCA04	Gulf of Maine, Portsmouth, NH (43°00'N, 70°19'W)
A. fundyense	WHOI-1	GTCA08	Gulf of Maine, Portsmouth, NH (43°00'N, 70°19'W)
A. fundyense	WHOI-1	GTCA28-9	Portsmouth, NH, Gulf of Maine (43°00'N, 70°19'W)
A. fundyense	WHOI-1	GTCN15	Mumford Cove, Groton, CT(41o20ïN,72o01ïW)
A. fundyense	WHOI-1	GTME20	Monhegan Island, ME (43°45'N, 69°19'W)
A. fundyense	WHOI-1	GTME23	Monhegan Island, ME (43°45'N, 69°19'W)
A. cf. fundyense	SCCAP	K-0270-1	Hobsons Bay, Australia
A. cf. fundyense	SCCAP	K-0275-7	Hobsons Bay, Australia
A. leei	JINAN	KD(J)930201	Dapeng Bay, South China Sea, People's Republic of China
A. margalefii	CAW	CAWD10	Bream Bay, New Zealand
A. margalefii	CSIRO	AMGDEO1	Derwent River, Tasmania, Australia
A. margalefii	CSIRO	AGNZ01	Bream Bay, New Zealand
A. margalefii	CSIRO	AGNZ02	Whangaparaoa, New Zealand

Alexandrium minutum	CAEN		Morlaix Bay, France
A. minutum	CAW	CAWD11	Anakoha Bay, New Zealand
A. minutum	CAW	CAWD12	Anakoha Bay, New Zealand
A. minutum	CAW	CAWD13	Croisilles Harbour, New Zealand
A. minutum	CCAP	NEPCC253	
A. minutum	CCMP	CCMP113	Ria de Vigo, Galicia, Spain
A. minutum	CN	Gonyau 7	Morlaix Bay, France
A. minutum	CSIRO	ALSP01	Ria de Vigo, Spain
A. minutum	CSIRO	ALSP02	Ria de Vigo, Spain
A. minutum	CSIRO	AMAD01-21	Port River, South Australia, Australia
A. minutum	CSIRO	AMAD24-9	Port River, South Australia, Australia
A. minutum	IPIMAR	St18	Óbidos Lagoon, Portugal
A. minutum	LME	LME18	Obidos Lagoon, Portugal
A. minutum	NEPCC	NEPCC253R	Obidos Lagoon, Portugal
A. minutum	NZOI	BOP02/HC	Bay of Plenty, New Zealand
A. minutum	SARU	AL1V-2V	Ria de Vigo, Galicia, Spain
A. minutum	TML	TK1	Tungkang fish pond, Taiwan
A. minutum	TML	TK2	Tungkang fish pond, Taiwan
A. minutum	TML	TK3	Tungkang fish pond, Tawain
A. minutum	VIGO	AL1V-3V	Ria de Vigo, Galicia, Spain
A. minutum	VIGO	18A	Portugal
A. minutum	VIGO	AMP1V-4V	Palma de Mallorca, Spain
A. minutum	VIGO	AMAD01	Australia
A. minutum	VIGO	AMAD06	Australia
A. minutum	VIGO	AMAD21	Australia
A. minutum	WHOI-1		Portugal
A. minutum	WHOI-1		Morlaix Bay, France
A. minutum	WHOI-1		Port River, South Australia, Australia
A. minutum	WHOI-1		Spain
A. minutum	WHOI-1	AM1-3	Morlaix Bay, France
A. minutum	WHOI-1	AMBOPO06	Tauranga Harbour, Bay of Plenty, New Zealand
A. minutum	WHOI-1	AMBOPO14	Tauranga Harbour, Bay of Plenty, New Zealand
A. minutum	WHOI-1	AMNZ01	Croisilles Hbr, New Zealand
A. minutum	WHOI-1	AMNZ02	Anakoha Bay, New Zealand
A. minutum	WHOI-1	AMNZ03	Anakoha Bay, New Zealand
A. minutum	WHOI-1	AMNZ04	Bay of Plenty, New Zealand
A. minutum	WHOI-1	TML-25	Tiawan
A. minutum	WHOI-1	TML-42	Tiawan
A. monilatum	CCMP	CCMP1492	Tampa Bay, FL, USA
A. monilatum	FLORIDA	CT87A6	Indian River Lagoon, FL, USA
A. monilatum	FLORIDA	CT87C6	Indian River Lagoon, FL, USA

Alexandrium monilatum	FLORIDA	CT87D3	Indian River Lagoon, FL, USA
A. monilatum	FLORIDA	CT87D5	Indian River Lagoon, FL, USA
A. monilatum	FLORIDA	RZ16B4	Tampa Bay, FL, USA
A. ostenfeldii	CAW	CAWD18	Croisilles Harbour, New Zealand
A. ostenfeldii	CAW	CAWD19	Croisilles Harbour, New Zealand
A. ostenfieldii	CAW	CAWD14	Kaitaia, New Zealand
A. ostenfieldii	CAW	CAWD15	Taharoa, New Zealand
A. ostenfeldii	CAW	CAWD16	Port Timaru, New Zealand
A. ostenfeldii	CAW	CAWD17	Port Wellington, New Zealand
A. ostenfeldii	SCCAP	K-0287	Limfjorden, Denmark
A. ostenfeldii	SCCAP	K-0324	Limfjorden, Denmark
A. ostenfeldii	WHOI-1		Limfjorden, Denmark
A. ostenfeldii	WHOI-1	AONZ01	Kaitaia, New Zealand
A. ostenfeldii	WHOI-1	AONZ02	Taharoa, New Zealand
A. ostenfeldii	WHOI-1	AONZ03	Wellington, New Zealand
A. ostenfeldii	WHOI-1	AONZ04	Timaru, New Zealand
A. ostenfeldii	WHOI-1	AONZ05-6	Croisilles Hbr, New Zealand
A. ostenfeldii	WHOI-1	K-0287	Limfjorden, Denmark
A. ostenfeldii	WHOI-1	K-0324	Limfjorden, Denmark
A. tamarense	CAW	CAWD20	Plymouth, England
A. tamarense	CAW	CAWD21	Ipswich, MA, USA
A. tamarense	CCAP	CCAPxxx	Ria de Vigo, Spain
A. tamarense	CCAP	CCAPxxx	Ipswich Bay, Gloucester, MA, USA
A. tamarense	CCAP	NEPCC403	Jericho Beach, Vancouver, British Columbia, Canada
A. tamarense	CCAP	NEPCC407	Jericho Beach, Vancouver, British Columbia, Canada
A. tamarense	CCAP	UW 2c*	(Not for Distribution)
A. tamarense	CCAP	UW 4*	(Not for Distribution)
A. tamarense	CCMP	CCMP115	Tamar Estuary, Plymouth, England
A. tamarense	CCMP	CCMP116	Ria de Vigo, Galicia, Spain
A. tamarense	CCMP	CCMP117	Ipswich Bay, Gloucester, MA, USA
A. tamarense	CCMP	CCMP118	Boothbay Harbor, ME, USA
A. tamarense	CCMP	CCMP1311	Perch Pond, Falmouth, MA, USA
A. tamarense	CN	MOG835	Onagawa, Japan
A. tamarense	CSIRO	ATBB01	Bell Bay, Tasmania, Australia
A. tamarense	CSIRO	CS300	Global Hope, Sanchompo,Korea
A. tamarense	JINAN	KD(J)930502	Hong Kong Sea
A. tamarense	JINAN	KD(J)920005	Dapeng Bay, South China Sea, People's Republic of China
A. tamarense	KAGAWA	KAGAWA56	Harima-Nada, Seto Inland Sea, Japan
A. tamarense	NEPCC	NEPCC71R	Patricia Bay, British Columbia, Canada
A. tamarense	NEPCC	NEPCC183	Tamar Estuary, Plymouth, England, UK
A. tamarense	NEPCC	NEPCC255R	Lummi Island, WA, USA

