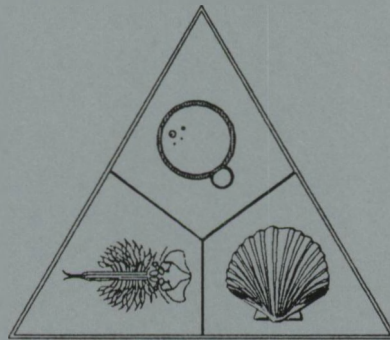


UNIVERSITEIT GENT
Fakulteit der Wetenschappen
Akademiejaar 1991 - 1992

BAKKERSGIST ALS SUBSTITUUT VOOR EENCHELLIGE WIEREN IN DE KWEEK VAN FILTREER-ORGANISMEN




BAKER'S YEAST AS SUBSTITUTE FOR MICRO-ALGAE IN THE CULTURE OF FILTER-FEEDING ORGANISMS

door
Peter Coutteau

Proefschrift voorgelegd tot het bekomen van de graad van Doctor in de Wetenschappen,
Groep Dierkunde

Promotor: Prof. Dr. A. Coomans
Co-promotor: Prof. Dr. P. Sorgeloos

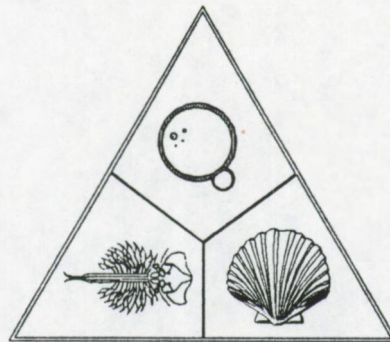
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opgedragen aan pa en schoonma

Preface

While writing this preface, I realized more and more that this study could only be accomplished thanks to the support from many people. As I may unintentionally skip somebody, I thank in the first place everybody who felt "forgotten" after reading this page.

I would like to thank Prof. Dr. A. Coomans and Prof. Dr. P. Sorgeloos for supervising this dissertation. I owe many thanks to Prof. Dr. P. Sorgeloos for offering me the facilities of the Laboratory of Aquaculture & Artemia Reference Center and the scientific backing, which were both a sine qua non to carry out this study.

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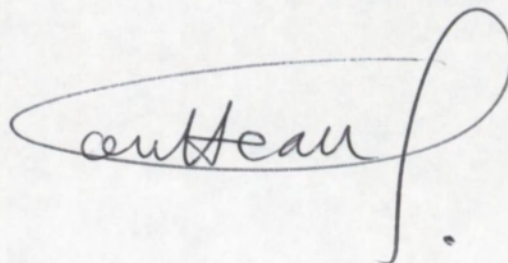
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Peter Couteau
16 december, 1991

A handwritten signature in dark ink, appearing to read 'Couteau', with a large, sweeping flourish extending from the end of the name.

FULL REFERENCE:

Coutteau, P., 1992.

Baker's yeast as substitute for micro-algae in the culture of filter-feeding organisms. PhD Thesis, University of Ghent, Belgium. 408 pp.

The yeast treatments described in Chapters IV, V, and VI of this thesis are protected by International Patents PCT/BE 89/00009 and EP-89870040.6 (old 09.03.89) "Feed for Aquaculture" filed in Europe, USA, Japan, Canada, Australia (various file numbers; pending; owned by Artemia Systems N.V./S.A., Baasrode, Belgium).

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LIST OF ABBREVIATIONS

<hr/> algal species <hr/>	
Bell	<i>Bellerochea polymorpha</i>
Cart	<i>Carteria chuii</i>
Chaecu	<i>Chaetoceros curvisetus</i>
Chaem	<i>Chaetoceros muelleri</i>
Croo	<i>Croomonas salina</i>
Dunam	<i>Dunaliella marina</i>
Dunap	<i>Dunaliella primolecta</i>
Isog	<i>Isochrysis galbana</i>
Phaeo	<i>Phaeodactylum tricornutum</i>
Skel	<i>Skeletonema costatum</i>
T-Iso	<i>Isochrysis galbana</i> , Tahitian strain
Tetra	<i>Tetraselmis suecica</i>
Thal	<i>Thalassiosira pseudonana</i> , Clone 3H
<hr/> preparations of <i>S. cerevisiae</i> <hr/>	
C-yeast	caked baker's yeast treated according to the standard cysteine treatment (see IV.4.) to improve the digestibility of the cell wall
T11, T12	laboratory-grown baker's yeast treated with cysteine at pH 11 and 12, respectively
UT	untreated laboratory-grown baker's yeast
<hr/> miscellanea <hr/>	
CV	coefficient of variation (%)
NA	data not available
N.D.	not detectable
H.D.	heteroscedastic data, analyzed as described in IX.2.8.
v	volume
WW	wet (=live) weight
DW	dry weight
CW	carbon weight
L	length (shell length of bivalve, body length of <i>Artemia</i>)
DGR, DGRΣ	daily growth rate (see IX.2.7.)
SAR	standard algal ration (= control diet of live microalgae)
2-ME	2-mercaptoethanol
CYS	L-cysteine hydrochloride
MET	DL-methionine

GENERAL INTRODUCTION

During the past decade aquaculture, more particularly the controlled production of fish and shellfish, has evolved from an artisanal or experimental activity into a successful bio-industry. In 1989 total aquaculture production amounted to over 11 million metric tons, representing an increase of 70% over the past 5 years (FAO, 1990). In comparison capture fisheries in the same time period increased by 14% only. For some species such as penaeid shrimp and salmon, 25% of the annual world consumption is generated through aquaculture production, *i.e.* about 500,000 metric tons of culture shrimp and 275,000 metric tons of salmon (New, 1991).

Larviculture nutrition, especially in the early larval stages, appears to be the major bottle-neck for the further industrial upscaling of the aquaculture of fish and shellfish (Sorgeloos *et al.*, 1991). The natural diet of most aquaculture fish, crustacean, and mollusc species consists of a wide diversity of phytoplankton species and/or zooplankton organisms found in great abundance in the natural environment. Feeding on natural plankton provides the best chances to meet the nutritional requirements of the developing larvae. However, relying on the collection of wild plankton as a larval food source in intensive aquaculture has proven not to be a reliable nor commercially feasible strategy. Over the past two or three decades trial and error approaches have resulted in the adoption of mainly three groups of live diets for the industrial larviculture of marine fish, crustaceans and molluscs:

- 1) different species of micro-algae (2-20 μm)
- 2) the rotifer *Brachionus plicatilis* (50-200 μm)
- 3) the brine shrimp *Artemia* (200-500 μm)

Phytoplankton comprise the base of the food chain in the marine environment. Therefore, it is not surprising that they play a central role in mariculture as a food source for all growth stages of bivalve molluscs, larval stages of some

crustacean species, and very early growth stages of some fish species (De Pauw & Persoone, 1988; Brown *et al.*, 1989). Algae are furthermore used to rear mass quantities of the other two groups of live feed, which serve in turn as food for late-larval and juvenile stages of crustaceans and fish (Fig. 1). Suitable algal species have been selected on the basis of their mass-culture potential, cell size, digestibility, and overall food value. Various culture systems have been developed to grow these food species on a large scale, ranging from extensive to highly controlled monoxenic intensive cultures. The latter are mostly preferred by aquaculturists as they allow more control over contamination and culture conditions, which are known to affect the nutritional value of the algae for the predator. However, the requirement for space, energy, nutrients and skilled labor to produce algae intensively results in production costs ranging from US \$ 160 to more than US \$ 200 per kg of dry biomass (De Pauw & Persoone, 1988). Furthermore, contamination and temporal variations in the algal food value still pose problems for any aquaculture operation depending on mass-cultures of unicellular algae.

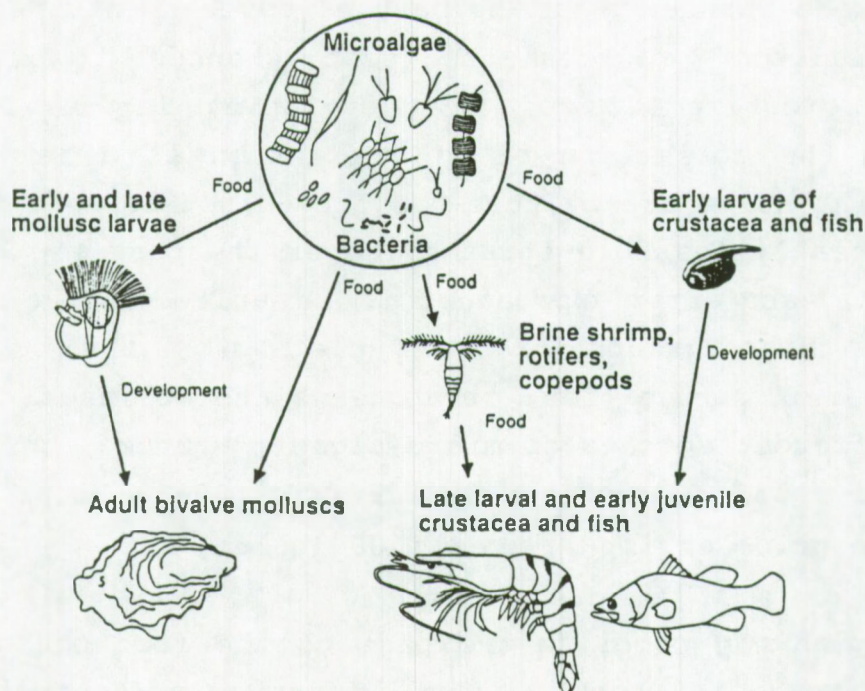


Fig. 1: The central role of micro-algae in marine aquaculture (from Brown *et al.*, 1989).

In order to overcome or reduce the problems and limitations associated with algal cultures, various investigators have attempted to replace algae by using artificial diets either as a supplement or as the main food source. Different approaches are being applied to reduce the need for on-site algal production, i.e. dried - heterotrophically grown algae (Gladue, 1991), preserved algal pastes (Donaldson, 1991), micro-encapsulated diets (Jones et al., 1984; Teshima et al., 1982), various microparticulate diets (Kanazawa et al., 1982), and yeast-based feeds (Urban & Langdon, 1984; Léger et al., 1987).

To date, the requirement for live algae in the mass-production of prey-organisms has been largely reduced. In this way, baker's yeast, marine yeasts and lipid-enriched yeast diets are now routinely used as a sole diet or in combination with the alga *Chlorella* for rearing the rotifer *B. plicatilis* (Fukusho et al., 1976; Hirata, 1980; Komis et al., 1991). The application of brine shrimp as a live food has been mainly limited to the use of the freshly-hatched nauplii, which excludes the requirement for a suitable food. Nevertheless, the use of on-grown and adult *Artemia* is attractive due to the larger prey size spectrum and higher nutritional value, and is essential for the farming of some marine species, such as lobsters (Léger et al., 1986). Although the cheapest source of brine shrimp biomass is from semi-natural biotopes and man-managed ponds, high-density culturing techniques have been developed to produce *Artemia* of a particular quality and size (Lavens, 1989). For the latter, cheap agricultural by-products are currently used as a food source instead of algae in order to cut feed costs (Sorgeloos et al., 1980; Platon & Zahradnik, 1987; Lavens, 1989). However, the variable culture success which is caused by the fluctuating composition of these waste-products, prompts a search for more reliable alternative diets (Lavens et al., 1987).

Also, considerable progress has been made in the replacement of live algae in the larval rearing of commercially important shrimp species. Partial replacement of live algae using micro-encapsulated (Jones et al., 1991) and yeast-based diets

(Naessens-Foucquaert et al., 1990) is now routine in hatcheries for penaeid prawn. By contrast, despite the extensive research efforts, reports on the use of artificial diets in the culture of bivalve molluscs are very scarce (Helm & Hancock, 1990).

The present work attempted to document the use of yeasts as a substitute for live algae in filter-feeding organisms. Because of their high protein content, suitable particle size and high stability in the water column, yeast cells have indeed interesting characteristics as a food for filter-feeders. Furthermore, as opposed to most of the other alternatives to live algae, yeasts can be mass-produced at a relatively low cost (Kihlberg, 1972). The potential of yeasts as a food in aquaculture has been proven by their successful application in the rearing of rotifers and some species of penaeid shrimp (see above). However, a limited nutritional value of yeasts was reported for various species of filter-feeders and attributed to their nutritionally deficient composition and/or undigestible cell wall (e.g. rotifers: Hirayama & Funamoto, 1983; *Artemia*: Johnson, 1980; bivalves: Epifanio, 1979a; Urban & Langdon, 1984; penaeid shrimp: Kittaka, 1969; Mock et al., 1980).

In the literature study it was attempted to give an overview of the results achieved in aquaculture when using yeasts as single-cell protein in particulate diets for filter-feeding organisms as well as in formulated feeds for fish (Chapter I). The knowledge on feeding and nutrition of the brine shrimp is outlined in Chapter II. Furthermore, an extensive review of the feeding biology in bivalve molluscs with special emphasis on the rearing of juvenile stages, is presented in Chapter III.

The poor growth and survival observed in *Artemia* fed baker's yeast inspired us to use these organisms as a test-system in the first experimental part of our study. Small scale experiments allowed to develop techniques for improving the nutritional value, more particularly the digestibility, of baker's yeast for the brine shrimp (Chapter IV). Furthermore, feeding, assimilation, and growth in *Artemia* fed yeast was studied using the traditional cell count method (Chapter V) as well as radiotracer techniques (Chapter VI). The improved yeast diet was

tested as a substitute for live algae for the controlled rearing of *Artemia* at laboratory scale (Chapter VII). In this way, the results of this study attempted to contribute to the knowledge of feeding and nutrition in the brine shrimp and to develop an artificial diet which could seriously reduce the need for live algae in the rearing of *Artemia* for research purposes. Furthermore, this knowledge eventually provides the basis for the development of a cost-effective and reliable diet for intensive rearing of the brine shrimp.

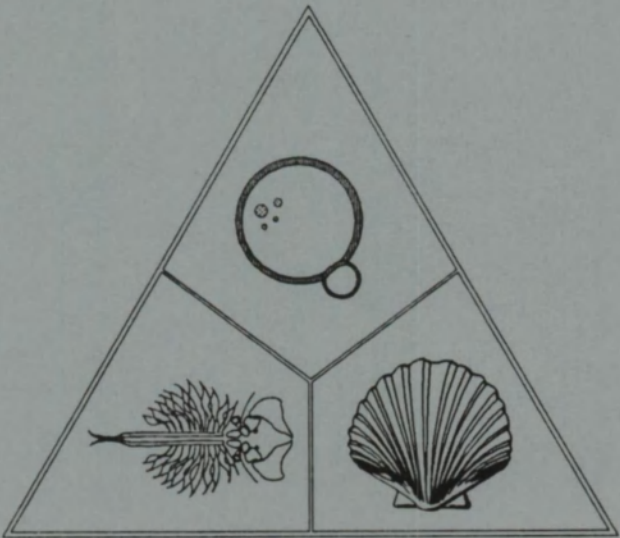
The use of yeasts as a food source for commercially important filter-feeding organisms was assessed in the second experimental part. Because of the relatively important requirement for algae and the limited success of artificial diets in the juvenile rearing of bivalve molluscs, the latter were selected as experimental animals. Growth experiments were run at various locations, using juveniles of different species of clams and oysters, to obtain a better understanding of the quantitative algal requirements of bivalve seed and to evaluate, and eventually improve, the nutritional value of yeast-based diets (Chapter IX). Finally, the extensive literature on artificial diets and our experimental findings were compared with experience "from the field" through an international inquiry among the operators of commercial and experimental bivalve hatcheries (Chapter X).