Alexandrium tamarense	NEPCC	NEPCC403	Jericho Beach, Vancouver, British Columbia, Canada
A. tamarense	NEPCC	NEPCC407R	Jericho Beach, Vancouver, British Columbia, Canada
A. tamarense	NEPCC	NEPCC412R	Jericho Beach, Vancouver, British Columbia, Canada
A. tamarense	NEPCC	NEPCC516	Jericho Beach, Vancouver, British Columbia, Canada
A. tamarense	NEPCC	NEPCC592R	Ipswich Bay, Gloucester, MA, USA
A. tamarense	NIES	NIES239	Harima-Nada, Seto Inland Sea, Japan
A. tamarense	NIES	NIES521	Hachinohe Harbor, Aomori, Japan
A. tamarense	PLY	PLY173	R. Lyhher, Cornwall, United Kingdom
A. tamarense	PLY	PLY173a	Tamar Estuary, Plymouth,United Kingdom
A. tamarense	SARU		Ipswich Bay, Gloucester, MA, USA
A. tamarense	UW	1	Ardtoe, Scotland
A. tamarense	UW	2	Ardtoe, Scotland
A. tamarense	UW	2c	Ardtoe, Scotland
A. tamarense	SCCAP	K-0049	England
A. tamarense	SCCAP	K-0055-6	Faroe Islands
A. tamarense	WHOI-1	AT1105	Tung Kang River mouth, Tung Kang, Taiwan
A. tamarense	WHOI-1	AT1204	Tung Kang River mouth, Tung Kang, Taiwan
A. tamarense	WHOI-1	ATCI01	Daya Bay, China
A. tamarense	WHOI-1	ATCI01-03	Daya Bay, China
A. tamarense	WHOI-1	ATCI01-1	Daya Bay, China
A. tamarense	WHOI-1	ATCI02	Daya Bay, China
A. tamarense	WHOI-1	ATCI02-1	Daya Bay, China
A. tamarense	WHOI-1	ATCI03	Daya Bay, China
A. tamarense	WHOI-1	ATCI03-1	Daya Bay, China
A. tamarense	WHOI-1	ATJP01	Japan
A. tamarense	WHOI-1	ATSL01	St. Lawrence Estuary
A. tamarense	WHOI-1	ATSL12	St. Lawrence Estuary (49°03'N. 68°20'W)
A. tamarense	WHOI-1	ATSL23	St. Lawrence Estuary
A. tamarense	WHOI-1	CU-1	Gulf of Thailand
A. tamarense	WHOI-1	CU-13	Gulf of Thailand
A. tamarense	WHOI-1	CU-15	Gulf of Thailand
A. tamarense	WHOI-1	CU-22	Gulf of Thailand
A. tamarense	WHOI-1	GT Port	Portugal
A. tamarense	WHOI-1	GT5-6	La CoruÒa Bay, Spain
A. tamarense	WHOI-1	GTCN02	Palmer Cove, Groton, CT (41°20'N, 72°00'W)
A. tamarense	WHOI-1	GTCN10	Palmer Cove, Groton, CT, Station C-2 (41°20'N)
A. tamarense	WHOI-1	GTCN16	Mumford Cove, Groton, CT (41°20'N, 72°01'W)
A. tamarense	WHOI-1	GTM253	Mitchell River, Orleans, MA (41°45'N)
A. tamarense	WHOI-1	GTME05	Deer Isle, ME (44°13'N, 68°40'W)
A. tamarense	WHOI-1	GTMR01	Mitchell River, Orleans, MA (41°40'N, 69°58'W)
A. tamarense	WHOI-1	GTPP	Perch Pond, Falmouth, MA (41°30'N, 70°35'W)

Alexandrium tamarense	WHOI-1	GTPP01-3	Perch Pond, Falmouth, MA (41°30'N, 70°35'W)
A. tamarense	WHOI-1	GTPP05-6	Perch Pond, Falmouth, MA (41°30'N, 70°35'W))
A. tamarense	WHOI-1	GTPP10	Perch Pond, Falmouth, MA (41°30'N, 70°35'W))
A. tamarense	WHOI-1	GTPPKF	Perch Pond, Falmouth, MA (41°30'N, 70°35'W))
A. tamarense	WHOI-1	I72/21-2	Presumed origin Muroran, Japan
A. tamarense	WHOI-1	I72/22-2	Presumed origin Muroran, Japan
A. tamarense	WHOI-1	I72/24-1	Presumed origin Muroran, Japan
A. tamarense	WHOI-1	K-0055	Suduroy, Tjaldavik, Faroe Islands
A. tamarense	WHOI-1	N239	Harima-Nada, Seto Inland Sea, Japan
A. tamarense	WHOI-1	ND-1	Noda Bay, Japan
A. tamarense	WHOI-1	° OF84423-D3	Ofunato Bay, Japan
A. tamarense	WHOI-1	OF875-17	Ofunato Bay, Japan
A. tamarense	WHOI-1	OF875-19	Ofunato Bay, Japan
A. tamarense	WHOI-1	OF875-8	Ofunato Bay, Japan
A. tamarense	WHOI-1	OK875-1	Okkirai Bay, Japan
A. tamarense	WHOI-1	OK905-5	Okkirai Bay, Japan
A. tamarense	WHOI-1	OK905-7	Okkirai Bay, Japan
A. tamarense	WHOI-1	PGT183	Tamar Estuary, Plymouth, UK
A. tamarense	WHOI-1	WKS-1	Tanabe Bay, Japan
A. tamarense	WHOI-1	GTLI21	Moriches Bay, Long Island, NY (40°45'N, 72°49'W)
A. tamarese	WHOI-1	GTMP	Mill Pond, Orleans, MA (41°47'N, 69°57'W)
A. tamarense	WHOI-1		Tamar Estuary, Plymouth, England
A. cf. tamarense	WHOI-1	369125	Alaska
A. cf. tamarense	WHOI-1	920501C	Dapeng Bay, China
A. cf. tamarense	WHOI-1	930201	Dapeng Bay, China
A. cf. tamarense	WHOI-1	930205	Dapeng Bay, China
A. cf. tamarense	WHOI-1	930205C	Dapeng Bay, China
A. cf. tamarense	WHOI-1	940101C1	Dapeng Bay, China
A. cf. tamarense	WHOI-1	940101C2	Dapeng Bay, China
A. cf. tamarense	WHOI-1	940102C1	Nanao Island, China
A. cf. tamarense	WHOI-1	940102C2	Nanao Island, China
A. cf. tamarense	WHOI-1	940102R	Nanao Island, China
A. cf. tamarense	WHOI-1	940201C1	Fujian Province, China
A. cf. tamarense	WHOI-1	940201R	Fujian Province, China
A. cf. tamarense	WHOI-1	Alex spp.HK	Hong Kong, China
A. cf. tamarense	WHOI-1	ATAG01-2	Region de patagonica, Argentina
A. cf. tamarense	WHOI-1	ATRU03-4	Mohovaya Inlet, Avachinskaya Guba, Russia
A. cf. tamarense	WHOI-1	ATSA01-2	St. Helen Bay, Cape Town, South Africa
A. cf. tamarense	WHOI-1	ATSK01A-B	Loon Mood, South Korea
A. cf. tamarense	WHOI-1	ATSK02	Loon Mood, South Korea
A. cf. tamarense	WHOI-1	ATUR01A-B	P. del Este, Uruguay

Alexandrium cf. tamarense	WHOI-1	ATUR02	P. del Este, Uruguay
A. cf. tamarense	WHOI-1	IP02	Alaska
A. cf. tamarense	WHOI-1	PI31	Alaska
A. cf. tamarense	WHOI-1	PI32	Alaska
A. cf. tamarense	WHOI-1	PW06	Prince William Sd. (60°03.0'N, 148°00.3'W)
A. tamarense/fundyense	WHOI-1	MR16B1,2,4	Laboratory hybrids
A. tamiyavanichi	WHOI-1	CU-18	Gulf of Thailand
A. tamiyavanichi	WHOI-1	CU-25	Gulf of Thailand
A. tamiyavanichi	WHOI-1	CU-5	Gulf of Thailand
A. tamiyavanichi	WHOI-1	CU-8	Gulf of Thailand
A. tamiyavanichi	WHOI-1	CU18	Cholburi Province, Gulf of Thailand
A. tamiyavanichi	WHOI-1	CU21	Cholburi Province, Gulf of Thailand
A. taylorii	VIGO	AM1V,4V	Costa Brava, Spain
A. taylorii	VIGO	AM7V-9V	Costa Brava, Spain
A. sp.	WHOI-1	JINAN	KD-(J)930205
A. sp.	WHOI-1	SBHS	4 Ardtoe
A. sp.	WHOI-1	SBHS	42 Belfast
A. spp.	WHOI-1	PW05	Prince William Sd. (60°03.0'N, 148°00.3'W)
A. spp.	WHOI-1	ATSW01	Gullmar Fjord, Essvik Sweden
Amphidinium carterae	AVD	LAC-1-KA-83	Kattegatt
A. carterae	CAW	CAWD22	Wellington Harbour, New Zealand
A. carterae	CAW	CAWD23	Marlborough Sounds, New Zealand
A. carterae	CAW	CAWD24	Plymouth, England, UK
A. carterae	CCMP	CCMP1314	Great Pond, Falmouth, MA, USA
A. carterae	CSIRO	CS-212	
A. carterae	IPIMAR	M-I-5	Sado Estuary, Portugal
A. carterae	NEPCC	NEPCC32	Great Pond, Falmouth, MA, USA
A. carterae	NEPCC	NEPCC629	Bamfield, British Columbia, Canada
A. carterae	NEPCC	NEPCC731	(unknown)
A. carterae	NIES	NIES331	Iriomote Island, Okinawa, Japan
A. carterae	NZOI	BGB12	Big Glory Bay, New Zealand
A. carterae	NZOI	BGB32/HC	Big Glory Bay, New Zealand
A. carterae	SCCAP	K-0406	Merimbula, Australia
A. cf. carterae	VIGO	A1V	Ria de Pontevedra, Galicia, Spain
A. klebsii	CCMP	CCMP1342	Knight Key, FL, USA
A. kelbsii	CCMP	CCMP1344	Knight Key, FL, USA
A. klebsii	CSIRO	CS-33	
Ceratium fusus	(no cultures?)		
Cochlodinium catenatum	(no cultures?)		
Coolia monotis	CCMP	CCMP302	Milford Sound, New Zealand
C. monotis	CCMP	CCMP304	Ria de Vigo, Galicia, Spain

Coolia monotis	CCMP	CCMP305	Knight Key, FL, USA
C. monotis	CCMP	CCMP1345	Knight Key, FL, USA
C. monotis	FLORIDA	000COO1	Florida, USA
C. monotis	FLORIDA	IBCM3V	Ria de Vigo, Spain
C. monotis	IPIMAR	M-III-35	Sado Estuary, Portugal
C. monotis	NIES	NIES343	Hachijo Island, Tokyo, Japan
C. monotis	VIGO	CM1V-5V	Ria de Vigo, Galicia, Spain
Dinophysis spp.	(no cultures)		
Gambierdiscus toxicus	CCMP	CCMP399	St. Bartts
G. toxicus	CCMP	CCMP400	Sargasso Sea, Bermuda
G. toxicus	CCMP	CCMP401	St. Bartts
G. toxicus	CCMP	CCMP403	Tahiti
G. toxicus	CCMP	CCMP1363	St. Bartts
G. toxicus	CCMP	CCMP1600	Australia
G. toxicus	CCMP	CCMP1648	Cozumel, Mexico
G. toxicus	CCMP	CCMP1649	Grand Cayman Islands
G. toxicus	CCMP	CCMP1650	Moorea
G. toxicus	CCMP	CCMP1651	Grand Cayman Islands
G. toxicus	CCMP	CCMP1652	Tahiti
G. toxicus	CCMP	CCMP1653	Hawaii, USA
G. toxicus	CCMP	CCMP1654	Mariana Island, Guam
G. toxicus	CCMP	CCMP1655	Martinique
G. toxicus	CCMP	CCMP1656	Pohnpel, FSM
G. toxicus	CCMP	CCMP1657	Palau
G. toxicus	COM	(various)	Mayotte, French Polynesia
G. toxicus	CSIRO	CS-232	North Queensland, Australia
G. toxicus	KAGAWA	KAGAWA189	Muroto-misaki, Kouchi, Japan
G. toxicus	NEPCC	NEPCC413	Bermuda
G. toxicus	NEPCC	NEPCC708R	Guam
G. toxicus	NEPCC	NEPCC746R	Bermuda
G. toxicus	WHOI-1		Gambier Island, French Polynesia
G. toxicus	WHOI-1		Lorrient, St. Barthelemy
G. toxicus	WHOI-1	MQ1	Fort-de-France Bay, Martinique
G. toxicus	WHOI-1	MQ2	Fort-de-France Bay, Martinique
G. toxicus	WHOI-1	SB01	Lorient, St. Barthelemy
G. toxicus	WHOI-1	T39	Hawaii
Gymnodinium breve	CAW	CAWD04	John's Pass, Florida, USA
G. breve	CCMP	CCMP718	Florida, USA
G. breve	FLORIDA	WILSON	John's Pass, Florida, USA
G. breve	FLORIDA	PBCC13	Corpus Christi, Texas, USA
G. breve	NIES	NIES140	Harima-nada, Seto Island Sea, Japan

Gymnodinium catenatum	CCMP	CCMP412-4	Ria de Vigo, Galicia, Spain
G. catenatum	CSIRO	GCDE01-3	Derwent River, Tasmania, Australia
G. catenatum	CSIRO	GCDE05-10	Derwent River, Tasmania, Australia
G. catenatum	CSIRO	GCHA01-4	Hastings Bay, Tasmania, Australia
G. catenatum	CSIRO	GCHU02	Huon River, Tasmania, Australia
G. catenatum	CSIRO	GCHU04-8	Huon River, Tasmania, Australia
G. catenatum	CSIRO	GCHU10-11	Huon River, Tasmania, Australia
G. catenatum	CSIRO	GCHU15-6	Huon River, Tasmania, Australia
G. catenatum	CSIRO	GCHU18-22	Huon River, Tasmania, Australia
G. catenatum	CSIRO	GCJP10	Senzaki, Japan
G. catenatum	CSIRO	GCPT01	Figueira da Foz, Portugal
G. catenatum	CSIRO	GCPT02-3	Aguda, Portugal
G. catenatum	CSIRO	GCSP01-09	Ria de Vigo, Spain
G. catenatum	FLORIDA	IBZVOI	Ria de Vigo, Spain
G. catenatum	IPIMAR	Ag89	Oporto litoral, Portugal
G. catenatum	IPIMAR	BFO93	Faro litoral, Algarve, Portugal
G. catenatum	IPIMAR	MS93	Formosa Lagoon, Portugal
G. catenatum	IPIMAR	Sg93	Sagres litoral, Algarve, Portugal
G. catenatum	IPIMAR	Sg94	Sagres litoral, Algarve, Portugal
G. catenatum	IPIMAR	Set94	Setubal litoral, Algarve, Portugal
G. catenatum	KAGAWA	KAGAWA98	Harima-Nada, Seto Inland Sea, Japan
G. catenatum	NEPCC	NEPCC663R	Ria de Vigo, Galicia, Spain
G. catenatum	LME	LME348	Sagres, Portugal
G. catenatum	SARU	GC7V	Ria de Vigo, Galicia, Spain
G. catenatum	SARU	GC9V	Ria de Vigo, Galicia, Spain
G. catenatum	SARU	GC19V	Ria de Vigo, Galicia, Spain
G. catenatum	SARU	GC21V	Ria de Vigo, Galicia, Spain
G. catenatum	VIGO	GC7V	Ria de Vigo, Galicia, Spain
G. catenatum	VIGO	GC9V	Ria de Vigo, Galicia, Spain
G. catenatum	VIGO	GC10V-13V	Ria de Vigo, Galicia, Spain
G. catenatum	VIGO	GC19V	Ria de Vigo, Galicia, Spain
G. catenatum	VIGO	GC21V	Ria de Vigo, Galicia, Spain
G. catenatum	WHOI-1	GC7B	Rìa de Vigo, Galicia, Spain
G. catenatum	WHOI-1	SE-GC	Senzaki Bay, Japan
G. lanskaya	DMMSU	U-3	Russia?
G. mikimotoi	CAW	CAWD05	Kushimoto, Japan
G. mikimotoi	NEPCC	NEPCC665R	Norway
G. mikimotoi	SCCAP	K-0286	Hobsons Bay, Australia
G. cf. mikimotoi	CAW	CAWD35	Foveaux Strait, New Zealand
G. nagasakiense	NIES	NIES249	Harima-Nada, Seto Inland Sea, Japan
G. nelsonii	MIL	Gymno-F	Oyster Bay, New York, USA

Gymnodinium pulchellum	CSIRO	GPPP01	Port Phillip Bay, Vic.,Australia
G. cf. pulchellum	CAW	CAWD02	Kawau Island, New Zealand
G. sanguineum	CAW	CAWD01	Marlborough Sounds, New Zealand
G. sanguineum	CAW	CAWD36	Akaroa, New Zealand
G. sanguineum	CCMP	CCMP417	Scripps Inst. Oceanography, LaJolla, CA, USA
G. sanguineum	CCMP	CCMP1321	Great South Bay, Long Island, NY, USA
G. sanguineum	CCMP	CCMP1593	Narragansett Bay, RI, USA
G. sanguineum	CSIRO	CS-35	LaJolla, CA, USA
G. sanguineum	FLORIDA	WR12A1	Florida Bay, FL, USA
G. sanguineum	IPIMAR	M-I-36	Sado Estuary, Portugal
G. sanguineum	NEPCC	NEPCC274	(unknown)
G. sanguineum	NEPCC	NEPCC354	Vancouver Island, British Columbia, Canada
G. sanguineum	UIO	G.sang	
G. sanguineum	NIES	NIES11	Harima-Nada, Seto Inland Sea, Japan
G. sanguineum	NIES	NIES141	Uchinomi Bay, Kagawa, Japan
G. uncatenum	CCMP	CCMP1310	Perch Pond, Falmouth, MA, USA
G. veneficum	PLY	PLY103	Tamar Estuary, Plymouth, United Kingdom
Gyrodinium aureolum	CCAP	CCAPxxx	
G. aureolum	CCMP	CCMP429	Sutton Harbour, Plymouth, England, UK
G. aureolum	CCMP	CCMP430	Oslofjorden, Norway
G. aureolum	PLY	PLY497a	Sutton Harbour, Plymouth, England, UK
G. aureolum	SCCAP	K-0260	Oslofjorden, Norway
G. aureolum	UIO		
G. aureolum	WHOI-1	PLY497A	Sutton Harbour, Plymouth, England, UK
G. cf. aureolum	IPIMAR	VS93	Albufeira Lagoon, Setubal, Portugal
G. galatheanum	CCMP	CCMP415-6	63° N, 10° E, Norway
G. galatheanum	CSIRO	GGLI03	Lake Illawarra
G. galatheanum	IPIMAR	Alg80	Faro litoral, Algarve, Portugal
G. galatheanum	IPIMAR	Esp87	Oporto literal, Portugal
G. galatheanum	NEPCC	NEPCC249R	Lummi Island, WA, USA
G. galatheanum	NEPCC	NEPCC408R	Jericho Beach, Vancouver, British Columbia, Canada
G. galatheanum	NEPCC	NEPCC555R	Station P, 49°5' N, 144°40' W
G. galatheanum	NEPCC	NEPCC734R	Oslofjorden, Norway
G. galatheanum	NEPCC	NEPCC735R	Oslofjorden, Norway
G. galatheanum	PLY	PLY517	Oslofjorden, Norway
G. galatheanum	SCCAP	K-0522-3	Oslofjorden, Norway
G. galatheanum	UIO		
G. galatheanum	WHOI-1	Gymgal	Oslofjorden, Norway
G. impudicum	CAW	CAWD03	Kawau Island, New Zealand
G. impudicum	VIGO	GY1VA-4VA	Valencia, Spain
G. impudicum	VIGO	GY5V	Ria de Vigo, Galicia, Spain