LITERATURE STUDY

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Chapter II: Feeding and nutrition in the brine shrimp *Artemia* 19

Chapter III: Feeding and nutrition in bivalve molluscs 23



Chapter I

THE USE OF YEAST AS SINGLE-CELL PROTEIN IN AQUACULTURE

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Chapter I

THE USE OF YEAST AS SINGLE-CELL PROTEIN IN AQUACULTURE

I.1. INTRODUCTION

Micro-organisms play an important role in aquaculture either as a direct or indirect source of food for the cultured organisms, as a causative agent of various diseases, as degrading agents of nitrogenous wastes in closed-cycle aquaculture, or as a source of fouling and oxygen consumption. The present paper is restricted to the first of these subjects: the role of micro-organisms, and more specifically yeasts as food for commercially important aquatic species. Single-Cell Proteins (SCP), in particular yeasts, have several advantages over plants and animals as food producers. They have short generation times, can easily be genetically modified, are produced on the basis of various raw materials and independently of the climate, and the production cost is relatively low (Kihlberg, 1972). For these reasons SCP have been considered as a potential source of proteins in animal feeding in general, and more particularly, as a substitute for fish meal in diets for intensive farming of fish. In addition, because of their single-cell characteristics and good properties in the water, yeasts have been evaluated as a substitute for unicellular algae in the culture of prey organisms, e.g. rotifers and brine shrimp, and of commercially important filter-feeding organisms, such as molluscs and penaeid shrimp larvae.

I.2. THE USE OF YEAST AS SINGLE-CELL PROTEIN IN FORMULATED DIETS FOR FISH

It has since long been recognized that in all fish diets, irrespective of the species, protein is quantitatively the most

important dietary nutrient (30 to 60 % of dry matter) and that fish meal remains the major source of dietary protein (20 to 60 % of fish diets). However, various attempts were made to reduce the high cost of this basic ingredient by the reduction of protein levels in the diets through the protein-sparing effects of other major nutrients, and by the use of alternative protein sources of good nutritional value. In this regard much research has been done on the possible use of microbial proteins in fish diets. A detailed review on the use of SCP in fish diets has been made by Tacon & Jackson (1985).

It has been shown that bacterial SCP can replace up to 40-50 % of the fish meal component in diets for *Tilapia* (Viola & Zohar, 1984; Davies & Wareham, 1988) and up to 80-100 % in feeds for rainbow trout (Kaushik & Luquet, 1980). Yeasts have been successfully incorporated into diets for rainbow trout allowing a replacement of up to 25-50% (Beck et al., 1979; Tiews et al., 1979). High inclusion levels (more than 35%) of alkane grown yeast (Beck et al., 1979) and brewer's yeast (Windell et al., 1974), however, lead to a significant reduction in growth rate.

Several authors have attributed the lower growth rates and food conversion efficiencies obtained with SCP diets to nutritional deficiencies or imbalances. The following amino acids have been found to have a beneficial effect on the growth of fish when supplemented into SCP-diets: cystine, arginine, lysine and methionine (Nose, 1974); L-lysine-HCl, DL-methionine, DL-tryptophan (Gropp et al., 1979); DL-methionine and DL-arginine (Beck et al., 1979); and L-methionine (Murray & Marchant, 1986). Furthermore, the high nucleic acid levels in SCP may have deleterious effects (Tacon & Cooke, 1980; Davies & Wareham, 1988).

On the other hand, various reports in literature indicated that the limited performance of yeast as the major protein source in fish feeds may be due to its low digestibility. Johnson et al. (1980) found that the cell wall of *Phaffia rhodozyma* is the main barrier which restricts the availability of astaxanthin and possibly other nutrients for *Salmo gairdneri*. In addition, Murray

& Marchant (1986) concluded that improved methods of cell processing would be required in order to increase the nitrogen and probably the whole cell digestibility of a mixed SCP diet, containing *Hansenula anomala*, *Candida kruzei* and *Geotrichum candidum*, for rainbow trout. When the yeast cells are first mechanically disrupted to release intracellular protein, baker's yeast can replace up to 50% of the total nitrogen in the diet of lake trout without deleterious effects on growth and feed conversion (Rumsey et al., 1990). On the contrary, Matty & Smith (1978) observed a higher growth and food conversion efficiency in rainbow trout with yeast (*Candida lipolytica*) in comparison with bacterial and algal protein. Also, effective enzymes for yeast digestion were detected in the digestive tract of several freshwater fishes and the ability of fish to digest yeast has been ascribed to the presence of chitin decomposing bacteria in the gut (Minami et al., 1972; Hibino et al., 1974).

I.3. THE USE OF YEASTS AS AN ALGAL SUBSTITUTE OR SUPPLEMENT FOR FILTER-FEEDING ORGANISMS

One of the main problems in aquaculture is the availability of the larval food. This consists often of unicellular algae, or small invertebrates e.g. rotifers or crustacean nauplii which in turn must be cultured on micro-algae. However, the mass-production of unicellular algae is labor-intensive and expensive (see III.4.1.). Furthermore, their variable nutritional quality and the risk for infections, culture crashes and blooms in rearing tanks involve important draw backs for the use of algae in larviculture operations. Because of their suitable particle size and high stability in the water column yeasts can easily be removed from suspension and ingested by filter-feeding organisms. Also, the rigid yeast cell wall prevents the leakage of nutrients into the culture medium and a subsequent deterioration of the water quality. The following section gives an overview of the most important results obtained with yeast-based algal substitutes for the prey organisms *Brachionus* (Rotifera) and *Artemia* (Anostraca), and for penaeid shrimp and molluscs.

I.3.1. Artemia

The availability of sufficient quantities of food organisms is a prerequisite for the successful larval rearing of marine fish and shrimp. This requirement is fulfilled for the branchiopod *Artemia* since its dormant embryos, so-called "cysts", can be harvested in large quantities from hypersaline lakes and solar saltworks all over the world. These cysts are available as a storable "off the shelf" live food, giving rise to free-swimming nauplii upon 24 hours of incubation in seawater. These freshly-hatched larvae can be given directly as a live source of food to the larvae of fish and shrimp. However, the use of ongrown and adult *Artemia*, produced in intensive culture systems, becomes more attractive (Lavens *et al.*, 1986; Léger *et al.*, 1986). In order to cut feed costs and to improve its applicability most of the high-density culturing of the brine shrimp rely on cheap agricultural by-products instead of live algae. However, it is understood that the use of yeast cells, because of their interesting properties, may further improve the culture success.

The use of yeasts as a substitute for micro-algae has already been described for sustaining laboratory cultures of *Artemia* for genetic and morphological studies (Bond, 1937; Weisz, 1946; Bowen, 1962; Bowen *et al.*, 1985). Yeasts have also been included as a protein source in mixed diets for the production of brine shrimp biomass, such as baker's yeast in combination with agricultural by-products (Talloon, 1978) or *Spirulina* (James & Makkeya, 1981), *Kluyveromyces* (Lavens *et al.*, 1987), brewer's yeast and methanol yeasts (Robin *et al.*, 1987). Furthermore, trials with various species of marine yeast mostly lead to poor culture results (Johnson, 1980; Nimmanit & Assawamunkong, 1985), unless the experiments were run in small scale systems with low animal densities (Shimaya *et al.*, 1967; Kawano *et al.*, 1976; Johnson, 1980). Only James *et al.* (1987a) reported high production yields with marine *Candida* yeast when culturing *Artemia* in 10 m³ batch cultures without water renewal. Most of the above studies do not allow the evaluation of the nutritional value of the yeast itself. Indeed, the latter forms only a component of a mixed diet or is subjected to microbial

decomposition and lysis, which in turn may lead to the development of algae and bacteria in the culture medium.

By contrast, few work has been done on the evaluation of pure yeast diets under highly standardized culture conditions. Preliminary trials in 5 l aquaria indicated that *Torula* yeast (*Candida utilis*) might be a promising food for cultivating *Artemia* (Blanco Rubio, 1987). Douillet (1987) obtained better growth and survival of *Artemia* fed on Fleischmann baker's yeast under xenic conditions in comparison with those kept bacteria-free. The latter author hypothesized that the development of favorable bacteria plays an essential role in the nutritional value and/or digestibility of the offered diet.

I.3.2. Rotifers

The rotifer *Brachionus plicatilis* is indispensable as an initial live food for raising larvae of many commercially important fish species (Lubzens, 1987; Kafuku & Ikenoue, 1983). As opposed to *Artemia* which is mostly fed as freshly-hatched nauplii, an adequate supply of rotifers relies on the continuous maintenance of mass cultures. Using unicellular algae (*Chlorella* sp.) as food for rotifers leads to high reproduction rates, but requires substantial place and labour, making the mass cultivation of *Brachionus* prohibitively expensive. Baker's yeast and several marine yeast species have been used successfully as a sole diet for the culture of the rotifer (Hirata & Mori, 1967; Furukawa & Hidaka, 1973; Matsuda *et al.*, 1980), although the culture success may be due to the release of nutritive elements during the decomposition of yeast, as well as to the phytoplankton and bacteria growing in the culture tanks utilizing the decomposed products as a nutritive source (Hirayama & Watanabe, 1973). When washed cells of baker's yeast were offered to rotifers cultured under axenic conditions, they did not have a nutritional effect on their population growth (Hirayama & Funamoto, 1983). The dietary value of baker's yeast could be improved by supplementing it with vitamin B 12 (Hirayama & Funamoto, 1983) and the fat-soluble vitamins A, D, and E (Satuito & Hirayama, 1986). These nutritional deficiencies have been considered as a potential cause for the instability of the mass

cultures of *Brachionus* fed on baker's yeast (Hirayama, 1987). Today, baker's yeast and marine yeasts are being used extensively in combination with *Chlorella* as food for mass production of the rotifer (Fukusho et al., 1976; Hirata, 1980; James et al., 1987b). The algal supplement improves the population growth as well as the nutritional value of the rotifer for fish larvae (James et al., 1983). Rotifers cultured exclusively with baker's yeast contain insufficient amounts of highly unsaturated fatty acids, mainly eicosapentaenoic acid (20:5 n3) and docosahexaenoic acid (22:6 n3), which are essential for marine fish larvae. Enrichment of the rotifers with these fatty acids can take place during the culture period by feeding them either baker's yeast in combination with an emulsion of cod liver oil (Watanabe et al., 1983), HUFA-supplemented yeast diets (Imada et al., 1979; Gatesoupe & Robin, 1981; Komis et al., 1989), or after the culture period by bioencapsulating them with specific enrichment products (Léger et al., 1989).

I.3.3. Penaeid shrimp

The first feeding stages of penaeid shrimp (zoea larvae) are filter-feeders that require micro-algae as a food source. Very few reports have been published with regard to the use of yeasts as a supplement or replacement for algae in the culture of penaeid shrimp. Baker's yeast has been found to be an inferior diet for larval *Penaeus japonicus* (Hudinaga & Kittaka, 1966; Kittaka, 1969). However, marine yeast (Furukawa, 1972), and baker's yeast in combination with soy cake (Hirata et al., 1975) were found suitable for larvae of *P. japonicus*. Also, Mock et al. (1980) obtained better growth with active dry baker's yeast than with the compressed caked form during feeding trials with larval *P. stylirostris*, although the control fed on frozen algae metamorphosed faster into postlarval stages. For the same species the feeding of HUFA-enriched Fleischmann yeast 7B lead to a delay of the larval development by 2 to 3 days compared to the algal control (Léger et al., 1985). These findings were possibly due to non-optimal feeding regimes and subsequent water quality problems. For *P. vannamei* the use of a new type of algal substitute on the basis of yeast resulted in significant better

postlarval survival and growth, and a higher resistance to starvation stress (Léger *et al.*, 1987). However, the same product could not be used as a total substitute in the larval culture of *P. monodon*, which still required an initial algae feeding period of two days (Léger, pers. comm. 1989). Since recent tests with an analogous, but more digestible product showed improved culture success, it is hypothesized that the "nutritional" problem is partially related to digestibility.

I.3.4. Molluscs

Bivalve molluscs are commonly fed mass-cultured unicellular algae during their hatchery and nursery phase. Production of these algae can account for 15 to 85 % of the operating costs of a hatchery (Bolton, 1982). The need for alternatives to monospecific algal cultures has encouraged a search for non-algal food materials, such as various microparticulate diets and yeasts. An extensive review of replacement diets for live algae in the culture of molluscs is presented in section III.4.

Epifanio (1979a) found that juvenile *Argopecten irradians*, *Mercenaria mercenaria*, and *Mytilus edulis* grew as fast or faster compared to algal controls when fed diets containing 50 % *Candida* yeast. By contrast, *Crassostrea virginica* was unable to utilize the yeast and the author attributed this to a digestibility problem. Diets containing more than 50 % yeast resulted in reduced growth of the first three species. Since this could not be explained by compositional differences between the diets, it might be due to a decrease in digestion efficiency when the yeast component is exceeding 50 %; the presence of the algae should therefore enhance the digestion of the yeast. In the same way it has been stated that the combination of an easily digested algal species with a relatively indigestible species results in a more effective digestion of the refractory species for at least some combinations of algal species (Epifanio, 1979b, 1983). However, when rice starch and kaolinite were added to the algae/yeast (50/50) diet, it resulted in an analogous growth of *C. virginica* than when 100 % algae were fed. This indicates that a deficiency or imbalance of nutrients in yeast may explain its poor nutritional value (Urban & Langdon, 1984). The beneficial

effect of the starch supplement suggests that the algae/yeast diets might be deficient in their caloric content. On the other hand, the addition of the silt kaolinite could have enhanced oyster growth by increasing filtration rate (Kjørboe *et al.*, 1980), by improving the delivery of soluble nutrients to the oysters via adsorption to the silt particles (Urban & Langdon, 1984), or even by increasing digestion efficiency (Murken, 1976).

The various reports in literature concerning the digestibility of yeast diets for bivalve molluscs reach conflicting conclusions. Portéres (1988) showed by means of transmission electronic microscopy that yeast cells were digested by juvenile *Tapes semidecussata* although yeast in multiplication phase was preferentially lysed. The same author found an assimilation rate of only 30 % for clams feeding on the yeast diet. By contrast, carbon and nitrogen assimilation efficiencies of *C. virginica* fed yeast were comparable with those of oysters fed on algae (Alatalo, 1980).

I.4. CONCLUSIONS

Instead of making an exhaustive review, an attempt was made to present the major results that have been obtained in aquaculture with yeast as a substitute either for fish meal in formulated fish diets or for algae in the culture of filter-feeding organisms. Nutritional deficiencies often restrict the degree of substitution by yeasts, although in some cases a complete substitution could be achieved. Furthermore, reports in the literature concerning digestibility of yeast seems to be contradictory. This indicates that the ability to digest yeast may differ according to the cultivated species, the species and strain of yeast, and the culture conditions (e.g. presence of algae in the culture medium).