Gymnodinium impudicum	VIGO	10B	Laguna de Fusaro, Italy
G. instriatum	CCMP	CCMP431	St. Andrè lagoon, Portugal
G. instriatum	CCMP	CCMP432	Obidos lagoon, Portugal
G. instriatum	NIES	NIES143	Shodo Island, Kagawa, Japan
G. instriatum	NIES	NIES354	Shimoda Harbor, Shizuoka, Japan
G. instriatum	WHOI-1	GyrIns177	St. Andrè lagoon, Portugal
G. instriatum	WHOI-1	GyrIns184	St. Andrè lagoon, Portugal
G. instriatum	WHOI-1	GyrIns228	St. Andrè lagoon, Portugal
Ostreopsis sp.	NEPCC	NEPCC273	Hawaii, USA
O. sp.	COM	OsMUR	Tuamotu, French Polynesia
O. sp.	KAGAWA	KAGAWA190	Muroto-misaki, Kouchi, Japan
Peridinium polonicum	NIES	NIES500	Shiogama, Miyagi, Japan
Prorocentrum compressum	CAW	CAWD30	Taharoa, New Zealand
P. compressum	CAW	CAWD31	Kaitaia, New Zealand
P. concavum	CCAP		
P. concavum	CCMP	CCMP683	Knight Key, FL, USA
P. emarginatum	COM	PemREU	St. Leu, Réunion Island
P. emarginatum	COM	PemMURx	Tuamotu, French Polynesia
P. emarginatum	FLORIDA	RZ19C6	Tampa Bay, FL, USA
P. hoffmannianum	FLORIDA	RZ1B10	Dry Tortugus, USA
P. hoffmannianum	FLORIDA	HN30A2	Montego Bay, Jamaica
P. hoffmannianum	FLORIDA	PL100A	Knight Key, FL, USA
P. hoffmannianum	FLORIDA	RZ1F1	Dry Tortugus, USA
P. hoffmannianum	FLORIDA	RZ1G8	Dry Tortugus, USA
P. hoffmannianum	FLORIDA	RZ2H4	Dry Tortugus, USA
P. hoffmannianum	FLORIDA	RZ1H9	Dry Tortugus, USA
P. lima	AVD	LAC-1	Lissabon?
P. lima	CAW	CAWD32	Spain
P. lima	CAW	CAWD33	Rangaunu, New Zealand
P. lima	CCAP	CCAP1136/11	Ria de Vigo, Spain
P. lima	CCAP	CCAP1136/9	
P. lima	CCAP	CCAP1136/9	Lincolnshire, England
P. lima	CCAP	CCAPxxx	
P. lima	CCMP	CCMP684	Ria de Vigo, Galicia, Spain
P. lima	CCMP	CCMP685-6	Ria de Vigo, Galicia, Spain
P. lima	CCMP	CCMP1368	Knight Key, FL, USA
P. lima	CN	PL2V,4V	Ria de Vigo, Galicia, Spain
P. lima	COM	PlMAR	Marseille, France
P. lima	COM	PlSAF	South Africa
P. lima	COM	PlMAYx	Mayotte Island, Comoros Archipelagos
P. lima	COM	PlMOO	Mooréa, French Polynesia

Prorocentrum lima	COM	PlMURx	Taumoto, French Polynesia
P. lima	FLORIDA	PL1V-4V	Ria de Vigo, Spain
P. lima	FLORIDA	RZ1C10-1	Dry Tortugas, FL, USA
P. lima	FLORIDA	RZ01G1,2,7	Dry Tortugas, FL, USA
P. lima	FLORIDA	TP99B3	Dry Tortugas, FL, USA
P. lima	FLORIDA	RZ01E4	Dry Tortugas, FL, USA
P. lima	FLORIDA	RZ1E10	Dry Tortugas, FL, USA
P. lima	FLORIDA	RZ1H10	Dry Tortugas, FL, USA
P. lima	FLORIDA	RZ01H3,7	Dry Tortugas, FL, USA
P. lima	FLORIDA	RZ01C2	Dry Tortugas, FL, USA
P. lima	FLORIDA	RZ01D4	Dry Tortugas, FL, USA
P. lima	IPIMAR	Pl-1	Vigo Ria, Galicia, Spain
P. lima	IPIMAR	P1-2	Vigo Ria, Galicia, Spain
P. lima	KAGAWA	KAGAWA151	Tokushima, Japan
P. lima	NEPCC	NEPCC514	(unknown)
P. lima	NEPCC	NEPCC712	Ria de Vigo, Galicia, Spain
P. lima	NIES	NIES517	Lake Obuchinuma, Aomori, Japan
P. lima	NZOI	RNH04	Rangaunu Harbour, New Zealand
P. lima	TML		Dapong Bay, Tawain
P. lima	VIGO	PL1V-2V	Ria de Vigo, Galicia, Spain
P. lima	VIGO	PL3V-4V	Ria de Pontevedra, Galicia, Spain
P. lima	VIGO	PL5V	Ria de Vigo, Galicia, Spain
P. lima	VIGO	PL6V-10V	Ria de Pontevedra, Galicia, Spain
P. maculosum	COM	PemREU	Réunion Island (Indian Ocean)
P. maculosum	COM	PmaMURx	Tuamotu, French Polynesia
P. mexicanum	CCMP	CCMP687	Knight Key, FL, USA
P. mexicanum	CCMP	CCMP1370	Knight Key, FL, USA
P. mexicanum	COM	PmeNOU	Nauméa, New Caledonia
P. mexicanum	COM	PmeQAT	Qatar
P. mexicanum	COM	PmS1	French Mediterranean coast
P. mexicanum	CSIRO	P. lima1	Wilson Inlet, WA, Australia
P. mexicanum	CSIRO	P. lima2	Wilson Inlet, WA, Australia
P. mexicanum	FLORIDA	CT58C3	Dry Tortugas, FL, USA
P. mexicanum	FLORIDA	PM300	Florida Bay, FL, USA
P. mexicanum	FLORIDA	PRMEX	Knight Key, FL, USA
P. mexicanum	FLORIDA	RZ03CL	Dry Tortugas, FL, USA
P. mexicanum	FLORIDA	RZ02E9	Dry Tortugas, FL, USA
P. mexicanum	NIES	NIES317	Harima-Nada, Seto Inland Sea, Japan
P. minimum	AVD	LAC-3	Lissabon?
P. minimum	AVD	LAC-4	Lissabon
P. minimum	AVD	LAC-5	Keil Bight (BAH ME 66)

Prorocentrum minimum	AVD	LAC-6	Kattegatt
P. minimum	AVD	LAC-9	Chesapeake Bay, Nova Scotia, Canada
P. minimum	CCAP		
P. minimum	CCAP		
P. minimum	CCMP	CCMP695	Florida Everglades, FL, USA
P. minimum	CCMP	CCMP696	East Massapequa, Long Island, NY, USA
P. minimum	CCMP	CCMP697	Norway
P. minimum	CCMP	CCMP698	Maquiot Bay, Brunswick, ME, USA
P. minimum	CCMP	CCMP699	Boothbay Harbor, ME, USA
P. minimum	CCMP	CCMP1329	Great South Bay, Long Island, NY, USA
P. minimum	CCMP	CCMP1529	2°40'S, 82°43'W, Equador
P. minimum	IPIMAR	St135	Óbidos Lagoon, Portugal
P. minimum	IPIMAR	B87	Óbidos Lagoon, Portugal
P. minimum	IPIMAR	Alb89	Albufeira Lagoon, Setubal, Portugal
P. minimum	MIL	Exuv	Great South Bay, Long Island, NY, USA
P. minimum	NEPCC	NEPCC96	Jericho Beach, Vancouver, British Columbia, Canada
P. minimum	NEPCC	NEPCC623	38°42.2'N 72°22.4'W
P. minimum	NIES	NIES237	Osaka Bay, Osaka, Japan
P. minimum	NIES	NIES238	Harima-Nada, Seto Inland Sea, Japan
P. minimum	PLY	PLY18	Plymouth Sound, England, UK
P. minimum	SCCAP	K-0010	Laholms, Bugten, Kattegat
P. minimum	SCCAP	K-0295	Lisboa
P. minimum	SCCAP	K-0336	Southern Kattegat
P. minimum	SCCAP	K-0355	Sweden
P. minimum	UIO		Oslofjord, Norway
P. minimum	WHOI-1	CCMP1329	Great South Bay, Long Island, NY
P. minimum	VIGO	PMIN	Ria de Vigo, Galicia, Spain
P. rostratum	VIGO	PR1V	Ria de Vigo, Galicia, Spain
P. triestinum	CCMP	CCMP700	Maquoit Bay, Brunswick, ME, USA
P. triestinum	FLORIDA	00PT05	Corpus Christi, Texas, USA
P. triestinum	NIES	NIES13	Osaka Bay, Osaka, Japan
P. triestinum	NIES	NIES219	Nomi Bay, Kochi, Japan
P. triestinum	VIGO	PT	Coruña, Galicia, Spain
P. triestinum	VIGO	PT2V	Ria de Vigo, Galicia, Spain
P. triestinum	VIGO	PT3V	Ria de Vigo, Galicia, Spain
P. triestinum	VIGO	PT5V	Ria de Vigo, Galicia, Spain
P. sp.	COM	(various)	French Polynesia
P. sp.	COM	(various)	Mayotte, New Caledonia
P. sp.	AVD	LAC-15	Denmark
P. sp.	JINAN	KD(J)93204	Dapeng Bay, South China Sea, People's Republic of China
Pyrodinium bahamense	WHOI-1		Sabah, Maylasia