Chapter II

FEEDING AND NUTRITION IN THE BRINE SHRIMP *ARTEMIA*

In view of the availability of extensive reviews on the feeding and nutrition of zooplankton in general (Omori & Ikeda, 1984; Peters, 1984) and *Artemia* in particular (Coutteau, 1987; Lavens, 1989), the present chapter has been limited to a short review of the knowledge of feeding in the brine shrimp.

Artemia starts to ingest food from the instar II stage onwards by using its larval antennae and mandibles as filtering organs (Barlow & Sleigh, 1980). During the post-embryonic development the filter-feeding function of the antennae and mandibles is gradually taken over by the thoracopods, which are multifunctional phyllopods used for locomotion, osmoregulation, respiration, and feeding (Schrehardt, 1987b). In juvenile and adult brine shrimp, the metachronial movement of the thoracopods causes a water flow by which nutrients are sucked into the mid-ventral food groove. Food particles are retained, concentrated, and transported through the food-groove to the mouth by the setulae on the protopodites and endites of the thoracic appendages (Barlow & Sleigh, 1980; Schrehardt, 1987ab). The development of the filter-feeding apparatus and the alimentary canal in *Artemia* has been well documented by scanning and transmission electron-microscopic studies (Schrehardt, 1987ab; Criel, 1991ab).

The brine shrimp is generally considered as a continuous, non-selective, obligate phagotrophic filter-feeder (Reeve, 1963b; Provasoli & Shiraishi, 1959; D'Agostino, 1980). The maximal size of particles which can be ingested was found to be 25-30 μm for nauplii and 40-50 μm for adult brine shrimp (Dobbeleir et al., 1980).

Earlier researchers demonstrated that feeding rate varies

as a function of food concentration according to a rectilinear type of functional response curve (Sushchenya, 1962; Reeve, 1963a; Yanase & Shiraishi, 1972), which is considered as typical for filter-feeding crustaceans (Frost, 1974). The maximal ingestion rate, measured under food-saturating conditions, was found to be determined by the physical limits of the feeding process, with a constant total volume of algal cells ingested irrespective of the algal species (Reeve, 1963a; Yanase & Shiraishi, 1972; Sick, 1976). However, more recent work showed that feeding rate in *Artemia* may also be influenced by the quality of the food (Samain *et al.*, 1981). The latter authors observed similar ingestion rates in terms of protein intake rather than volume in *Artemia* which were fed either of two batches of the same algal species differing in protein content.

The relation between digestive enzyme activity and food concentration has been studied extensively in *Artemia* by Samain and co-workers. Based on experimental work with the brine shrimp, Samain & Moal (1982) proposed a model which related digestive enzyme activity with food ingestion and the nutritional requirements of marine zooplankton. The latter authors thus offered an explanation for the apparently conflicting reports in literature of positive, negative or non-significant correlations between digestive enzyme activity and food concentration. Substrate-specific induction of the digestive enzymes in response to the composition of the diet is believed to result in a balancing of the assimilation of carbohydrates and proteins according to the requirements of the brine shrimp (Samain *et al.*, 1981). A mechanism for this digestive enzyme regulation was proposed by Samain *et al.* (1985), who hypothesized that the haemolymph levels of sugars and amino-acids trigger the induction of, respectively, carbohydrase and protease activity. In this way, a depletion of the haemolymph concentration due to an increase of the animal's requirements or a decrease of the input from the feeding process, would be compensated by an increase of the enzyme activity.

The nutritional requirements of *Artemia* have been investigated under axenic conditions by Provasoli, D'Agostino,

and co-workers (reviewed by D'Agostino, 1980). Rearing *Artemia* in monoxenic cultures allowed to identify algal species that could function as the primary source of nutrients in bacteria-free media (Provasoli et al., 1959). Furthermore, the absolute nutritional requirements for carbohydrates, proteins, cholesterol, lipids and various vitamins were determined by growing brine shrimp aseptically in a biphasic artificial medium (Provasoli & D'Agostino, 1969; Provasoli & Pintner, 1980).

Micro-organisms play an important role in the nutrition of brine shrimp in xenic cultures. The nutritional value of live algae (Gibor, 1956) as well as various dried foods, including yeast, rice bran, and *Spirulina* (Douillet, 1987), was found to be lower in bacteria-free than in xenic cultures. Provasoli et al. (1959) showed that bacteria and other algae can successfully supplement species of algae that alone cannot support the growth of brine shrimp. Furthermore, the success of intensive cultures fed micronized agricultural by-products may largely depend on the establishment of a suitable microflora, which supplements the nutritional deficiencies in the food (D'Agostino, 1980).

Chapter III

FEEDING AND NUTRITION IN BIVALVE MOLLUSCS

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Chapter III

FEEDING AND NUTRITION IN BIVALVE MOLLUSCS

III.1. INTRODUCTORY NOTE ON BIVALVE TAXONOMY

Most malacologists (*e.g.* Abbott, 1974) separate the bivalves into six subclasses based primarily upon the type of gill structure, the structure of the teeth that make up part of the hinge connecting the two valves, the life habit, and the number and relative size of the adductor muscles. The majority of the commercially important bivalves belong to two subclasses, the Pteriomorpha and the Heterodonta (Table 1).

The species designations of many bivalve species have been changed over the years. The Manila clam, *Tapes philippinarum* (Adams & Reeve, 1850), has been described in the literature as *Tapes japonica* (Deshayes, 1853), *Tapes semidecussata* (Reeve, 1859), and *Venerupis japonica* (Deshayes, 1853).

Table 1: Taxonomic position of some commercially important bivalve genera (taxonomic system according to Barnes, 1980).

Phylum Mollusca
Class Bivalvia
Subclass Pteriomorpha
Order Mytiloida
Family Mytilidae (mussels)
<i>Mytilus, Modiolus</i>
Family Pteriidae (winged oysters)
<i>Pinctada, Pteria</i>
Family Pectinidae (scallops)
<i>Pecten, Chlamys, Argopecten</i>
Family Ostreidae (oysters)
<i>Crassostrea, Ostrea</i>
Subclass Heterodonta
Order Veneroida
Family Cardiidae (cockles)
<i>Cardium</i>
Family Tridacnidae (giant clams)
<i>Tridacna</i>
Family Mactridae
<i>Spisula</i>
Family Veneridae (Venus clams)
<i>Mercenaria, Tapes</i>
Order Myoidea
Family Myacidae
<i>Mya</i>
Family Hiatellidae
<i>Panope</i>

III.2. FEEDING AND GROWTH OF LAMELLIBRANCHIATE BIVALVES WITH SPECIAL REFERENCE TO SELECTING OPTIMAL FOOD LEVELS AND THE DEVELOPMENT OF ARTIFICIAL DIETS FOR THE CULTURE OF SEED

III.2.1. Introduction

For more than 60 years (e.g. Savage, 1925) researchers have tried to unravel the feeding mechanisms in bivalve molluscs. The intent of this chapter is not to present an exhaustive review of the numerous publications on filtration biology, food ingestion, assimilation and growth of bivalves, but to emphasize some aspects which have direct relevance to their culture, with particular reference to the selection of optimal food levels and the development of artificial diets. We will focus primarily on the feeding biology of postset through adult stages of the bivalves' life cycle (see Bayne, 1983; Pechenik, 1987; Cragg & Crisp, 1991; for reviews on larval feeding and growth).

For comprehensive reviews on suspension-feeding in bivalves we refer to Winter (1978; focus on mussels), Reid (1983), Bayne & Newell (1983), Malouf & Bricelj (1989; focus on clams), and Bricelj & Shumway (1991; focus on scallops).

III.2.2. Filter-feeding mechanism

Water currents and ciliary transport

The evolution of filter-feeding in bivalve molluscs was coupled with the modification of the gills into filtration organs equipped with cilia for creating water currents, and for collecting and transporting food particles. For many years taxonomy of bivalves was based on the structure and function of the gills, resulting in the obsolete subclasses Protobranchia, Lamellibranchia, and Septibranchia. All commercially important bivalves belong to the Lamellibranchia and have highly developed respiratory and suspension-feeding organs composed of filaments that are folded and attached to adjacent filaments by cilia (filibranch gill, e.g. scallops and mussels) or by tissue connections (eulamellibranch gill, e.g. oysters and clams).

The lamellibranch gills (Fig. 2) bear cilia to create a water current (lateral cilia) and to collect (laterofrontal cilia) food particles, which are trapped in mucous secretions. The frontal cilia transport the retained particles to the marginal grooves which are ciliary tracts on the gills that carry the food strings anteriorly via the labial palps to the mouth (Reid, 1983).

The actual mechanisms by which the cilia accomplish water transport and filtration are a subject of continued controversy. One mechanism ascribes retention of particles to a filtering mesh provided by the latero-frontal cirri, which beat in synchrony and remove particles from the water (Moore, 1971). Alternatively, according to the model proposed by Jørgensen (1981) based on hydromechanical principles, particles are concentrated by steep velocity gradients occurring at the boundary zone between surface and through currents in the gills.

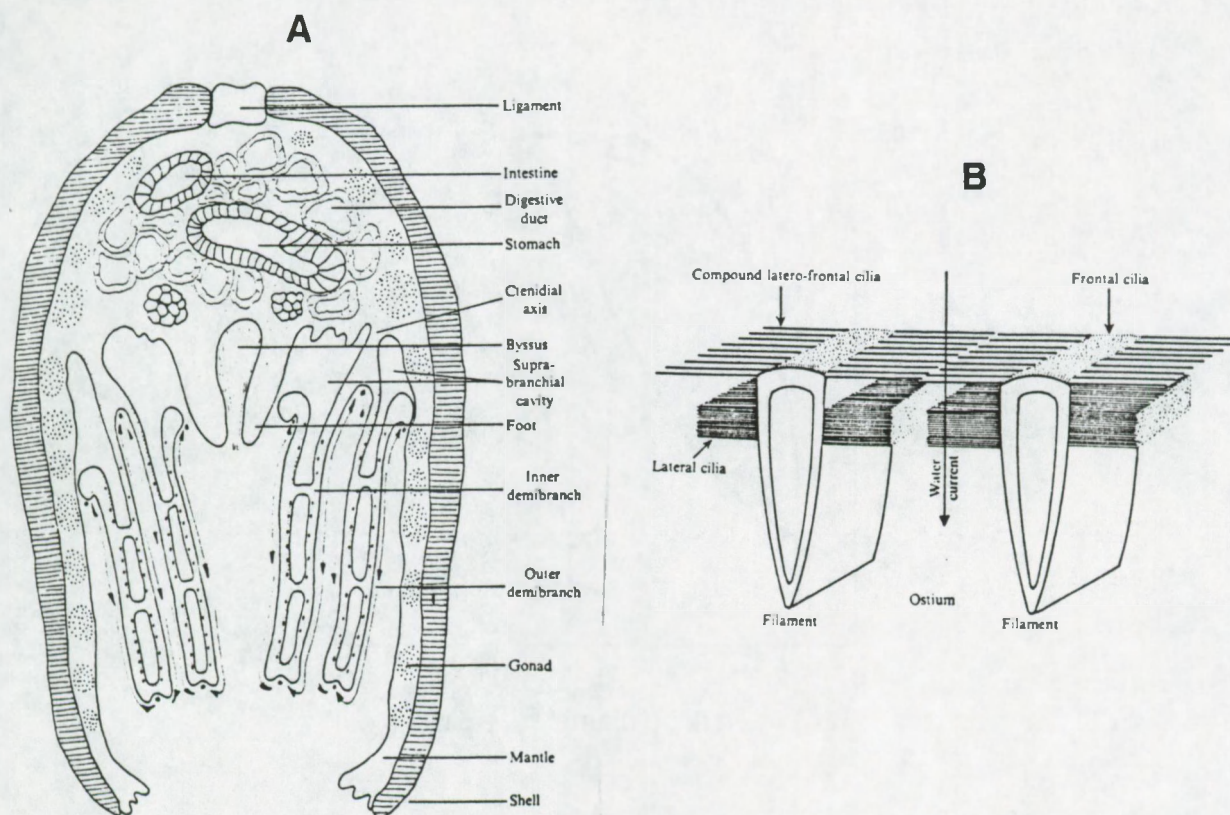


Fig. 2: A schematic representation of the lamellibranchiate gill showing the directions of ciliary tracts on a transverse section of the gill (A) and the position of the cilia on the filaments (B) (from Bayne *et al.*, 1976).

Retention efficiency and size limits of particles

The rate at which food is filtered from suspension is determined by the rate of water transport through the gills (pumping rate) as well as the efficiency with which particles are retained by the gills. Most post-settlement stages of eulamellibranch bivalves with well developed laterofrontal cirri (e.g. mussels, clams) retain particles above 3-4 μm with 100 % retention efficiency (Møhlenberg & Riisgård, 1978; Riisgård, 1988; Sprung & Rose, 1988). In contrast, the critical size for efficient particle retention is ca. 7 μm in bivalves with simple laterofrontal cilia, such as scallops (Fig. 3). Retention efficiency drops with decreasing particle size to 35-90 % for 2 μm particles, depending on the species (Møhlenberg & Riisgård, 1978).

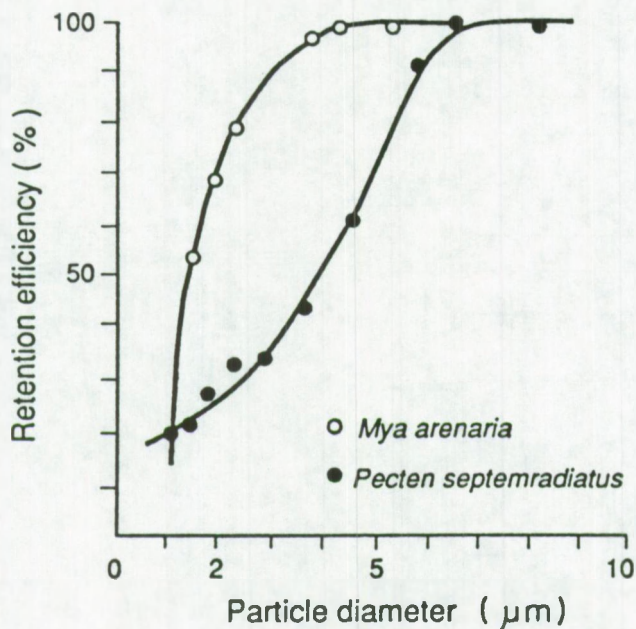


Fig. 3: Retention efficiency (%) as a function of particle size in a typical pectinid (*Pecten septemradiatus*) and a bivalve with well-developed laterofrontal cilia (e.g. *Mya arenaria*) (from Møhlenberg & Riisgård, 1978).

The ability of bivalves to filter particles smaller than 2 μm is less well documented, aside from the various reports on the retention of bacteria (see III.3.3.). Jørgensen (1975) found a retention efficiency for 1- μm sized latex beads amounting to only 10% of that for 6 μm particles in *M. edulis*. Wright *et al.* (1982) demonstrated in field and lab experiments that of *Mytilus edulis*, *Mya arenaria*, and *Geukensia demissa*, only the latter species was capable of efficient clearance of marine bacterioplankton (87% of the particles $\leq 1 \mu\text{m}$). This was attributed to a closer spacing and more lateral overlapping of the latero-frontal cirri in the gills of *G. demissa* compared to other species.