Pyrodinium bahamense var. compressu	MSIUP	PBCMZRC	Bamban Bay, Masinloc, Zambales, The Philippines
P. bahamense var. compressu	MSIUP	PBCBMRC	Bataan, Manila Bay, The Philippines
Scrippsiella trochoidea	CCMP	CCMP1331	Plymouth, England, UK
S. trochoidea	CCMP	CCMP1599	DaYia Bay, People's Republic of China
S. trochoidea	JINAN	KD(J)930102	Dapeng Bay, South China Sea, People's Republic of China
S. trochoidea	FLORIDA	000SB4	Ria de Vigo, Spain
S. trochoidea	NEPCC	NEPCC15	Point Atkinson, WA, USA
S. trochoidea	NEPCC	NEPCC602	Los Angeles, CA, USA
S. trochoidea	PLY	PLY104	Plymouth Sound, England, UK
S. trochoidea	PLY	PLY452	Oslofjord, Norway
S. trochoidea	PLY	PLY452a	S.W. England, U.K.
S. trochoidea	UTEX	UTEX1017	Plymouth, England, UK
S. spp.	CCMP	CCMPxxxx	
S. sp.	JINAN	KD(J)930103	Dapeng Bay, South China Sea, People's Republic of China
S. sp.	VIGO	EXQ7V	Ria de Vigo, Galicia, Spain
S. sp.	VIGO	S2V	Ria de Vigo, Galicia, Spain
S. sp.	VIGO	S3V	Ria de Vigo, Galicia, Spain
PELAGOPHYCEAE			
Aureococcus anaphagefferens	CCMP	CCMP1706-7	Long Island, NY, USA
A. anaphagefferens	CCMP	CCMP 1708	USA
"Texas Brown Tide Organism"	CCMP	CCMP1502-5	Gulf of Mexico, TX, USA
"Texas Brown Tide Organism"	CCMP	CCMP1507-10	Gulf of Mexico, TX, USA
"Texas Brown Tide Organism"	CCMP	CCMP1681	Gulf of Mexico, TX, USA
PRYMNESIOPHYCEAE			
Chrysochromulina acantha	CAEN	78	Les Glénans, France
C. acantha	CAEN	78bis	Cotentin, France
C. acantha	PLY	PLY326	English Channel\
C. brevifilum	PLY	PLY143	English Channel
C. brevifilum	UTEX	UTEX985	English Channel
C. camella	CAEN	56	Bayonne, France
C. camella	CAEN	56bis	La Rochelle, France
C. camella	PLY	PLY297	English Channel
C. campanulifera	UIO	J10	
C. chiton	PLY	PLY146	English Channel
C. chiton	UTEX	UTEX982	English Channel
C. cymbium	CAEN	75	Luc-sur-Mer, France
C. cymbium	PLY	PLY321	English Channel

Chrysochromulina cymbium	PLY	PLY365	Plymouth Sound, England, UK
C. cf. cymbium	NEPCC	NEPCC306	Pacific Ocean, 49°52'N 142°40W
C. ephippium	CAEN	72	Concarneau, France
C. ericina	CCMP	CCMP281	Pacific Ocean, 49°36'N 140°37'W
C. ericina	CCMP	CCMP282-3	Gulf of Maine, ME, USA
C. ericina	NEPCC	NEPCC109	Pacific Ocean, 49°52'N 142°40 W
C. ericina	UIO	AU-eri	Austevoll, Bergen, Norway
C. herdlensis	CAEN	77	Cotentin, France
C. herdlensis	CCMP	CCMP284	Pacific Ocean, 49°52'N 142°40'W
C. hirta	UIO	AU-hi1	Austevoll, Bergen, Norway
C. kappa	PLY	PLY'K'	Port Erin, Isle of Man, UK
C. cf. kappa	UIO	EN3	Oslofjord, Norway
C. leadbeaterii	UIO	ERIK	Lofoten, Norway
C. leadbeaterii	UIO	TJE	Lofoten, Norway
C. minor	PLY	PLY304	English Channel
C. polylepis	AVD	LAC-14	
C. polylepis	CCMP	CCMP285-7	Kristineberg, Sweden
C. polylepis	UIO	A	Skagerrak, 59° N, 10°45' E
C. polylepis	UIO	B11	Skagerrak, 59°N, 10°45' E
C. polylepis	UIO	B1511	Skagerrak, 59° N, 10°45' E
C. polylepis	UIO	K	Skagerrak, 58°30' N, 9°30' E
C. polylepis	UIO	S	Skagerrak, 58°30' N, 9°30' E
C. polylepis	UIO	T	Skagerrak, 58°30' N, 9°30' E
C. polylepis	UIO	W	Skagerrak, 58°30' N, 9°30' E
C. polylepis	SCCAP	K-0259	Kattegatt, Sweden
C. polylepis	WHOI-1	Chry p.	
C. quadrikonta	CAEN	70	Concarneau, France
C. quadrikonta	CAW	CAWP04	Waimangu Point, New Zealand
C. quadrikonta	CCMP	CCMP1642	Australia
C. cf. simplex	UIO	JomfB	Jomfruland, Norway
C. spinifera	PLY	PLY328	English Channel
C. strobilus	PLY	PLY43a	English Channel
C. strobilus	UTEX	UTEX981	English Channel
C. cf. strobilus	CAEN	74	Bayonne, France
C. sp.	CAEN		Concarneau
C. sp.	CAEN		Luc-sur-Mer, 1993
C. sp.	IPIMAR	M-1-46	Sado Estuary, Portugal
C. spp.	CCMP	CCMPxxxx	
C. sp.	UIO	K11	Arendal, Norway
C. sp.	UIO	G7	Skagerrak, Norway
C. spp.	PLY		

Phaeocystis pouchetii	CAW	CAWP14	Marlborough Sounds, New Zealand
P. pouchetii	CSIRO	CS-165	Port Hacking, New South Wales, Australia
P. pouchetii	CSIRO	CS-188	Prydz Bay, Antarctica
P. pouchetii	CSIRO	CS-239	Port Esperance, Tasmania, Australia
P. pouchetii	CSIRO	CS-240	PLY540
P. pouchetii	CSIRO	CS-242-3	Prydz Bay, Antarctica
P. pouchetii	CSIRO	CS-244-5	Davis Base, Antarctica
P. pouchetii	NEPCC	NEPCC225	49°52'N 142°40 W
P. pouchetii	NIES	NIES388	Hachijo Island, Tokyo, Japan
P. pouchetii	WITS		South Africa
P. sp.	CCMP	CCMP1524	Thailand
P. sp.	CCMP	CCMP1528	Gardiner Bay, Espanola
P. sp.	CCMP	CCMP627	29°15' N, 85°54' W
P. sp.	CCMP	CCMP628	6°45' N, 53°19' W
P. sp.	CCMP	CCMP629	Gulf Stream, Atlantic Ocean
P. sp.	NEPCC	NEPCC452	
P. sp.	SCCAP	K-0494	CCMP628, Surinam
P. sp.	SCCAP	K-0495	CCMP629,Gulf of Mexico
P. sp.	SCCAP	K-0496	CCMP627,Gulf of Mexico
Prymnesium annuliferum	CAEN	47	Pont-Lorois, France
P. calathiferum	CAEN	53bis	Martinique
P. calathiferum	CCMP	CCMP707	Northland, New Zealand
P. calathiferum	NZOI	AKH04/HC	Akaroa, New Zealand
P. nemamethecum	WITS		South Africa
P. parvum	CAEN	45	Plestin-les-Grèves, France
P. parvum	CAEN	45bis	La Réunion Island
P. parvum	CAW	CAWP10	(Univ. Copenhagen)
P. parvum	CCMP	CCMP708	
P. parvum	FACHB		Tianjin, People's Republic of China
P. parvum	IPIMAR	Alv77	Alvor Lagoon, Algarve, Portugal
P. parvum	IPIMAR	M-I-38	Sado Estuary, Portugal
P. parvum	IPIMAR	Zb-b-1	Sado estuary, Portugal
P. parvum	MIL	Prym	Plymouth, England
P. parvum	NEPCC	NEPCC673	Millport, Isle of Cumbrae, Scotland, UK
P. parvum	PLY	PLY94	R. Blackwater, Essex, England, UK
P. parvum	PLY	PLY94a	English Bay, BC, Canada
P. parvum	PLY	PLY94b	Israel
P. parvum	SAG	B127.79	
P. parvum	SCCAP	K-0081	
P. parvum	UIO		
P. parvum	UTEX	UTEX995	R. Blackwater, Essex, England, UK

Prymnesium parvum	WITS		South Africa
P. patelliferum	CAEN	36	Sallenelles, France
P. patelliferum	CAEN	36bis	Honfleur, France
P. patelliferum	CAW	CAWP12	(Univ. Copenhagen)
P. patelliferum	CCMP	CCMP709	England, U.K.
P. patelliferum	CSIRO	CS-288	The Fleet, Dorset, England, UK
P. patelliferum	UIO		
P. patelliferum	UIO	N	Hylsfjord, Norway 59°33' N, 6°24' E
P. patelliferum	PLY	PLY 527	The Fleet, Dorset, England, UK
P. patelliferum	WITS		South Africa
P. saltans	IPIMAR	M-I-37	Sado Estuary, Portugal
P. cf. saltans	CAEN	27	Chausey Island, France
P. cf. saltans	CAEN	27bis	Tunis, Tunisia
P. zebrinum	CAEN	29	Le Logeo, France
P. zebrinum	CAEN	29bis	Gatteville, France
P. sp.	CAEN	52bis	Balearic Islands
P. sp.	CAEN	64	Tunis, Tunisia
P. sp.	CAWT		?University of Copenhagen
P. spp.	CCMP	CCMP	
P. spp.	NEPCC		
P. sp.	UIO		

RAPHIDOPHYCEAE

Chattonella antiqua	CAW	CAWR01	Seto Inland Sea, Japan
C. antiqua	CSIRO	CS-331	Harima-Nada, Seto Inland Sea, Japan
C. antiqua	FLORIDA	000NIES236	Japan
C. antiqua	NIES	NIES1,114	Harima-Nada, Seto Inland Sea, Japan
C. antiqua	NIES	NIES2	Osaka Bay, Osaka, Japan
C. antiqua	NIES	NIES83-4	Hiketa, Seto Inland Sea, Japan
C. antiqua	NIES	NIES85	Shodo Island, Kagawa, Japan
C. antiqua	NIES	NIES86	Uranouchi Bay, Kochi, Japan
C. antiqua	NIES	NIES113	Naoshima Island, Kagwa, Japan
C. antiqua	NIES	NIES114	Harima-Nada, Seto Inland Sea, Japan
C. antiqua	NIES	NIES161,557	Hiroshima Bay, Hiroshima, Japan
C. antiqua	NIES	NIES558	Mikawa Bay, Aichi, Japan
C. antiqua	SCCAP	K-0248	Not for distribution
C. globosa	no cultures?)		
C. marina	NIES	NIES3	Osaka Bay, Osaka, Japan
C. marina	NIES	NIES14	Harima-Nada, Seto Inland Sea, Japan
C. marina	NIES	NIES115	Kinko Bay, Kagoshima, Japan

Chattonella marina	NIES	NIES116	Harima-Nada, Seto Inland Sea, Japan
C. marina	NIES	NIES117	Naoshima Island, Kagawa, Japan
C. marina	NIES	NIES118	Harima-Nado, Seto Inland Sea, Japan
C. marina	NIES	NIES121	Kagoshima Bay, Kagoshima, Japan
C. marina	NIES	NIES558	Maizuru Bay, Kyoto, Japan
C. minima	(no cultures?)		
C. ovata	NIES	NIES603	Harima-nada, Seto Island Sea, Japan
C. verruculosa	(no cultures?)		
C. sp.	CCMP	CCMP218	Harima, Japan
C. sp.	UTEX	UTEX2111	Harima, Japan
Fibrocapsa japonica	CAW	CAWR02	Leigh, New Zealand
F. japonica	CAW	CAWR03	Tsuda Bay, Kagawa, Japan
F. japonica	CCMP	CCMP1661	Port Philip Bay, Victoria, Australia
F. japonica	CSIRO	NIES-136	Tsuda Bay, Kagawa, Japan
F. japonica	NIES	NIES136	Tsuda Bay, Kagawa, Japan
F. japonica	NZOI	HAG05/HC	Hauraki Gulf, New Zealand
F. japonica	NZOI	ORE09	Orewa, New Zealand
F. japonica	SCCAP	K-0247	Hobsons Bay, Port Phillip Bay, VI, Australia
F. japonica	UTEX	UTEX2162	Pt. Loma, CA, USA
Heterosigma akashiwo	NIES	NIES9,10	Harima-Nada, Seto Inland Sea, Japan
H. akashiwo	NIES	NIES145	Nomaike, Kagoshima, Japan
H. akashiwo	NIES	NIES146	Shido Bay, Kagawa, Japan
H. akashiwo	NIES	NIES293	Onagawa Bay, Miyagi, Japan
H. akashiwo	NIES	NIES4	Fukuyama Bay, Hiroshima, Japan
H. akashiwo	NIES	NIES5	Gokasho Bay, Mie, Japan
H. akashiwo	NIES	NIES6	Osaka Bay, Osaka, Japan
H. akashiwo	NIES	NIES9	Harima-Nada, Seto Inland Sea, Japan
H. akashiwo	NIES	NIES561	Mikawa Bay, Aichi, Japan
H. akashiwo	NZOI	BGB06	Big Glory Bay, New Zealand
H. akashiwo	NZOI	BGB12/HC	Big Glory Bay, New Zealand
H. akashiwo	NZOI	MAR15	Marborough Sounds, New Zealand
H. carterae	CAEN		La Rochell, France
H. carterae	CAEN		Camaret, France
H. carterae	CAW	CAWR04	West Lake, South Australia, Australia
H. carterae	CAW	CAWR05	Big Glory Bay, New Zealand
H. carterae	CAW	CAWR06	Leigh, New Zealand
H. carterae	CA	CAWR07	New Zealand
H. carterae	CAW	CAWR08	Ruakaka, New Zealand
H. carterae	CAW	CAWR09	Nelson Harbour, New Zealand
H. carterae	CCMP	CCMP452	Long Island Sound, NY, USA
H. carterae	CCMP	CCMP1595	Narragansett Bay, RI, USA

Heterosigma carterae	CCMP	CCMP1596	Narragansett Bay, RI, USA
H. carterae	CN		
H. carterae	CSIRO	CS-169	West Lake, South Australia, Australia
H. carterae	CSIRO	CS-39	
H. carterae	CSIRO	CS-96	
H. carterae	IPIMAR	Cs82	Cascais Bay, Portugal
H. carterae	IPIMAR	Oc80	Litoral Setubal, Portugal
H. carterae	IPIMAR	Alb86	Albufeira Lagoon, Setubal, Portugal
H. carterae	FLORIDA	000LUT	Narragansett Bay, RI, USA
H. carterae	MIL	OL	
H. carterae	NEPCC	NEPCC102R	English Bay, British Columbia, Canada
H. carterae	NEPCC	NEPCC522R	Jericho Beach, Vancouver, British Columbia, Canada
H. carterae	NEPCC	NEPCC560R	Long Island Sound, Milford, CT, USA
H. carterae	NEPCC	NEPCC625R	Genoa Bay, British Columbia, Canada
H. carterae	NEPCC	NEPCC630R	Long Island Sound, Miford, CT. USA
H. carterae	NEPCC	NEPCC759	(unknown)
H. carterae	PLY	PLY12a	English Channel
H. carterae	PLY	PLY239	New Zealand
H. carterae	PLY	PLY461	Oslofjord, Norway
H. carterae	SAG	B46.89	
H. carterae	SCCAP	K-0246	
H. carterae	UIO		Gulf of Naples, Italy
H. carterae	UTEX	UTEX2005	York River, Gloucester Point, VA, USA
H. carterae	VIGO	HA1V-2V	Ria de Arosa, Galicia, Spain
H. carterae	VIGO	CHATON	Coruña, Galicia, Spain
H. carterae	WHOI-1	Olisto MX	Long Island Sound
Olisthodiscus luteus	NIES	NIES15	Tamano, Okayama, Seto Inland Sea, Japan

Table 25.3. The culture collection codes from Table 25.2 and their corresponding culture collections - see Table 25.1.

code	collection	country
AVD	AVD for Marine Ekologie	Sweden
CAEN	Caen Algal Culture Collection	France
CAW	Cawthron Microalgae Culture Collection	New Zealand
CCAP	Culture Centre for Algae and Protozoa	United Kingdom
CCMP	Provasoli-Guillard Natl Center for Culture of Marine Phytoplankton	United States of America
CN	Centre de Nantes	France
COM	Centre d'Oceanologie de Marseille	France
CSIRO	CSIRO	Australia
DMMSU	Department of Microbiology, Moskow University	Russia
FACHB	Freshwater Algal Collection, Institute of Hydrobiology	People's Republic of China
FLORIDA	Florida DNR Culture Collection	United States of America
IPIMAR	Instituto PortuguÍs de InvestigaÁ„o Maritma	Portugal
JINAN	Institute of Hydrobiology Algal Collection, Jinan University	People's Republic of China
KAGAWA	Akashiwo Research Institute of Kagawa Prefecture	Japan
LME	Lab. Microbiologia Experimental	Portugal
MBARI	Monterey Bay Aquarium Research Institute	United States of America
MIL	Millford Laboratory, NOAA	United States of America
MSIUP	Marine Science Institute of the University of the Philippines	The Philippines
NEPCC	Northeast Pacific Culture Collection	Canada
NIES	National Institute for Environmental Studies	Japan
NIVA	Norwegian Institute for Water Research	Norway
NZOI	New Zealand Oceanographic Instutute	New Zealand
PLY	Plymouth Marine Laboratory	United Kingdom
SAG	Sammlung von Algenkulturen	Germany
SARU	Swansea Algal Reseach Unit	United Kingdom
SCCAP	Scandinavian Culture Collection of Algae and Protozoa	Denmark
TML	Tungkang Marine Laboratory	Taiwan
UIO	University of Oslo	Norway
UPLB	University of the Philippines-Los Banos	The Philippines
UTEX	University of Texas Culture Collection	United States of America
UW	University of Westminster	United Kingdom
VIGO	Instituto Espanol de Oceanografia	Spain
WHOI-1	Don Anderson's Collection, Woods Hole Oceanographic Institute	United States of America
WITS	University of Witwatersrand Culture Collection	South Africa

26. Agencies and Addresses: International and Regional Organizations with programmes or activities on harmful microalgae

H.O. Enevoldsen

This Chapter aims at facilitating communication between scientists, managers, and the various international or regional organizations which are running programmes on harmful microalgae. Activities and the scope of science and management programmes continuously evolves and changes, therefore only very basic information is included. For detailed up-to-date information the relevant organization should be contacted directly.

Only organizations which have harmful microalgae as an explicit priority and are running activities for a longer period are included. In addition to these, several organizations or international development agencies have shorter or longer single projects related to HAB on a case-to-case basis.

An important supplement to this Chapter is the IOC International Directory of Experts in Toxic and Harmful Algae (edited by A.W.White).

GOVERNMENTAL ORGANIZATIONS

INTERGOVERNMENTAL OCEANOGRAPHIC COMMISSION (IOC) of UNESCO

IOC Harmful Algal Bloom Programme

Background:
A Harmful Algal Bloom Programme (HAB) was initiated by IOC Member States as an activity under the joint IOC-FAO Ocean Science in Relation to Living Resources Programme (OSLR). Through a number of international workshops, a programme plan was prepared to cover educational, scientific, and operational aspects of harmful algae. Programme activities are continuously developed, and since 1992 implemented jointly by IOC Member States, the IOC Secretariat, and co-sponsoring organizations through the IOC Intergovernmental Panel on Harmful Algal Blooms (IPHAB).