Reid (1983) concluded on the basis of gastric contents that there is a considerable interspecific variation in the upper size limits of ingested particles from 10 μm for *Macoma calcaria* to 300-400 μm for *Macoma secta* and *Tridacna gigas*. Scallops are capable of consuming relatively large particles as demonstrated by the gut contents of *Placopecten magellanicus* which ranged from 10 to 350 μm (Shumway *et al.*, 1987). However, the removal of corn flour particles in the size range of 2 to 10 μm by the Sydney rock oyster was most efficient for particles less than 5 μm in diameter (Wisely & Reid, 1978). Feeding various pure clay suspensions to *Crassostrea gigas* showed that only kaolinite (modal size 3-4 μm) was efficiently retained, while illite (62% of the particles $< 2.5 \mu\text{m}$) and montmorillonite (63% $> 5 \mu\text{m}$) were, respectively, not retained or rejected (Sornin *et al.*, 1988). Gabbott *et al.* (1976) showed by means of microcapsules that the median diameter of the ingested particles increased with shell length of *Mytilus edulis* (shift from 16 to 50 μm for mussels of a length of 0.8 to 4.5 cm). Microcapsules greater than 100 μm were largely rejected by all mussel size classes.

Bivalve larvae capture food particles using the preoral cirri of the velum and are efficient in retaining particles that are smaller than the threshold size of juveniles and adults. Walne (1965), using ^{32}P -labelled algae, demonstrated that cells in the size range 3-10 μm diameter were equally well ingested by the larvae of *O. edulis*. Also, Gallager *et al.* (1988) showed that

larvae of *M. mercenaria* can ingest particles between 0.5 and 8 μm with equal efficiency. Conversely, Riisgård *et al.* (1980) showed that 13-day old veligers of *M. edulis* retained particles of 1 to 2 μm diameter with only 20% of the efficiency with which particles of 2.5 to 3.5 μm were removed.

Particle selection and pseudofaeces production

Above a threshold particle concentration bivalves are able to regulate ingestion by rejecting the excess of filtered particles at the labial palps and eliminating them as pseudofaeces through the inhalant siphon (see also III.2.3.). Selective feeding is then possible by discriminating between particle types and selectively rejecting particles as pseudofaeces.

Earlier authors believed that bivalves are able to preferentially ingest nutritious food particles and to reject large and dense mineral particles (reviewed by Jørgensen, 1966). Subsequently, this was challenged by several researchers (reviewed by Winter, 1978), such as Foster-Smith (1975b) who showed that three bivalve species were not capable of selective ingestion of *P. tricornutum* from a mixture of this alga with alumina particles. Conversely, more recent work has again demonstrated that bivalves can discriminately ingest algae from mixed algal-sediment suspensions (Kiørboe & Møhlenberg, 1981; Newel & Jordan, 1983) as well as from mixed algal cells of similar size (Ten Winkel & Davids, 1982; Shumway *et al.*, 1985) by selective rejection of particles as pseudofaeces. The efficiency of selection was found to vary according to the species and the origin of the bivalves, and could be correlated with the length of the labial palps (Kiørboe & Møhlenberg, 1981). It should be emphasized that this "pre-ingestive" selection is possible only when pseudofaeces are produced. In this way, species that regulate their ingestion rate at higher food concentrations by copious pseudofaeces production (*e.g.* mussels, oysters, surf clams; see III.2.3.), show a higher selection efficiency than species that primarily reduce their filtration

rate.

So far, the mechanism for pre-ingestive particle selection is not well understood. Moreover, the evidence for the latter initiated a controversy on the widely accepted role of mucus in the feeding mechanism of bivalves, *i.e.* embedding particles retained by the gills for transport as food-loaded mucous strings to the mouth and oesophagus, where the strings are pulled into the stomach by the windlass action of the crystalline style (Barnes, 1980). It is indeed hard to envisage any mechanism for selection of particles bound in mucous strings. Kiørboe & Møhlenberg (1981) observed that only particles rejected as pseudofaeces were embedded in mucus, while particles in the oesophagus were freely suspended. However, the latter was again contradicted by the observations of Beninger & Benhalima (1990). Newell & Jordan (1983) hypothesized that changes in the viscosity of the mucus on the labial palps would allow the temporary formation of individual particles which can be subjected to testing by chemoreceptors, and selected for ingestion or re-incorporation in the mucus prior to rejection as pseudofaeces.

Elucidation of the basis for selection could be of major importance in determining the optimal characteristics (*e.g.* size, density, surface) of particles used in artificial diets, such as yeast and microcapsules (Malouf & Bricelj, 1989). Very little is known about the selection criteria of suspension-feeding bivalves, although they appear to select rather on the biochemical composition than on the specific gravity and the size of particles (see review by Newell & Jordan, 1983).

III.2.3. Clearance rate and ingestion rate in relation to food concentration

Definitions and formulas

In discussing the feeding activity of bivalves, the terms clearance rate (= filtration rate) and pumping rate (= ventilation rate) are often confused. Clearance rate (CR) is defined as the volume of water filtered completely free of

particles per unit time, while pumping rate is the volume of water flowing through the gills per unit time. Clearance rate and pumping rate only have the same value if all particles are completely (100%) retained by the gills. The pumping rate can be determined directly by isolating and measuring the exhalant flow (e.g. Hildreth, 1976), while the clearance rate is calculated from the removal of suspended particles from a known volume of water per unit of time (e.g. Winter, 1973). A general equation for calculating clearance rate, assuming a constant pumping rate and a retention of the particles with 100% efficiency, is given by Coughlan (1969):

$$CR = \frac{V}{n t} \left[\ln \frac{C_0}{C_t} - \ln \frac{C'_0}{C'_t} \right]$$

where CR= clearance rate per animal; V= volume of suspension; n = number of animals; C_0 , C'_0 = initial, and C_t , C'_t = final (after time t) concentrations of, respectively, experimental and control (without animals) vessel.

At concentrations below the threshold for pseudofaeces production the ingestion rate (IR) can be calculated directly from the clearance rate, if the concentration of suspended particles (C) is known, by the equation:

$$IR = CR \times C$$

In the case pseudofaeces are produced, the latter product equals the rate at which particles are filtered out of suspension before rejection at the labial palps and can be called "intake rate" (Foster-Smith, 1975a). The actual ingestion rate is then given by:

$$IR = (CR \times C) - P$$

where P= rate of pseudofaeces production

Concepts of the relationships between clearance rate, ingestion rate and food concentration

Bivalves are able to restrict the amount of food ingested when exposed to increasing suspended particle loads by (a) reducing the clearance rate and/or (b) increasing the proportion of material rejected in pseudofaeces (Foster-Smith, 1975a). Clearance rate is varied by regulating the pumping rate as well as the efficiency with which the particles are retained at the gills (Winter, 1978).

From the literature it seems that, depending on the species, either of these mechanisms is dominating. Foster-Smith (1975a) found that the European clams *Cerastoderma edule* and *Venerupis pullastra* primarily regulated ingestion in response to higher particle concentrations through reducing their clearance rates, while the principal regulatory mechanism in *Mytilus edulis* was increasing pseudofaeces production (Fig. 4). Furthermore, strong inverse relationships between clearance rate and particle concentration, indicating that ingestion is mainly adjusted by mechanism (a), were demonstrated for *Mercenaria mercenaria* (Bricelj & Malouf, 1984) and various pectinids (reviewed by Bricelj & Shumway, 1991). In contrast with the above mentioned venerid clams, surf clams (*Spisula subtruncata*) showed a clearance rate which was independent of the concentration of *Phaeodactylum tricornutum* within the range tested (6.9 - 31 cells μl^{-1} ; Møhlenberg & Kiørboe, 1981).

The type of food particle may also direct the response of the bivalve in either one of the two mechanisms. Epifanio & Ewart (1977) observed a rapid decrease of filtration rate with concentration and, as a result, a nearly constant removal rate of *Thalassiosira pseudonana* and *Carteria chuii*. In contrast, oysters did not reduce their clearance rate when fed gravimetrically equivalent suspensions of *Isochrysis galbana*, and thus produced large amounts of pseudofaeces. Furthermore, Griffiths (1980) could even demonstrate a higher filtration rate in the mussel *Choromytilus meridionalis* on algal cultures of *Dunaliella primolecta* in the exponential growth phase than on

cultures that had been in stationary phase for several days.

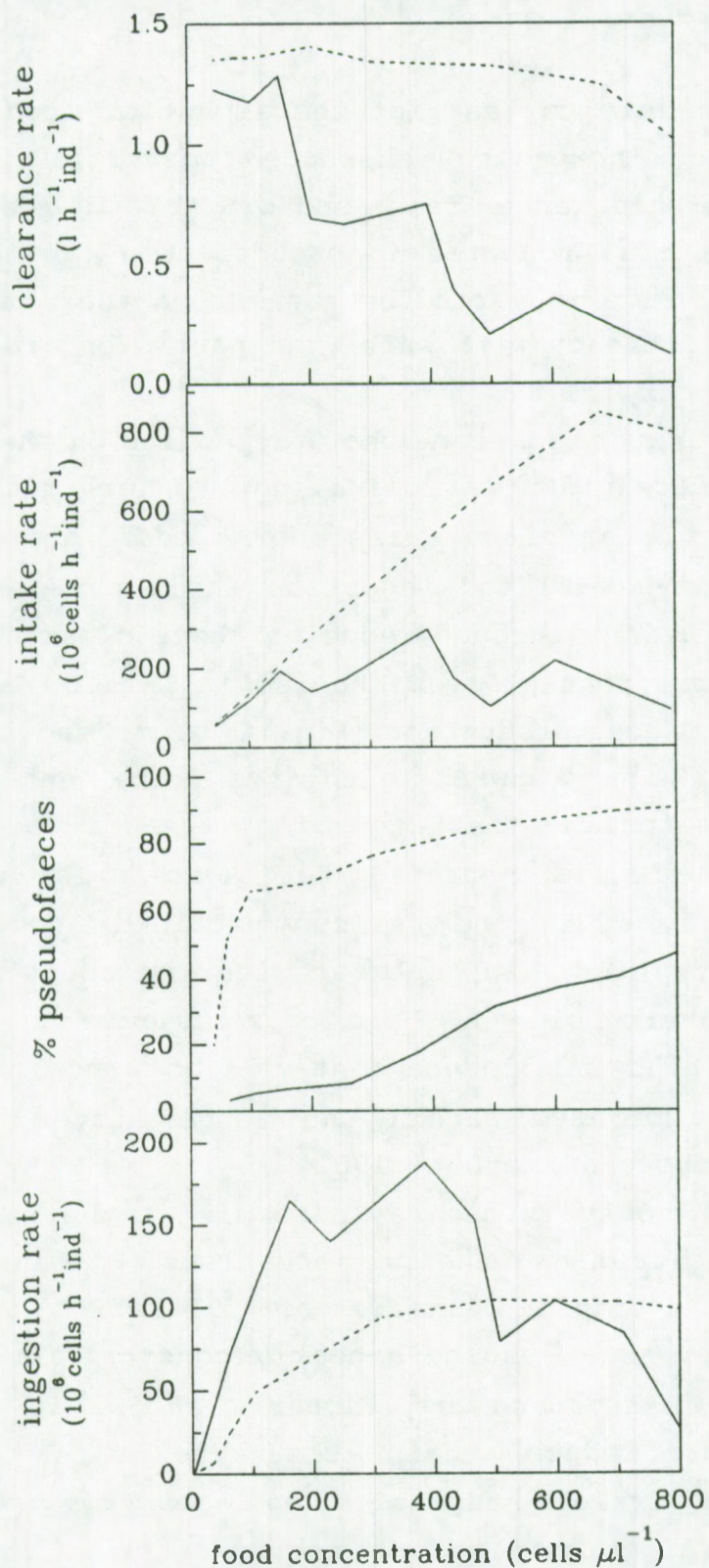


Fig. 4: Clearance rate, intake rate, pseudofaeces production, and ingestion rate in relation to concentration of *Phaeodactylum tricornutum* in *Venerupis pullastra* (—) and *Mytilus edulis* (----) (modified from Foster-Smith, 1975a).

The concept of Foster-Smith (1975a) implicates that bivalves can maintain a constant ingestion rate between a lower ("satiation point") and an upper threshold concentration, which do not necessarily coincide with the concentrations at which the reduction of clearance rate and, respectively, pseudofaeces production is initiated (e.g. *M. edulis*, Fig. 4). In this respect, the model differs from that proposed by Winter (1978; Fig. 5). At extremely high food concentrations, the combined action of mechanisms (a) and (b) may even result in a decrease of the ingestion rate (e.g. *V. pullastra* feeding at concentrations above 500 *P. tricornutum* cells μl^{-1} , Fig. 4). Conversely, several authors observed a further increase of ingestion rate at higher food concentrations (Thompson & Bayne, 1974, Winter & Langdon, 1976; Langton *et al.*, 1977; Goldstein & Roels, 1980; Griffiths, 1980). In some cases this may be due to evaluating insufficiently high particle loads (e.g. Winter & Langdon, 1976) or neglecting the production of pseudofaeces.

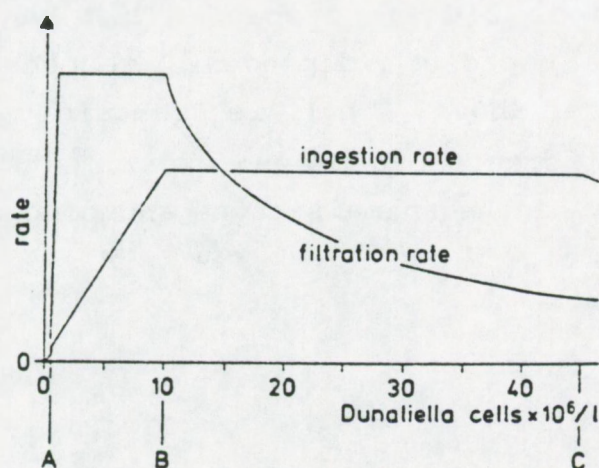


Fig. 5: Relationship between particle concentration, clearance rate and ingestion rate for a lamellibranchiate bivalve (modified from Winter, 1978). Thresholds are minimum concentrations for initiating filtration ("A"), reducing filtration ("B"), and pseudofaeces production ("C").

A third mechanism for controlling ingestion at higher food concentrations, *i.e.* reducing the time spent pumping by a discontinuous feeding activity, is less well documented. Several authors have demonstrated variations in clearance rate during the course of feeding experiments (Winter, 1973; Schulte, 1975; Epifanio & Ewart, 1977; Griffiths, 1980; Sprung & Rose, 1988), though it is difficult to relate the duration of the rest periods to the food concentration. Epifanio & Ewart (1977) found that the clearance rate of *C. gigas* is more constant at the low algal concentrations ($< 3 \mu\text{g DW/ml}$). *M. edulis* showed interruptions in filtration for up to one minute at concentrations of 50 to 100 *Platymonas suecica* cells μl^{-1} (Schulte, 1975).