Goal:
'To foster the effective management of, and scientific research on harmful algal blooms in order to understand their causes, predict their occurrences, and mitigate their effects'.

Activities:
Educational activities include training courses on taxonomy, toxin determination, and monitoring of harmful algae, individual study grants, publication of a newsletter on toxic algae and algal blooms (*Harmful Algae News*), manuals, guides and directories on various topics related to harmful marine microplankton.

Scientific activities include an ICES-IOC Working Group on Harmful Algal Bloom Dynamics, a SCOR-IOC Working Group on the Physiological Ecology of Harmful Algal Blooms, Regional Science Planning Workshops, and related pilot projects and workshops. To facilitate implementation of training and capacity building activities, IOC Science and

Communication Centres on HAB are established at the University of Copenhagen and at the Vigo Oceanographic Centre (IEO), Spain.

Operational activities include initiatives directed towards improved resource protection, monitoring, public health and seafood safety.

At the regional level, the IOC Sub-Commission for the Western Pacific, WESTPAC, has a specific Task Team on HAB.

Harmful Algae News, the *IOC International Directory of Experts in Toxic and Harmful Algae*, the *IOC Manual on Harmful Marine Microalgae*, and various reports related to the HAB Programme, can be obtained free of charge upon request to the IOC Secretariat or the IOC HAB Centre.

Liaisons:
Joint activities with WHO, FAO, UNEP, ICES, SCOR, ITS, IUPAC, and Member State institutions.

Focal points:

Intergovernmental Oceanographic Commission
Harmful Algal Bloom Programme Office
UNESCO
1, rue Miollis
75732 Paris cedex 15
France
Tel.: (33) (1) 45683983
Fax.: (33) (1) 40569316
Tlm.: hab.ioc@unesco.org

IOC Regional Secretariat
for WESTPAC
196 Phaholyothin Road
Chatuchak, Bangkok 10900
Thailand
Tel.: (66) 25796000
Fax.: (66) 25796001

IOC Regional Office for IOCARIBE
Apartado Aereo 1108
Casa del Marques de Valdehoyos
Cartagena
Colombia
Tel.: (57) 53646399
Fax.: (57) 53646399

IOC Science and Communication Centre
on Harmful Algae
University of Copenhagen
Oster Farimagsgade 2D
DK-1353 Copenhagen K
Denmark
Tel.: (45) 33134446
Fax.: (45) 33134447
Tlm.: hab@bot.ku.dk

FOOD AND AGRICULTURAL ORGANIZATION OF THE UNITED NATIONS (FAO)

Background and Activities:
Previously joint programme with IOC.

FAO no longer has specific activities on HAB, but will continue focus on practical aspects of the problem, in particular through cooperation with WHO.

Liaisons:
FAO-WHO Joint Expert Committee on Food Additives (JECAF), CODEX Alimentarius Programme, GESAMP.

Focal point:

Food and Agriculture Organization of the United Nations
Fishery Resources and Environment Division
Room NF 517, Vialle delle Terme di Caracalla
00100 Rome
Italy
Tel.: (39) (6) 52253454
Fax.: (39) (6) 52253020
Telex 610181 FAO I

WORLD HEALTH ORGANIZATION (WHO)

International Programme on Chemical Safety (IPCS)

Background:
The Programme was set up to provide assessment of the risks to human health and the environment from exposure to chemicals, whatever their origin, man-made or natural.

Goal:
To provide guidance to Member States on how to use such assessments, and to strengthen national capabilities to prevent and treat harmful effects.

Activities:
In 1984 the IPCS published an Environmental Health Criteria document (No. 37) on Aquatic (Marine and Freshwater) Biotoxins covering toxicology of the most important biotoxins such as Paralytic Shellfish Poisons (PSP), ciguatera toxins, Diarrhetic Shellfish Poison (DSP), etc. A 1994 revised version is in preparation.

IPCS-WHO publishes the series Environmental Health Criteria (EHC), and Health and Safety Guides (HSGs).

Liaisons:
Joint training activities with IOC. Represented in the IOC Intergovernmental Panel on Harmful Algal Blooms. FAO-WHO Joint Expert Committee on Food Additives (JECAF).

Focal point:

International Programme on Chemical Safety
World Health Organization
CH-1211 Geneva 27
Switzerland
Tel.: (41) (22) 7912111
Fax.: (41) (22) 7881949

INTERNATIONAL MARITIME ORGANIZATION (IMO)

Background:
There is evidence world-wide that aquatic organisms have been trans-located via ships' ballast water. This has resulted in significant environmental and human health problems. Many IMO Member States have expressed their concern on the introduction through ballast water discharges of toxic planktonic algae (primarily dinoflagellates) in their coastal areas, resulting in human poisoning and fatalities from eating contaminated seafood, the periodic closure of shellfish farms, red tides and considerable loss of material income due to restricted exports.

Goal:
The Assembly of IMO by Resolution A 18/Res.724 adopted "International Guidelines for Preventing Introduction of Unwanted Aquatic Organisms and Pathogens from Ships' Ballast Water and Sediment Discharges". It should be noted that these voluntary Guidelines have so far been implemented in only a small number of IMO Member States. Several countries do not consider the ballast water problem to be significant and express the need for more research, with a view to gathering evidence that toxic algae (dinoflagellate cysts) can indeed be transferred through ballast water.

The Marine Environment Protection Committee of IMO in 1993 established a Working Group with a view to further developing mandatory guidelines as a basis for a new annex to the MARPOL 73/78 Convention. The Committee further requested the Working Group to investigate the possibility of an international symposium on the subject of transfer of species, including issues covered by the Guidelines.

Activities:
Collection of information and data on options for preventing or minimizing the introduction of unwanted species, e.g. by exchange of ballast water on the high seas, treatment of ballast water on board ship, discharges in port in reception facilities and treatment on land.

Development of standardized methods which could form the basis for an internationally agreed certification system.

Liaisons:
IMO Working Group on Introductions and Transfers of Marine organisms, joint activities with IOC and ICES.

Focal point:

International Maritime Organization
Mr. Manfred K. Nauke
Marine Environment Division
4 Albert Embankment
London SE1 7SR
United Kingdom
Tel.: (44) (71) 7357611
Fax.: (44) (71) 5873210

INTERNATIONAL COUNCIL FOR THE EXPLORATION OF THE SEA (ICES)

Background:
Because harmful algal blooms present problems quite distinct from those of normal blooms due to their potentially serious economic and social impacts, they require special attention. ICES plays a major role for its member countries in cooperating on harmful algae-related problems because of the organization's interdisciplinary capabilities.

Activities:
The joint ICES-IOC Working Group on Harmful Algal Bloom Dynamics (joint sessions with the ICES Working Group on Shelf Seas Oceanography), is the main focus on harmful algae in ICES, but related areas include the ICES Working Group on Phytoplankton Ecology, the Biological Oceanography Committee, and the Advisory Committee on the Marine Environment.

Liaisons:
Joint Working Group with IOC.

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GOVERNMENTAL NON-SPECIALIZED ORGANIZATIONS WHICH FUND REGIONAL HAB RESEARCH AND CAPACITY BUILDING

THE EUROPEAN UNION, COMMISSION OF THE EUROPEAN COMMUNITIES (CEC)

Background:
The CEC has a broad range of programmes which includes, or potentially includes, projects on harmful and toxic algae. The main programmes of interest are; Marine Science and Technology Programme (MAST), Measurements and Testing Programme (M&T, former Community Bureau of Reference, BCR), Human Capital and Mobility Programme, Environment Programme (ENV), and the Environment Institute (Ispra). For detailed information contact the relevant focal point.

Liaisons:
Joint training activities with IOC.

Focal points:

DG XII - Measurements and Testing Programme (M&T)

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DG VI - Reference Laboratories for Monitoring of Marine Biotoxins

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ASSOCIATION OF SOUTH-EAST ASIAN NATIONS (ASEAN)

Background:
ASEAN consisting of Brunei Darussalam, Indonesia, Malaysia, Philippines, Singapore, and Thailand, has since 1991 received support from the Canadian International Development Agency (CIDA) through the ASEAN-Canada Cooperative Programme on Marine Science

(CPMS). The implementation and coordination of the programme is facilitated by the Canadian Executing Agency (CEA) based in Vancouver Canada, and the Project Execution Centre (PEC) located in Kuala Lumpur, Malaysia. This project focuses on institutional strengthening, training and research on marine sciences for the ASEAN region. It provides assistance to each ASEAN nation to develop national and regional marine science programs by providing expert assistance in program design and implementation, and development of technical capabilities. Three different activities focus on the development of environmental criteria, pollution monitoring and red tide capabilities.

Goal:
The Red Tide component aims to maintain the integrity of the resource and economic base and enhance protection of human health. Specifically, the goal of ASEAN-Canada CPMS is to enhance ASEAN scientific and management capabilities both individually and for the region as a whole. The programme provides: training on a country specific and regional basis; technical assistance to in-country activities; practical attachments for specific scientific subjects related to the taxonomy and toxicity of HAB organisms; opportunities for higher education in related subjects; and equipment and logistical support to assist with the implementation of in-country activities.

Activities:
The main activities include field methods and monitoring strategies; taxonomy of HAB organisms; marine toxin detection methods, especially mouse-bioassay procedures; development of public awareness materials and information; development of a framework for red tide response and management, as a tool for planning to meet national and regional needs; information management; a regional alert and information network for HAB events; technical and logistical support to in-country activities which focus on specific HAB organisms and/or areas of interest such as monitoring of harmful species, effects of nutrients, culturing of *Pyrodinium bahamense* var.*compressum*, occurrence of ciguatera, mechanisms affecting HAB occurrence in shrimp ponds, low level toxin detection methods, information management and development of capabilities for toxin detection. CPMS publishes *The South East Asian Harmful Algae Bulletin* (SEAHAB). For detailed information on current activities the Project Execution Centre should be contacted.