The existence of a minimal food level required to initiate filtration (level "A", Fig. 5) was evidenced by Riisgård & Randløv (1981), who observed shell closure and reduced filtration rates in *M. edulis* at concentrations below 1500 *P. tricornutum* cells/ml. Exceeding this threshold for feeding implicates the switching from a low "quiescent" metabolic rate to a higher rate, necessary for ventilation (Thompson & Bayne, 1974).

From Table 2 it is clear that a considerable variation is found in the literature with regard to the effect of cell concentration on rates of filtration, ingestion and pseudofaeces production of various bivalve species. This can be partially explained by the species dependent response to higher concentrations (see above), but is certainly also due to methodological differences between experiments (*e.g.* size, origin, and history of the animals; type and concentration range of the food particle).

Table 2: Threshold particle concentrations for clearance rate, ingestion rate and pseudofaeces production in various bivalve species.

bivalve species (+initial size) [§]	diet [‡]	food concentration range tested [†]		minimum concentration ($\mu\text{g DW ml}^{-1}$) for			source
		10^3 cells ml^{-1}	$\mu\text{g DW ml}^{-1}$ (*)	reducing clearance rate	initiating pseudofaeces production	maximizing ingestion rate	
<i>Mytilus edulis</i> (DMW=100-2000 mg)	Tetra	0.5 - 25	0.03 - 1.65	CR constant	> 1.65	IR increasing	Thompson & Bayne (1974)
<i>Mytilus edulis</i> (L=50 mm)	Phaeo Isog	Phaeo and Isog:	Phaeo: 0.17 - 14.5*	11.9 - 15.3 CR constant	0.2 - 0.3 0.4 - 0.6	2.6 NA	Foster-Smith (1975a) (selected data)
<i>Venerupis pullastra</i> (L=40 mm)	Phaeo Isog			2.6 - 3.4 9.4 - 11.8	0.9 - 1.3 1.2 - 1.8	8.5 - 11.9 NA	
<i>Cerastoderma edule</i> (L=40 mm)	Phaeo Isog		Isog: 0.24 - 20.0*	1.7 - 3.4 4.7 - 7.1	0.7 - 0.9 1.8 - 2.4	3.44 NA	"
<i>Mytilus edulis</i> (L=40 mm)	Tetra	0.3 - 150	0.07 - 33*	CR decreasing	9.5	9.5	Schulte (1975)
<i>Mytilus edulis</i> (L=20 mm)	Dunam	10 - 40	0.53 - 2.13	NA	> 2.13	IR increasing	Winter & Langton (1976)
<i>Mytilus edulis</i> (DMW=35 mg, L=19 mm)	Dunam	10 - 60	0.53 - 3.21	CR decreasing	2.13 \leq \leq 3.21	1.07 - 2.14	Winter (1976)
<i>Crassostrea virginica</i> (WW=15 g)	Cart	29 - 206	3.1 - 22.0	CR decreasing	\geq 10: copious production for all algae	NA	Epifanio & Ewart (1977)
	Croo	12 - 200	1.12 - 18.6	CR decreasing			
	Isog	95 - 738	2.23 - 17.3	CR max. at 4.7			
	Thal	100 - 800	1.32 - 10.6	CR decreasing			
<i>Mytilus edulis</i> (L=17-70 mm)	seston	NA	10 - 320	CR decreasing	2.6 - 5.0	NA	Widdows <i>et al.</i> (1979)
<i>Choromytilus meridionalis</i> (L=45 mm)	Dunap	0.2 - 50	0.02 - 5.60	> 0.04: CR constant	5.6	IR increasing	Griffiths (1980)
<i>Mytilus edulis</i> (L=16-20 mm)	Phaeo	0.5 - 105	0.01 - 1.79*	0.51	NA	NA	Riisgård & Randløv (1981)
<i>Mytilus chilensis</i> (L=13-76 mm)	Dunam	15 - 40	0.80 - 2.14	CR decreasing	> 2.14	IR increasing	Navarro & Winter (1982)

§: L= shell length, DMW= unit dry meat weight, WW=unit live weight

‡: see Table of abbreviations (xv) for explanation of abbreviated species names

†: cell concentrations represent continuous loads

*: when algal dry weight (DW) data were not provided by the author, an approximate value was calculated using the following conversion factors ($\mu\text{g DW cell}^{-1}$): Tetra = 220 (Walne & Spencer, 1974); Isog = 23.5 (Epifanio & Ewart, 1977); Phaeo = 17 (Riisgård & Randløv, 1981)

III.2.4. Absorption efficiency, growth efficiency, and growth rate in relation to food concentration and ration

III.2.4.1. Definitions and formulas

Physiological energetics

Physiological energetics is concerned with the study of energy balance within individuals not only in terms of the acquisition and expenditure of energy, but also the efficiency with which it is converted from one form to another. The basic energy budget equation of Winberg (1956) gives the relationship between the primary source of input, *i.e.* food ingestion (I), and energy expenditures, including faecal losses (F), excretory products (U), respiratory loss (R) and energy invested in production (P):

$$I = P + R + F + U$$

Several parameters relevant to the energy balance can be calculated from the equation such as absorbed ration ($Ab = I - F$), absorption efficiency ($AbE = Ab/I$), assimilated ration ($A = Ab - U = R + P$) and assimilation efficiency ($AE = A/I$). The energy available for production after respiration and excretion have been subtracted from absorption is referred to as scope for growth [$= P = Ab - (R + U)$]. The proportion of the ingested ration that is invested in production is referred to as total gross growth efficiency ($K_1 = P/I$), while the proportion of the absorbed ration converted to production is termed total net growth efficiency ($K_2 = P/Ab$).

Besides the great diversity in methods described in the literature to determine growth efficiencies of bivalves (based on calories, organic dry weight, organic nitrogen, organic carbon,...), there is a general lack of uniformity in definitions. Production in bivalves is the sum of reproductive output, somatic tissue growth and shell production. Most of the K_1 and K_2 values recorded in the literature are based on somatic production rather than on total production ("gross and net somatic growth efficiency"). Furthermore, some authors (*e.g.* Laing & Millican, 1986) defined net growth efficiency as the

proportion of the assimilated ration turned into production (P/A). When pseudofaeces are produced, the term F equals the losses as biodeposits (= pseudofaeces + faeces) (e.g. Deslous-Paoli *et al.*, 1990) and I is then the food quantity removed from the water rather than the food ingested. For recent reviews on bioenergetics in bivalve molluscs we refer to Bayne & Newell (1983), Griffiths & Griffiths (1987), and Thompson & MacDonald, (1991).

Estimators of growth

Growth may be defined as a change, either positive or negative, in the size of an individual organism or in the mean size of a population (Malouf & Bricelj, 1989). Various measures are used to express the size of an individual bivalve: linear measures such as shell length (maximum anterior-posterior dimension, parallel to the hinge) and shell height (maximum dorsal-ventral dimension, perpendicular to the hinge), volume measures, or weight measures such as whole live weight or weight of soft tissues (wet, dry, or organic weight). For a comparison of advantages and disadvantages of these estimators of growth we refer to Malouf & Bricelj (1989).

Regardless of how size is estimated, growth can be expressed either as an absolute or as a relative change in size. Although absolute growth rate is mostly applied in culture systems (e.g. mm per month), it does not allow comparisons between animals of different sizes. In this regard, various formulas have been proposed to calculate dimensionless relative growth rates.

When growth is exponential, an instantaneous growth rate (k) can be calculated as (e.g. Urban *et al.*, 1983):

$$k = \frac{1}{t_2 - t_1} \ln\left(\frac{W_2}{W_1}\right)$$

where W_1 and W_2 are, respectively, the initial and the final weight (or other measure of size); $t_2 - t_1$ is the elapsed time (mostly in days). The k coefficient can be multiplied by 100 to express growth as percent per day.

When the growth increment $W_2 - W_1$ is small and the time interval $t_2 - t_1$ is short, k can be approximated by the average relative growth rate (ARGR), calculated as (Malouf & Bricelj, 1989):

$$ARGR = \frac{W_2 - W_1}{(W_2 - W_1) \times 0.5 \times (t_2 - t_1)}$$

Finally, growth can be computed directly as a daily growth rate (DGR), expressed as percent increase per day (Gutierrez, 1990):

$$DGR = \left(\sqrt[t_2 - t_1]{\frac{W_2}{W_1}} - 1 \right) \times 100$$

The comparison of the three formulas to determine the relative growth rate in a hypothetical test case (Fig. 6) demonstrates that DGR is slightly overestimating k at higher growth increments, but that the use of ARGR is only valid at very low growth rates.

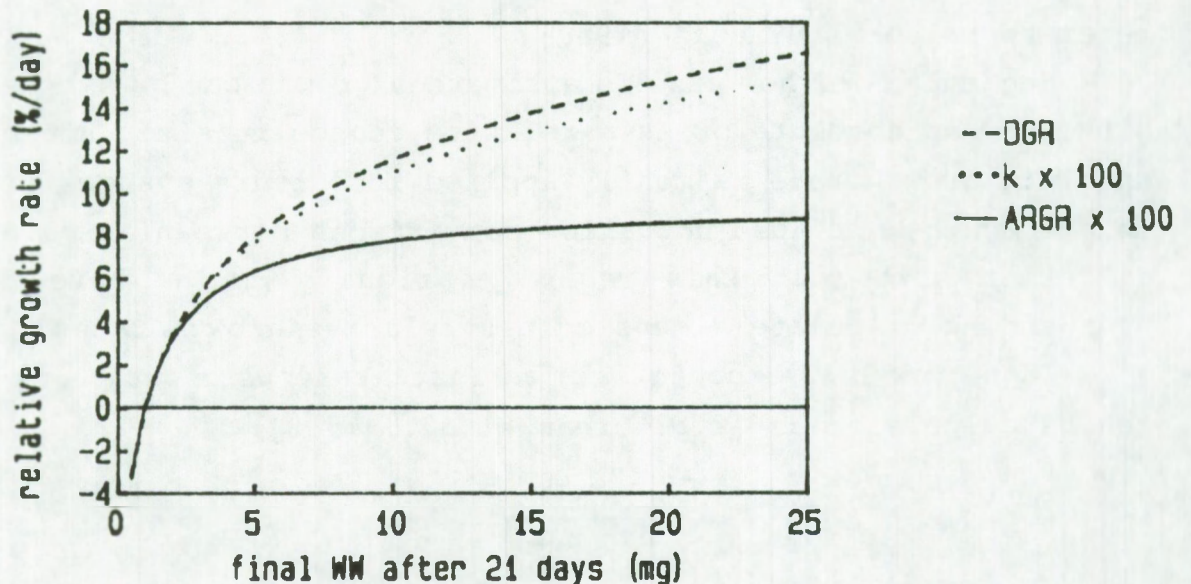


Fig. 6: Calculation of relative growth rate using various formulas in a hypothetical test case (initial weight = 1 mg, final weight after 21 days = 0.5 to 25 mg).

III.2.4.2. Absorption efficiency

The efficiency with which the ingested ration is utilized is referred to as absorption (AbE) or assimilation efficiency (AE), depending on whether the loss term excretion (U) is included, respectively, excluded. Since excretion is only a minor component in the energy budget, e.g. less than 3% of the energy consumed by mytilids (Navarro & Winter, 1982, Deslous-Paoli *et al.*, 1990), values of AbE and AE are practically identical.

For bivalves it is widely accepted that the absorption efficiency is not dependent on body size (Winter, 1978; Thompson & Bayne, 1974; Navarro & Winter, 1982), but mainly determined by the quality and the quantity of the food ingested. A decrease in AE with increasing food concentration has been demonstrated for various species (*Mytilus edulis*: Thompson & Bayne, 1972, 1974; *Aulacomya ater*: Griffiths & King, 1979; *Choromytilus meridionalis*: Griffiths, 1980; *Mytilus chilensis*: Navarro & Winter, 1982; *Argopecten irradians*: Kuenstner, 1988). Also, an inverse relationship between the amount of organic matter in the seawater and AE could be recorded during a study of the energy budget in situ for a cultured population of *M. edulis* on bouchots (Deslous-Paoli *et al.*, 1990). A considerable variation is found between the literature data when expressing the concentration ranges over which AE decreases in gravimetrically equivalent units (Fig. 7).

Since the assimilation efficiency is determined by the amount of food ingested rather than by the food concentration, the decrease in AE is expected to be coupled to the increase of the ingestion rate with rising food concentrations, which in turn depends on the bivalve species (see III.2.3.). Few data document how AE varies within and beyond the range of food concentrations where ingestion is maintained constant, thus relating the assimilation efficiency to the thresholds for filtration, ingestion, and pseudofaeces production. Indeed, most assimilation studies are performed at particle loads below the threshold for pseudofaeces production in order to estimate ingestion rate directly from cell clearance. In this regard, it is difficult to

explain that *M. edulis* (Thompson & Bayne, 1974) and *C. meridionalis* (Griffiths, 1980) initiated pseudofaeces production at a concentration well above that at which AE fell to zero.

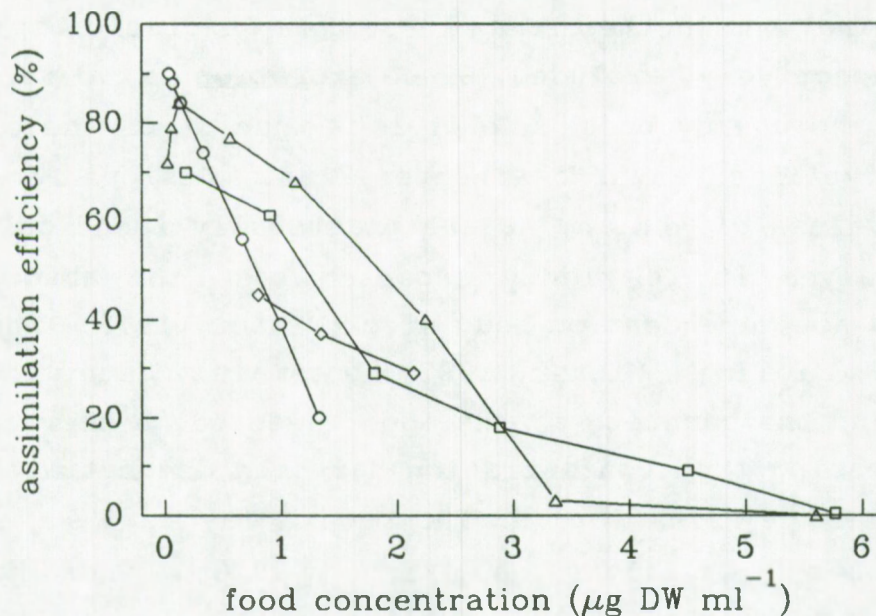


Fig. 7: Effect of algal concentration on assimilation efficiency in various mytilids. Data modified from Thompson & Bayne, (1974; o, *M. edulis* fed *Tetraselmis suecica*), Griffiths & King (1979; □, *Aulacomya ater* fed *Dunaliella primolecta*), Griffiths (1980; Δ, *Choromytilus meridionalis* fed *Dunaliella primolecta*), Navarro & Winter (1982; ◊, *Mytilus chilensis* fed *Dunaliella marina*).