Focal points:

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NON-GOVERNMENTAL ORGANIZATIONS:

SCIENTIFIC COMMITTEE ON OCEAN RESEARCH (SCOR)

Background:
As a part of its mandate to promote international collaboration in marine science, SCOR establishes small working groups with terms of reference which must be accomplished within three to four years. In the field of harmful algae SCOR has identified the physiological factors involved in the occurrence of harmful algae as one scientific topic in which progress could be made through the efforts of a SCOR Working Group.

Activities:
SCOR-IOC Working Group on the Physiological Ecology of Harmful Algal Blooms. WG 97 works in close cooperation with the IOC Harmful Algal Bloom Programme and the ICES-IOC Working Group on Harmful Algal Bloom Dynamics.

Liaisons:
Joint Working Group with IOC. Represented in the IOC Intergovernmental Panel on Harmful Algal Blooms.

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INTERNATIONAL SOCIETY FOR TOXINOLOGY (IST)

Background:
IST is a scientific organization devoted to research on toxins from animals, plants and micro-organisms. This research includes chemistry, biochemistry, pharmacology, toxicology, clinical aspects and therapy of envenomation. IST has 700 members in 57 countries.

Goal:
One of the goals is research on toxic algae, including red tides, ciguatera poisoning, etc. An important aspect is exchange of results in meetings of the Society, e.g. satellite symposia on marine pharmacology.

Activities:
Major international meetings of the Society have been held regularly. The Society edits its own journal TOXICON (founded 1962).

Liaisons:
Joint activities with WHO, ESF, ISF, and State institutions in the countries of our members.

Focal points:

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INTERNATIONAL UNION FOR PURE AND APPLIED CHEMISTRY (IUPAC)

Commission on Food Chemistry

Background:
The IUPAC Commission on Food Chemistry is composed of eight working groups whose activities focus on mycotoxins, aquatic biotoxins, elemental analysis, natural toxins (plant), halogenated hydrocarbon environmental contaminants, animal drugs, and effects of food processing. The Aquatic Biotoxins Working Group is the unit within the Commission on Food Chemistry charged with the responsibility of coordinating all IUPAC activities associated with contamination of food and water with aquatic biotoxins, including phycotoxins.

Goal:
The primary purpose of the Commission on Food Chemistry is to sponsor projects fostering international cooperation and participation.

Activities:
The Commission sponsors a series of symposia on mycotoxins and/or phycotoxins, and each working group plans and implements projects involving chemical methodology, collaborative studies of such methods, surveys of foodstuffs for analytes of interest to the international community, etc., which are summarized and published.

Other previous projects of the Commission, relating to aquatic biotoxins, which have resulted in publications, include worldwide regulations for phycotoxins, phycotoxins of public health concern, status of methods for the detection of seafood toxins, and an inter-laboratory study of the immunobead assay for ciguatoxins. Proceedings of symposia and status reports for projects are sponsored by IUPAC and published in selected scientific publications.

The Aquatic Biotoxins Working Group of the Commission initiate and conduct research on aquatic biotoxins in food and water, initiate and conduct method performance studies of selected methods of analysis for aquatic biotoxins in food and water, address the issue of criteria of purity and stability of aquatic biotoxins, collect and evaluate data relative to incidence, regulations, levels and sources of contamination of food and water with aquatic biotoxins, collaborate with other international organizations involved in dealing with the consequences of human exposure to aquatic biotoxins, and plan and conduct symposia on aquatic biotoxins.

Liaisons:
Close cooperation with AOAC International on method and validation studies. Coordination with Codex Alimentarius-FAO, and Commission of the European Communities-BCR. Member of the IOC Intergovernmental Panel on Harmful Algal Blooms.

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ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (AOAC)

Methods Committee on Natural Toxins

Background:
The AOAC Official Methods Programme coordinates the development and validation of chemical and microbiological analytical methods by expert scientists working in their government, industry, and academic laboratories worldwide. Candidates for AOAC Official Methods status are subjected to a collaborative study involving a minimum of eight laboratories and conducted according to an internationally harmonized and recognized protocol. The proposed methods are reviewed first by an expert reviewer (Called the General Referee), statistical and safety advisors, and then a broader committee of experts, the Methods Committee on Natural Toxins. Adopted methods are published in the Journal of AOAC International. The Methods Committee on Natural Toxins is responsible for guiding and coordinating the development and validation of analytical methods for the detection, identification, and/or quantitation of mycotoxins, plant toxins, and seafood toxins in food. Those methods demonstrating sufficient accuracy, precision, and specificity will be recommended for adoption by AOAC International as AOAC Official Methods.

Goal:
To provide methods of analysis for natural toxins for which performance characteristics such as accuracy, precision, sensitivity, range, specificity, and similar attributes have been determined and tested under typical laboratory applications.

Activities:
Review and recommend General Referee topics, such as General Referee on Seafood Toxins, and Associate Referee topics such as Ciguatera, Cyanobacterial Peptide Toxins, Domoic Acid, and Diarrhetic, Neurotoxic and Paralytic Shellfish Poisoning Toxins. Review proposed methods and collaborative study protocols, such as Solid Phase Immunobead Assay Method for Ciguatoxins in Fish, Liquid Chromatographic Method for Domoic Acid in Shellfish, etc.. Publish General Referee reports, summarizing activities and literature for the year, in the AOAC Journal. Sponsor special interest symposia at the AOAC INTERNATIONAL annual meeting, such as Analytical Methods for Seafood Toxins. Publish peer-reviewed reports of research in the Journal of AOAC INTERNATIONAL, such as comparisons of mouse bioassay and cell bioassays for ciguatoxins; liquid chromatographic methods for domoic acid; detection of marine toxins using reconstituted sodium channels; detection methods based on radioimmunoassay; and pre-column oxidation techniques for determining saxitoxin.

Liaisons:
Joint activities with IUPAC Commission of Food Chemistry; International Standards Organization; Royal Society of Chemistry Analytical Methods Committee; Nordic Committee

for Food Analysis, Codex Committee for Methods of Analysis and Sampling; and numerous others.

Focal points:

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Corrigendum to IOC-HAB Manual

Cover:

The *Alexandrium* cell illustration is optically reversed; the correct illustration appears on p. 293, Fig.15.

Cover page:

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Chapter 3. Culture Methods.

Figure legend 3.2. (p. 48) ; item D, Latex tubing should be 1/4 inch (6.35mm)
Table 3.2 . (p.57); K_2CRO_4 concentration (first column, last line) should be 0.0194 [This error is also in the original Guillard and Hargraves 1993 paper].

Chapter 5. Post-column derivatization HPLC methods for Paralytic Shellfish Poisons.

The specific toxicity values for dcGTX2 and dcGTX3 are erroneous.

p.83, line 16 from below should read: For the calculation of toxicity from HPLC chromatograms, the following values of specific toxicity (MU/micromole) were determined: GTX1 (2468), GTX2 (892), GTX3 (1584), GTX4 (1803), GTX5 (160), dcGTX2 (382), dcGTX3 (935), C1 (15), C2 (239), C3 (33), C4 (143), STX (2483), neoSTX (2295) and dcSTX (1274).

Chapter 10 A. *In vitro* biochemical and cellular assays.

p.188, last line: competitive binding assay
p.191, line 3 from below: 0.89 ± 0.07 nM
p.192, figure legend 10.5: competitive binding curves
p.200, line 16 from below: 0.65 micrometer Duropore membrane (not mm).
p.200, line 8 from below: volume-200 microlitre (not mL),
amount of radioactivity-50 microCi (not mCi)
p.200, line 7 from below: 50 microCi / (39 microCi / nmol);
STX concentration= 6.40 microM (not mM)
p.200, line 4 from below: 3 microL [^{3}H] STX (not mL)
p.201. The Table on the top of the page should read:

		conc.of standard	conc.in assay
15 microL	100 microM STX + 235 microL HEPES/NaCl buffer	6.00×10^{-6}	1.0×10^{-6}
50 microL	6.00×10^{-6} solution + 450 microL buffer	6.00×10^{-7}	1.0×10^{-7}
50 microL	6.00×10^{-7} solution + 450 microL buffer	6.00×10^{-8}	1.0×10^{-8}
25 microL	6.00×10^{-7} solution + 475 microL buffer	3.00×10^{-8}	5.0×10^{-9}
50 microL	3.00×10^{-8} solution + 200 microL buffer	6.00×10^{-9}	1.0×10^{-9}
50 microL	6.00×10^{-9} solution + 450 microL buffer	6.00×10^{-10}	1.0×10^{-10}
Reference		Buffer only	0

p.201, line 2 in text below Table: 100 microL aliquots (not mL)
p.201, line 6 from below: 35 microL [^{3}H] STX (not mL)
p.201, line 5 from below: 35 microL STX standard (not mL)
p.201, line 4 from below: 135 microL synaptosome preparation (not mL)
p.202, line 2: 200 microL of ice-cold HEPES buffer (not mL)
Note that ALL OTHER "milli" (m)-designations in Chapter 10 are correct.

Chapter 15. Taxonomy of Harmful Dinoflagellates

p.289. The illustrations in Figures 15.10 i-j depict *Gyrodinium impudicum* (not *Gymnodinium catenatum*) . A correct illustration for *Gymnodinium catenatum* appears in Fig. 15.5 (p. 287).