The differences revealed by Fig. 7 can also be partially due to the use of different algal species, which are likely to differ in digestibility for the various bivalves. In this way, Peirson (1983) showed by using ¹⁴C labelling techniques that *A. irradians* absorbed various algal species at a concentration of 2 mm³/l with a high efficiency (e.g. *Thalassiosira pseudonana*: AbE = 89.9%), except *Chlorella autotrophica* (AbE = 17.4%). Bricelj et al. (1984a) demonstrated by means of the ⁵¹Cr:¹⁴C twin tracer technique that gut passage time of *Pseudoisochrysis paradoxa*, a chlorophyte which was absorbed with high efficiency (AbE = 82%), was significantly longer than that of algae which were inefficiently utilized (e.g. *Nannochloris atomus*, *Stichococcus* sp.) by *M. mercenaria*. The latter authors postulated that the

hard clam is capable of sorting various algal species in their passage through the gut and egesting poorly digestible algae more rapidly. Also bivalve veligers are unable to digest certain algal species as revealed by epifluorescence microscopy (*Dunaliella primolecta*, *Platymonas suecica* for *Pecten maximus*: Le Pennec & Rangel-Davalos, 1985; *Chlorella autotrophica* for *C. virginica*: Babinchak & Ukeles, 1979).

Finally, absorption efficiency is influenced by the physiological condition of the animal and, as a result, by the experimental circumstances. Thus, in *C. meridionalis* starvation for 3 weeks shifted the curve for AE as a function of algal concentration to the left, so that AE declined to zero at 20 *D. primolecta* cells μl^{-1} instead of 30 cells μl^{-1} (Griffiths, 1980).

III.2.4.3. Growth

Successful rearing of bivalve molluscs depends upon the delivery of an adequate food ration. Although the qualitative aspect of the food requirements (see III.3.) has been treated in numerous publications, the relationship between ration size and growth has not been adequately studied for many bivalves. An optimal feeding regime requires knowledge of the range of food concentrations that can be allowed to occur in the system as well as the ration that supports rapid growth. The food concentration, through its effect on the mechanisms for feeding and digestion, will determine the ration ingested per animal and the efficiency with which the latter will be utilized. The limits for the food concentration will in turn force restrictions upon the culture technology (e.g. feeding frequency in batch culture, flow rate in flow through systems, animal density) for feeding a suitable ration.

Growth in relation to food concentration

The combined effect of saturation of food ingestion (see III.2.3.) and reduced absorption efficiency (see III.2.4.) at high algal densities is that growth rates of bivalves are often

maximized at intermediate cell concentrations (Table 4A). Furthermore, this is corroborated by determinations of the scope for growth from energy balances of various mytilid species (Table 4B). In most studies, maximum growth rates and scope for growth were obtained at algal concentrations ranging from 1 to 3 μg dry weight ml^{-1} .

Walne (1970) evaluated the food value of 25 algal species at various cell concentrations for the juvenile culture of six bivalves belonging to the genera *Ostrea*, *Crassostrea*, *Mercenaria*, and *Mytilus*. He concluded that the cell density at which greatest growth was obtained was related to the median cell volume of the algae and approximated 5,000 μm^3 algal material μl^{-1} (or less when the cell volume was smaller than 10 μm^3), being equivalent to for example 90 *I. galbana* cells μl^{-1} . A manual for the hatchery rearing of oysters recommended algal concentrations of 60 to 100 *I. galbana* cells μl^{-1} (Wilson, 1981).

In practice, concentrations of mixed algal cultures are expressed as "Isochrysis equivalents", giving one cell of each species the value of a certain number of cells of *Isochrysis galbana* (Tahitian strain) based on its cell volume and empirical nutritional value (Table 3). In flow-through systems for seed culture of clams and oysters, culturists recommend maintaining the algal concentration in the feeding current between 50 and 150 "Isochrysis equivalents" μl^{-1} (Bayes, Dravers, pers. comm., 1990).

Table 3: Empirical index of food value for various algal species utilized in the seed culture of clams and oysters (Bayes, Dravers, pers. comm., 1990).

Species	Index of food value (Isochrysis equivalents)
<i>Chaetoceros calcitrans</i>	0.3
<i>Isochrysis galbana</i> (T-Iso)	1
<i>Isochrysis galbana</i>	1
<i>Monochrysis lutheri</i>	1
<i>Chaetoceros gracilis</i>	3
<i>Skeletonema costatum</i>	3
<i>Tetraselmis suecica</i>	10

Table 4: Growth rate (A) and scope for growth (B) in relation to food concentration for various bivalve species

bivalve species (+initial size) [§]	diet [†]	concentration [†] range examined (10 ³ cells ml ⁻¹)	food concentration [†] for maximum growth		temperature (°C)	source
			10 ³ cells ml ⁻¹	µg DW ml ⁻¹ (*)		
A: EMPIRICAL GROWTH STUDIES						
<i>Mercenaria mercenaria</i> (L=0.6 mm)	Tetra Isog	2.5 - 50 5 - 200	15 - 20 75 - 100	3.3 - 4.4* 1.76 - 2.35*	21 - 23	Walne (1970)
<i>Mercenaria campechiensis</i> (WW=0.1 g; L=7.6 mm)	T-Iso	10 - 500 (inflow) (flow-through)	50 - 100 (inflow) 25 - 60 (outflow)	1.18 - 2.35* (inflow) 0.60 - 1.41* (outflow)	23 - 28	Goldstein & Roels (1980)
<i>Mytilus edulis</i> (DMW=22 mg; L=20 mm)	Dunam	10 - 40	40	2.13	12	Winter & Langton (1976)
<i>Ostrea edulis</i> (L=0.5-0.6 mm)	Isog Tetra Skel	5 - 150 1 - 30 5 - 100	25 - 50 10 - 30 50 - 75	0.59 - 1.18* 2.2 - 6.6* 2.61 - 3.92*	21 - 23	Walne (1970)
<i>Hinnites multirugosus</i> (L=1 mm)	Isog	42 - 330 cells µl ⁻¹ (2days) ⁻¹	168 cells µl ⁻¹ (2days) ⁻¹	3.95 µg ml ⁻¹ (2days) ⁻¹ *	18	Cary <i>et al.</i> (1981)
<i>Placopecten magellanicus</i> (H=2 mm)	Isog (2 strains) + Chaem (1:1:1 cell number)	12 - 87 cells µl ⁻¹ day ⁻¹	45 cells µl ⁻¹ day ⁻¹		10	Hollett & Dabinett (1989)
B: ENERGY BUDGET STUDIES						
<i>Aulacomya ater</i> (DMW=540 mg, L=50 mm)	Dunap	0 - 32	16	2.88	12.5	Griffiths & King (1979)
<i>Choromytilus meridionalis</i> (DMW=300 mg, L=45 mm)	Dunap	0.4 - 50.0	20	2.24	12	Griffiths (1980)
<i>Mytilus chilensis</i> (range: DMW=20-3000 mg, L=13-76 mm)	Dunam	15 - 40	15	0.80	12	Navarro & Winter (1982)
<i>Mytilus edulis</i> (range: DMW=100-2000 mg)	Tetra	0.5 - 20	15	0.99	15	Thompson & Bayne (1974)
<i>Mytilus edulis</i> (L=16-20 mm)	Phaeo	0 - 26	26	0.44	15	Riisgård & Randløv (1981)

§: L= shell length, H= shell height, DMW= unit dry meat weight, WW=unit live weight

‡: see Table of abbreviations (xv) for explanation of abbreviated species names

†: cell concentrations represent continuous loads, unless tabulated value is followed by a unit of measure

*: when algal dry weight (DW) data were not provided by the author, an approximate value was calculated using the following conversion factors (pg DW cell⁻¹):

Tetra = 220 (Walne & Spencer, 1974); T-Iso/Isog = 23.5 (Epifanio & Ewart, 1977); Skel = 52.2 (Brown, 1991)

Growth in relation to ration size

The effect of the food concentration on growth is in fact mainly a reflection of the influence of the amount of food ingested. Earlier work examining the effect of various culture conditions on the growth of *O. edulis* juveniles revealed that the amount of food available per spat was far more important than any other factor explored (Walne & Spencer, 1974).

Various methods are used in the literature to express ration of bivalves. In order to maintain the link of ration with the concentration at which the food is presented, some authors refer to the cell density which is obtained daily by batch feeding a constant amount of food, independently of the size of the seed (e.g. Langton & McKay, 1976; Hollett & Dabinett, 1989). However, the latter method complicates comparisons between experiments which differ in density and size of the seed, and the algal species fed. For this reason, food rations are often expressed as daily weight-specific rations, such as number of cells (Pruder et al., 1976) or dry weight (Urban et al., 1983) of algae per live weight of bivalves. It should be emphasized that the exactness of a daily ration expressed in units of dry weight strongly depends on the accuracy of the dry weight analysis of the phytoplankton used. Feeding the animals a constant amount of food per individual results in a decrease of the weight-specific ration as the animal grows. In this way, Walne & Spencer (1974) obtained maximum growth of *O. edulis* by feeding a ration of 10^7 *Tetraselmis* cells per spat per 21-day culture period. Although the latter authors attempted to compensate for growth by feeding 20%, 30%, and 50% of the total ration during the first, second, and third week respectively, the weight-specific ration decreased from 35% to 2% of oyster live weight. Similarly, if a constant weight-specific ration is desired throughout the experiment, rations that are based on the initial seed biomass should be frequently adjusted in proportion to growth. Such adjustments are especially important in growth experiments with juvenile bivalves in which weight-specific growth rates are high, resulting in substantial decreases in weight-specific rations over short

periods of time (Urban *et al.*, 1983).

In the late seventies, researchers at the University of Delaware tried to quantify the amount of algae required to culture the American oyster *Crassostrea virginica* from egg to marketable size under optimal conditions (Pruder *et al.*, 1976; 1977; Epifanio & Ewart, 1977; Epifanio, 1979a). For this, they assumed that maximal growth would be obtained when the animals were fed the maximum ration, *i.e.* as much as they could filter from the water when excess food was available. The maximum ration is directly proportional to the filtration rate at a given food concentration and, in turn, the filtration rate (F) is related to body size (W) by the allometric equation $F = aW^b$ (with b generally between 0.62 and 0.82; see reviews by Winter, 1978; Malouf & Bricelj, 1989; Bricelj & Shumway, 1991). A weight-normalized ration Y could then be described by the equation

$$Y = R/W = a W^{b-1}$$

where Y is the daily algal ration (expressed as cell numbers or dry weight) per gram of live oyster weight, R and a are daily rations for an oyster weighing, respectively, W and 1 gram, and $b-1$ is a constant. Similar allometric equations relating maximum ration to body size have been derived by Winter (1978) for mytilids. Based on maximal clearance of different algal species by *C. virginica* of varying size and origin, various values for a and $b-1$ have been proposed (Table 5). Figure 8 shows that the rations predicted by these equations, which are based on measurements of maximally three different oyster sizes within the range of 0.5 to 100 g, differ considerably within the same range.

So far, growth of oysters on rations derived from these equations has not yet been verified experimentally. Furthermore, the predictive value of these equations for oysters weighing less than 1 g is very doubtful, since they were derived from experiments using mainly larger animals. Urban *et al.* (1983) concluded that the ration for maximum growth of the American oyster in the size range of 11 to 64 mg was probably greater than that predicted by the equations of Pruder, Epifanio, and co-workers (Fig. 8). On the other hand, the predicted rations reach unlikely high values for oysters smaller than 10 mg live weight.

Table 5: Reported equations for calculating the maximum ration of *C. virginica*.

equation	units of Y (g oyster WW) ⁻¹ day ⁻¹	basis	source
$Y = 5.3 W^{-0.41}$	<i>Thalassiosira pseudonana</i> x 10 ⁸	combination of data for lab-grown juveniles (0.5 g; 26-28 °C) and oysters from the wild (10-233 g, 100g, 20-22 °C)	Pruder et al. (1976)
$Y = 8.2 W^{-0.21}$	<i>Thalassiosira pseudonana</i> / <i>Isochrysis galbana</i> (50/50 by cell numbers) x 10 ⁸	lab-grown oysters (30 g, 18-20 °C; 3 g, 20-22 °C; 0.5 g, 26-28 °C)	Pruder et al. (1977)
$Y = 0.01 W^{-0.41}$	g dry weight algae	lab-reared oysters (15 g, 20 °C) fed <i>T. pseudonana</i> and <i>Carteria chuii</i>	Epifanio & Ewart (1977)
$Y = 0.01 W^{-0.33}$	g dry weight algae	exponent in equation of Epifanio & Ewart (1977) adjusted on theoretical basis	Epifanio (1979a)

.....Pruder et al. (1977)
----Epifanio & Ewart (1977)
——Epifanio (1979a)

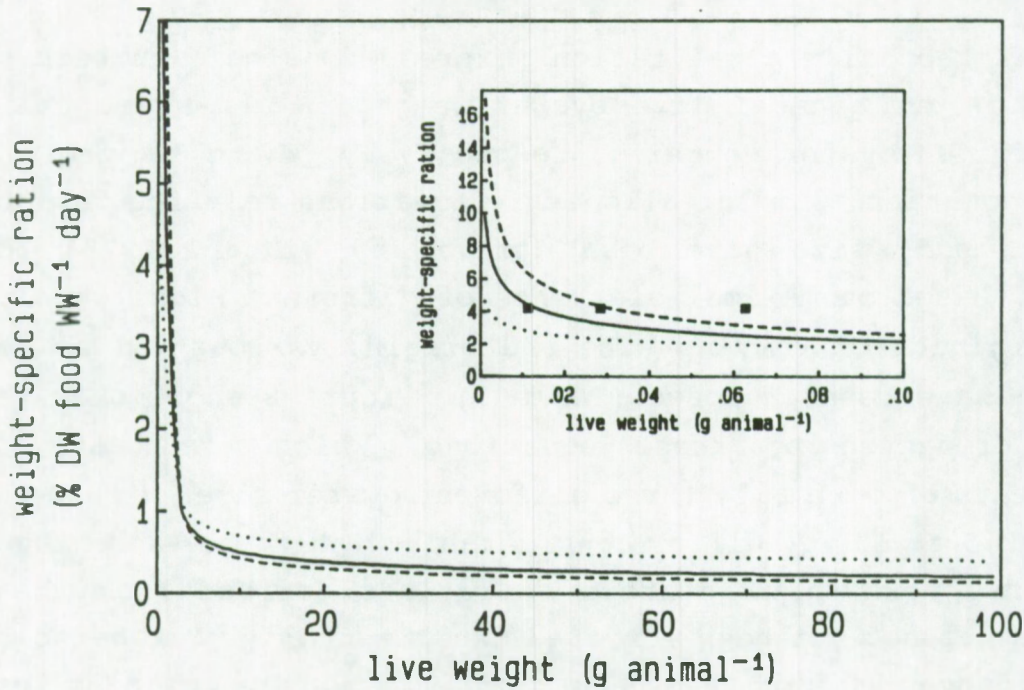


Fig. 8: A comparison of the weight-specific daily rations derived from the equations for determining the maximum ration of *C. virginica* (Table 5).

The ration predicted by the formula of Pruder et al. (1977) was converted to dry weight by assuming 1.32 10⁻¹¹ g/*T. pseudonana* cell (Epifanio & Ewart, 1977). The squares on the magnified part of the graph represent the empirical values of Urban et al. (1983; initial daily ration for maximum growth versus initial live weight for each week of the 3-week experiment).

Because optimal rations for maximum bivalve growth will vary according to the culture conditions, they must be determined through empirical growth studies which integrate culture conditions with the physiological, as well as nutritional, requirements of the bivalves for maximum growth (Urban *et al.*, 1983). In this regard very few data are available from the literature and, moreover, information required to calculate weight-specific rations is often lacking. The data published by Urban and co-workers were recalculated to show the relationship between growth rate and initial daily ration for *C. virginica* (11 to 28 mg initial live weight; Fig. 9). Maximum growth was obtained at the highest initial daily ration of 4.6% which was, in spite of the weekly adjustments of ration to growth, equivalent to an effective ration of only 2.8% (Urban *et al.*, 1983). Similar data for *M. mercenaria* juveniles (25 mg live weight) are available for food rations lower than 1% (Urban & Pruder, in press).

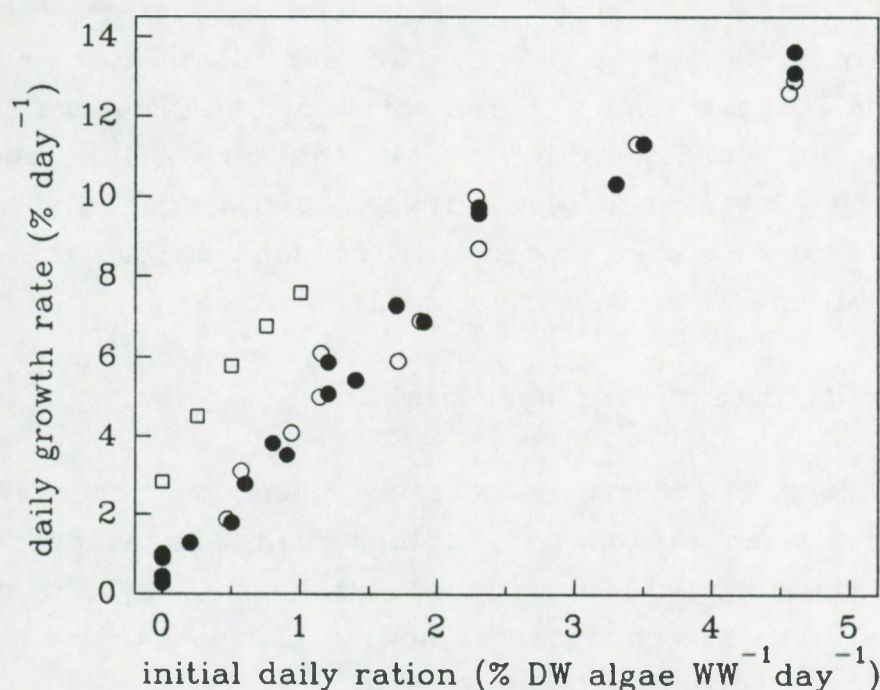


Fig. 9: Daily growth rate (III.2.4.1.) in relation to initial algal ration for *C. virginica* (●●) and *M. mercenaria* (□) fed a mixture (50/50 on dry weight) of *T. pseudonana*, clone 3H, and *I. galbana*, clone T-IS0. Data modified from Urban *et al.* (1983: ●), Urban & Langdon (1984: ○), and Urban & Pruder (in press: □).

Furthermore, the optimal ration appears to depend upon the species and culture conditions of the algae making up the diet. Enright et al. (1986a) evaluated the nutritional value of 16 phytoplankton species fed individually to *O. edulis* (5-25 mg initial live weight) at rations ranging from 0.1 to 6.0% of the oyster live weight and found that optimal algal rations for growth differed according to the algal species. In the same way, Epifanio & Ewart (1977) demonstrated that maximum daily rations removed from suspension by *C. virginica* (15 g live weight) varied from 0.4% for *T. pseudonana* and *Carteria chuii* to 1.5% for *I. galbana*. Growth of *O. edulis* juveniles fed nutrient limited cultures of *C. gracilis* was saturated at a feeding ration of $\pm 2.5\%$ DW WW⁻¹ day⁻¹, whereas that of oysters fed algae grown in a complete medium showed a maximum between 2.5 and 4.9% (Enright et al. (1986b)).

Food rations used in practice are often exceeding those reported to be optimal in the literature. Helm (1990a) recommended a daily food supply equivalent to 57 g dry weight of algae per kg live weight of oyster and clam spat held in recirculation systems. In a Spanish hatchery, the routine feeding strategy aims at a daily ration of 4% algal dry weight per live weight of seed (Gutierrez, pers. comm., 1990). Possibly, higher feed rations are required in order to compensate for losses due to settling of the food in larger scale systems.

Growth under different feeding regimes

Most research efforts have been spent on revealing the optimal food concentrations or rations that are necessary for obtaining maximum growth in bivalves. However, very few studies are concerned with the question of how to present a fixed amount of food in order to obtain best growth.

Winter & Langton (1976) demonstrated that the growth rates of *M. edulis* that were fed at three constant food concentrations were about twice as high as those of mussels ingesting the same food rations per day under discontinuous feeding regimes (fed the same daily ration at 1-h or 4-h intervals). As a result, the

latter authors postulated that growth in artificial aquaculture systems can be maximized by maintaining a constant relatively high food concentration (between level B and C, see Fig. 5). This would reduce the energy expended in filtration, avoid the formation of pseudofaeces, and exclude rest periods in feeding and digestion due to food depletion. Conversely, Langton & McKay (1976) revealed that discontinuous feeding (once daily, 6 h on/6 h off, or 3 h on/3 h off) gave better growth in spat of *C. gigas* than continuous feeding of the same amount of food. The latter authors attributed this to a higher food consumption and feeding efficiency when the oysters are exposed to peak concentrations than when feeding at constant, though lower food levels, and a possible energy saving during the rest periods when food concentration was below the threshold to stimulate feeding.

These apparently contradictory findings may be explained by the methodological differences between the experiments. Winter & Langton (1976) compared the growth of mussels that ingested similar rations under different feeding regimes¹, whereas Langton & McKay (1976) tried to relate growth to the way of presenting the same amount of food. In the latter case, the spreading of the food ration over a continuous regime will probably have reduced the ingestion rates due to the lower food concentrations, in turn causing the lower growth rate. However, it remains inexplicable why growth of *M. edulis*, ingesting the same ration at lower concentrations, was two times lower on the discontinuous feeding regime than on the continuous regime. It is unlikely that this is due to a reduction of energetic costs for the process of filter-feeding, since the latter counts for only a small fraction of the standard metabolism (3.0 to 7.6% in *M. chilensis*, Navarro & Winter, 1982).

Besides the effect on the feeding physiology, the feeding regime plays an important role in the availability of the food, especially when the particles tend to clump and settle out of suspension. The limited food availability in cultures of *C.*

¹e.g. a food ingestion of $\pm 2 \text{ mg mussel}^{-1} \text{ day}^{-1}$ was obtained by maintaining a constant food level of 2.13 mg/l or, for the discontinuous regimes, by feeding daily either 24 times 0.36 mg/l, or 6 times 1.66 mg/l.

virginica batch-fed microgel particles thus resulted in significantly lower growth compared to continuously fed oysters (Langdon & Siegfried, 1984).

III.2.4.4. Growth efficiency

The gross growth efficiency (K_1), equivalent to the percent increase in biomass per unit of ingested ration, provides a measure of the most efficient and cost-effective use of food in aquaculture systems. Gross growth efficiencies reported in the literature vary between and within species, depending on animal size and on the quantity and quality of food ingested as well as on the different methods used for measuring K_1 . Estimates based on organic nitrogen usually result in higher values of K_1 than those based on calories, dry weight or organic carbon (Malouf & Bricelj, 1989). On the other hand, estimating growth on the isolated meat may result in a considerable underestimation of gross growth efficiencies, due to excluding the increase of the organic content of the shell (Urban *et al.*, 1983). The literature summary in Table 6 shows that in most studies K_1 is maximized at the lowest or moderate food concentrations or ingested rations examined. Maximal gross growth efficiencies, ranging from 30 to 60%, are mostly observed at food concentrations between 0.5 and 1.0 μg dry weight ml^{-1} , and decrease with increasing body size (Thompson & Bayne, 1974; Navarro & Winter, 1982).

Table 6: Gross growth efficiency in relation to food concentration and ration for various species of bivalves

bivalve species (+initial size [§])	diet	range of food concentrations (10 ³ cells ml ⁻¹) [‡] or rations [†] examined	basis for calculating K ₁	K ₁ (%) min - max value	food concentration or ration [†] for maximum K ₁		source
					10 ³ cells ml ⁻¹	µg DW ml ⁻¹ (*)	
MUSSELS							
<i>Choromytilus meridionalis</i> (DMW=300 mg, L=45 mm)	Dunap	0.2 - 50.0	calories	-238 - 55	5 - 10	0.56 - 1.12	Griffiths (1980)
<i>Mytilus chilensis</i> (DMW=20 mg, L=13 mm)	Dunam	15/25/40	calories	38 - 58	15	0.8	Navarro & Winter (1982)
<i>Mytilus edulis</i> (DMW=258 mg, L=32 mm)	mixed diatoms	535 µg C l ⁻¹ (average)	organic carbon	10.0	NA	NA	Tenore <i>et al.</i> (1973)
<i>Mytilus edulis</i> (DMW=100-2000 mg)	Tetra	0.5 - 20	calories	-484 - 53	5 - 15	0.33 - 0.99	Thompson & Bayne (1974)
<i>Mytilus edulis</i> (DMW=200-300 mg)	Dunam and/or Isog	0.78 - 3.00 mg DW mussel ⁻¹ day ⁻¹	dry weight	-2.8 - 28.0	NA	3 mg DW mussel ⁻¹ day ⁻¹	Winter & Langton (1976)
<i>Mytilus edulis</i> (L=16-20 mm)	Phaeo	0/1.6/3/26	calories	15 - 36	3	0.051	Riisgård & Randløv (1981)
CLAMS							
<i>Mercenaria campechiensis</i> (WW=0.1g; L=7.6 mm)	T-Iso	10 - 500	organic nitrogen	21.5 - 36.1	50/27 (in/outflow)	1.2/0.6* (in/outflow)	Goldstein & Roels (1980)
<i>Mercenaria mercenaria</i> (DMW=737 mg; L=4.3 mm)	mixed diatoms	575 µg C l ⁻¹ (average)	organic carbon	23.7	NA	NA	Tenore <i>et al.</i> (1973)
<i>Tapes japonica</i> ** (DMW=33 mg; L=14 mm)	Chaecu + Bell	3 food levels	protein-N	36.2 - 48.4	lowest food level	NA	Langton <i>et al.</i> (1977)
OYSTERS							
<i>Ostrea edulis</i> (WW=±0.2 mg; L=1 mm)	Tetra	0.5 - 2.4 mg spat ⁻¹ (21 days) ⁻¹	dry weight	10 - 20	NA	0.5 mg spat ⁻¹ 21 days ⁻¹	Walne & Spencer (1974)
<i>Crassostrea virginica</i> (DMW=425 mg, L=4.8 mm)	mixed diatoms	595 µg C l ⁻¹ (average)	organic carbon	18.4	NA	NA	Tenore <i>et al.</i> (1973)
<i>Crassostrea virginica</i> (DMW=63 mg)	Thal/Isog/Tetra (single or 1:1 cell volume)	1 ration tested (initial conc: 10 ⁷ µm ³ ml ⁻¹)	organic carbon	neg. - 36	NA	NA	Romberger & Epifanio (1981)
<i>Crassostrea virginica</i> (WW=11.3 mg)	Thal + T-Iso (1:1 DW)	0.2 - 2.8% DW WW ⁻¹ day ⁻¹	DOW oyster/DW algae	-37.7 - 22.6	NA	1.4 - 2.3% DW WW ⁻¹ day ⁻¹	Urban <i>et al.</i> (1983)
SCALLOPS							
<i>Placopecten magellanicus</i> (H=2 mm)	Isog + Chaem (2:1 cell number)	12 - 87 cells µl ⁻¹ day ⁻¹	calories	5 - 25	12 - 45 cells µl ⁻¹ day ⁻¹	NA	Hollett & Dabinett (1989)

§: L= shell length, H= shell height, DMW= unit dry meat weight, WW=unit live weight

‡: see Table of abbreviations (xv) for explanation of abbreviated species names

†: unit of measure is tabulated for every record

‡: unless other unit of measure is specified

*: approximate value, based on conversion factor: T-Iso = 23.5 pg DW cell⁻¹ (Epifanio & Ewart, 1977)**: = *Tapes philippinarum*

A model describing the general relationship between growth, growth efficiency and ingested ration was proposed by Thompson & Bayne (1974; Fig. 10). When estimated over a wide range of ration levels, K_I adopts a negative value if the animals are fed less than the maintenance ration, increases dramatically with increasing ration until a maximum is attained at the optimum ration, and then decreases with further increase in ration. However, growth rate is maximized at an even larger ingested ration which, although less efficiently utilized, provides a maximum of energy available for production. The model is supported by empirical relationships between K_I and food ration for *M. edulis* (Thompson & Bayne, 1974), *Mercenaria campechiensis* (Goldstein & Roels, 1980) and *C. virginica* (Urban *et al.*, 1983). The maintenance ration is equivalent to the amount of food necessary to maintain a constant weight and, when expressed as a percentage of body weight, decreases with body size (Winter & Langton, 1976).

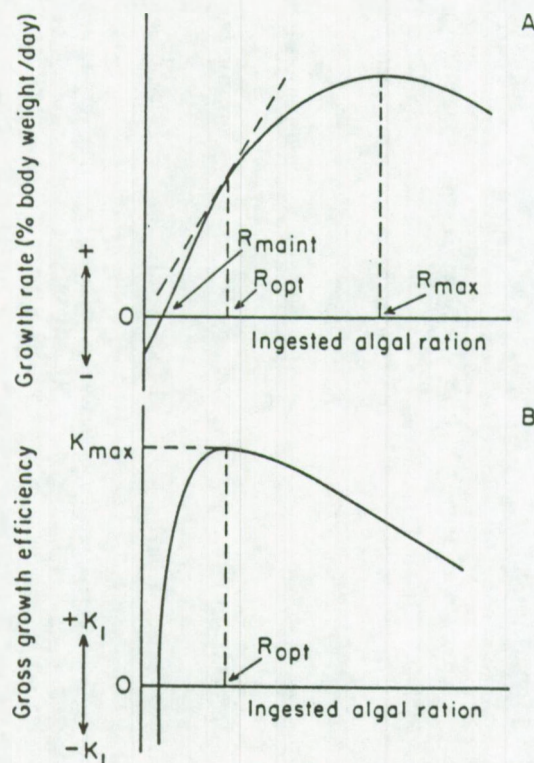


Fig. 10: Generalized relationship between growth rate and algal ration (A), and gross growth efficiency and algal ration (B) according to Thompson & Bayne (1974) and Bayne & Newell (1983) (from Malouf & Bricelj, 1989).

III.2.5. Effect of temperature on feeding

The effect of temperature on the filtration rate of bivalves is well documented in the literature. The temperature coefficient Q_{10}^2 , which provides a measure for the dependence of clearance rate on temperature, varies according to the species and the temperature range studied, being generally higher at a lower temperature range (reviewed by Winter, 1978; Malouf & Bricelj, 1989). In *M. edulis*, clearance rate is independent of temperature ($Q_{10} \approx 1$) between 10 and 20 °C, while a considerable reduction is observed below 10 °C, or above 25 °C. Similarly, depending on the thermal tolerance limits of the species, a range where clearance was not influenced by temperature was found for *Argopecten irradians* (10 - 26 °C: Kirby-Smith, 1970) and *Mya arenaria* (5 - 20 °C: Lowe & Trueman, 1972). Conversely, *Mercenaria mercenaria* exhibited an increase of filtration rate with increasing acclimation temperature between 12 and 25 °C (Hibbert, 1977).

Very little is known about the mechanism which enables bivalves to regulate filtration rate as a function of temperature. Recently, Jørgensen *et al.* (1990) ascribed the increase of pumping rate with temperature in *M. edulis* to the decrease of viscosity of the water rather than to the increased beat frequency of the lateral cilia on the gills.

Winter (1978) found significantly higher assimilation efficiencies in *Modiolus* and *Arctica islandica* at 20 °C than at 4 or 12 °C. Furthermore, the latter author believed that with an increase of temperature there is a shift towards a higher and wider range of food concentrations at which maximum amounts of food are assimilated.

2:

$$Q_{10} = \left(\frac{CR_1}{CR_2} \right)^{\frac{10}{T_1 - T_2}}$$

where CR_1 and CR_2 are the clearance rate at temperatures T_1 and T_2 , respectively.

III.2.6. Effect of suspended sediments on feeding and growth

Suspended sediments may result in either positive or negative effects on bivalve growth, depending on the species, the available food quantity and quality, as well as the type and concentration of sediments. Winter (1976) showed that growth of *M. edulis* increased when suspended silt (12.5 mg l^{-1}) was added to constant concentrations of *Dunaliella marina* within the range of 20 to 40 cells μl^{-1} . Similarly, Murken (1976) reported that growth of *M. edulis* fed a mixed diet of fish-waste water and algae improved significantly by the addition of silt. However, when Winter (1976) added the algae and silt discontinuously at 4 h intervals, the addition of silt only influenced the increase of shell weight, while dry-tissue weight was not significantly affected. Growth enhancements by the addition of low concentrations of bottom sediments (less than $5\text{--}10 \text{ mg l}^{-1}$) to a diet of *Phaeodactylum tricornutum* were reported in both *M. edulis* (Kiørboe et al., 1981) and *Spisula subtruncata* (Møhlenberg & Kiørboe, 1981). Ali (1981) reported a stimulation of growth in *Crassostrea virginica* by supplementing oxidized silt, but only for oysters fed the highest algal ration tested. In order to enhance growth in feeding experiments, kaolinite has been added to algal suspensions (Enright et al., 1986a) as well as artificial diets (Langdon & Bolton, 1984, Urban & Langdon, 1984). The latter authors showed that growth of *C. virginica* was improved by the addition of kaolinite to an algal diet supplemented with yeast and rice starch, although they observed the contrary when the silt was added to the algae/yeast diet alone. Furthermore, Bricelj et al. (1984b) demonstrated that growth in *Mercenaria mercenaria* was not significantly affected by sediment concentrations up to 25 mg l^{-1} , and at $44 \text{ mg silt l}^{-1}$ growth was even reduced by 16% relative to controls fed pure algae.

Suspended inorganic material may raise the concentration of the total particulate matter above the threshold for pseudofaeces production and thus decrease the amount of organic matter ingested. However, this "dilution" effect, which results in a

decline of the energy available for production (Widdows *et al.*, 1979), is counteracted by selective ingestion of particles and the various mechanisms through which silts may affect the feeding and digestion of bivalves: increasing clearance rate, utilization of the organic matter originating from the silt, and increasing absorption efficiency of the ingested algae (Kiørboe *et al.*, 1981).

The effects of silts on the feeding activity varies according to the bivalve species. Low levels of bottom sediments stimulated clearance rate in *M. edulis* (12.5 mg l⁻¹: Winter, 1976; 3.5-5 mg l⁻¹: Kiørboe *et al.*, 1980, 1981), but did not affect or depressed feeding activity in *M. mercenaria* (5 mg l⁻¹: Bricelj & Malouf, 1984). Furthermore, high sediment loads reduced clearance rates to a larger extent in hard clams than in surf clams, mussels and oysters. In this way, the clearance rate of *Spisula subtruncata* was independent of sediment concentrations up to 25 mg l⁻¹ (Møhlenberg & Kiørboe, 1981). Loosanoff (1962) found that the pumping rate of *C. virginica* decreased by 57% to over 90% at silt concentrations of, respectively, 100 to 4,000 mg l⁻¹. At 40 mg silt l⁻¹ clearance rate in the hard clam was already more than halved (Bricelj & Malouf, 1984). The latter authors attributed these interspecific differences to differential strategies in regulating ingestion. Species which regulate ingestion primarily by producing pseudofaeces, such as mussels, oysters, and surf clams, would be better adapted to cope with high suspended sediment loads than species such as clams and scallops, which control ingestion mainly by reducing clearance rate (see III.2.3.).

Silts can enhance the amount of food ingested not only through the stimulation of the filtering activity and subsequently algal ingestion, but also by delivering an additional food source to the bivalve. The organic content of suspended bottom sediments used in feeding experiments amounted to 11.8% of dry weight (Bricelj & Malouf, 1984). In their experiments with natural silt, Kiørboe *et al.* (1981) allocated 20 to 30% of the assimilated organic matter in *M. edulis* to the suspended sediments. Furthermore, clay particles such as

kaolinite can adsorb a substantial fraction of dissolved organic material and may thus improve the delivery of soluble nutrients to bivalves (Urban & Langdon, 1984).

Some authors hypothesized that the presence of silt in the stomach of bivalves increases the utilization of the ingested food due to a grinding effect of the silt particles (Murken, 1976; Kiørboe et al., 1981). Alternatively, the dilution of the algae in inorganic matter results in reduced algal ingestion rates, which in turn may cause an increase of algal absorption efficiency (Bricelj & Malouf, 1984). However, few experimental data are available to confirm these hypotheses. Bricelj (1984) found that additions of ashed silt to a diet of *Pseudoisochrysis paradoxa* had no effect on the absorption efficiency of algae by *M. mercenaria*. The higher assimilation efficiencies which were observed in *M. edulis* in the presence of silt were at least partially due to the assimilation of organic matter derived from the silt (Kiørboe et al., 1981). Furthermore, the absorption efficiency of organic matter is inversely related to the fraction of inorganic particulates in the suspension (Vahl, 1980; Bricelj & Malouf, 1984). This may be explained by the lower efficiency with which sedimentary organic material is absorbed (22% versus 82% for phytoplankton, Bricelj & Malouf, 1984).

III.2.7. The effect of flow rate on feeding and growth

In the culture of bivalves, a suitable water current is required to stimulate feeding, provide food and oxygen, and remove metabolites and faeces.

Water flow may directly influence feeding either by stimulation or by inhibition. A positive correlation between clearance rate and flow rate was found only below a critical flow rate and attributed to an underestimation of filtration rate due to the recirculation of exhalant water at low flow rates (Hildreth & Crisp, 1976; Møhlenberg & Riisgård, 1979; Rodhouse & O'Kelly, 1981). On the other hand, extremely high current velocities can inhibit the feeding activity and eventually result in reduced growth. Growth of scallops decreased at current speeds

exceeding approximately 6 cm s^{-1} (*Argopecten irradians*: Kirby-Smith, 1972) or 10 cm s^{-1} (*Placopecten magellanicus*: Wildish et al., 1987). The latter authors ascribed the growth inhibition to a reduction of clearance rate (e.g. to 50% of the optimum at a flow of about 40 cm s^{-1}) due to the build up of a pressure differential between inhalant and exhalant apertures that interferes with filtration. For a review on the effect of high current speeds on the feeding and growth of bivalves we refer to Bricelj & Shumway (1991). Studies evaluating the effect of extremely high flow speeds are of limited relevance for aquaculture systems where they are rarely encountered. For instance the various volume flow rates and stocking densities which were tried by Manzi et al. (1986) to culture the hard clam in an upflow system resulted in flow velocities between 0.06 and 0.5 cm s^{-1} .

In flow-through aquaculture systems water flow rate, in addition to food concentration, determines the food ration. As a result, growth is expected to vary with flow rate in a similar way as with food ration, and optimal flow rates will depend on the stocking density and the food load of the inflowing water. The latter might explain the deviating values reported in the literature with regard to the optimal flow rate (expressed as volume per min per live weight of seed) for growth of bivalve seed. In this way, optimum growth of oyster spat in outdoor pumped upwelling systems occurred at flow rates of $20\text{--}30 \text{ l min}^{-1} \text{ kg}^{-1}$ in fertilized seawater and $30\text{--}50 \text{ l min}^{-1} \text{ kg}^{-1}$ in unfertilized seawater (Spencer et al., 1986). Studies on the nursery culture of seed of *M. mercenaria* using natural seawater showed a positive correlation between growth and flow rate in raceways (flow rates of 56 to $1483 \text{ l min}^{-1} \text{ kg}^{-1}$; Hadley & Manzi, 1984) as well as in experimental upflow units (flow rates of 7 to $118 \text{ l min}^{-1} \text{ kg}^{-1}$; Manzi et al., 1986). The latter authors found high growth rates at flow rates equal to or greater than $29 \text{ l min}^{-1} \text{ kg}^{-1}$ when water temperature averaged 22°C , whereas a flow rate of $10 \text{ l min}^{-1} \text{ kg}^{-1}$ was required at lower temperatures ($15\text{--}17^\circ\text{C}$). Bayes (1981) recommended flow rates of $1 \text{ l min}^{-1} \text{ kg}^{-1}$ in autumn and spring, and

five times higher values in summer for the nursery culture of oysters and clams in forced upwelling systems using impounded seawater.

Several formulas have been proposed to adjust flow rate as a function of food availability and stocking density in flow-through systems. The flow rate (FR, l h⁻¹) required to achieve a certain reduction in food concentration can be calculated using the equation:

$$FR = \frac{CR \times C_0}{C_i - C_0} \quad (\text{Hildreth \& Crisp, 1976})$$

where CR is the clearance rate (l h⁻¹), C_i and C_o are, respectively, the inflow and outflow concentrations.

Rodhouse & O'Kelly (1981) developed equations to predict the flow requirements of oysters cultured in an upwelling column for realizing 90% stripping of the algae (FR, ml min⁻¹ g⁻¹) between 10 and 20 °C:

$$\begin{aligned} FR &= (0.47 + 0.04 T) WW^{-0.26} && (O. edulis) \\ FR &= (-0.92 + 0.17 T) WW^{-0.32} && (C. gigas) \end{aligned}$$

where T is the ambient temperature (°C), WW is the mean oyster live weight. For the same oyster species, Spencer (1988) showed that the percentage of particles removed from the water (P: %) decreased with increasing flow rate (FR: ml min⁻¹ g⁻¹), particle concentration in the inflowing water (C_o: particles mm⁻³), and oyster live weight (W: mg animal⁻¹); and increased with increasing temperature (T: °C), according to equations of the form:

$$P = a - b \ln FR - c \ln W - d C_o + e T$$

The importance of temperature (T) on the flow rate requirement is further highlighted by the expression proposed by Drinkwaard (1981):

$$FR = T \text{ m}^3 \text{ h}^{-1} (100 \text{ kg})^{-1}$$

There exists some controversy with regard to the minimum food level that should persist in the effluent to obtain maximal growth. For the nursery culture of surf clams in raceways, flow rates should be adjusted so that at least $1 \mu\text{g l}^{-1}$ of chlorophyll-a remained in the outflow (Rhodes *et al.*, 1981). Kirby-Smith (1972) reported that growth of the bay scallop was reduced when more than 40% of the available chlorophyll-a was utilized. Growth of oyster seed declined drastically when more than 20% of the particles were removed from the water flowing through an upwelling column (Spencer, 1988). In the same way, Manzi *et al.* (1986) suggested that growth of hard clams in an upflow nursery system may be reduced if more than 20% of the ambient chlorophyll-a is removed. Conversely, Malinowski & Siddall (1989) found that natural seawater, which lost 17% of its chlorophyll-a content after passage through an initial group of clams, could support maximum growth rates of an additional, equivalent biomass of hard clams. The latter authors concluded that physical (such as maximum velocities of flow necessary to achieve uniform flow) rather than biological (such as food availability) factors determine maximum stocking density and critical flow rates in upflow nursery systems.

III.3. BIVALVE NUTRITION

III.3.1. Introduction

Since Martin (1927) identified phytoplankton as the main food source of bivalves, many investigators have tried to unravel their nutritional requirements. Pioneering researchers tested a wide range of algal species and demonstrated that not all species were equally successful in supporting growth of a particular bivalve (Davis & Guillard, 1958; Walne, 1970). Furthermore, diets consisting of mixtures of several algae generally yielded better growth of bivalve larvae and juveniles (Epifanio *et al.*, 1976; Helm, 1977; Epifanio, 1979b). The nutritional value of an algal species depends on its ingestibility (*i.e.* the acceptability of the cell dimensions), the production of toxic metabolites, the digestibility of the cell wall, and the biochemical composition. Especially the latter criterium has been studied by innumerable authors who attempted to correlate bivalve growth with quantitative and/or qualitative differences in algal composition. In this way, valuable information was compiled on the nutritional value of various algal species (*e.g.* Enright *et al.*, 1986a) and strains (*e.g.* Helm & Laing, 1987) for many species of bivalves. Also, some of these studies revealed that modifications of the culture conditions of algae, such as nutrient, light and temperature limitation, altered their biochemical composition and nutritional value for bivalves (Wikfors *et al.*, 1984; Enright *et al.*, 1986b; Ukeles & Wikfors, 1988). However, the complexity and variability of the composition of live algae made it difficult to separate the effect of the various, often interdependent, biochemical components on the bivalve and to identify its nutritional requirements (reviewed by Webb & Chu, 1983; Brown *et al.*, 1989). Furthermore, the development of an adequate tool to study nutrition in bivalves is hampered by the difficulties in presenting microparticulate food to filter-feeders without degradation and clumping of particles, leakage of nutrients, and proliferation of bacteria. Nevertheless, the use of artificial diets as an incomplete diet (*e.g.* Castell & Trider, 1974;

Langdon, 1983) or as a supplement to deficient algal diets (e.g. Langdon & Waldock, 1981) revealed valuable information about, for instance, the lipid requirements of bivalves. Progress in the development of artificial diets, in which each individual nutrient can be independently varied, is required to complete the still very limited knowledge of marine bivalve nutrition.

III.3.2. Nutritional requirements

protein and amino acids

Differences in the food value of various algal species could not be correlated with variation of the protein quantity or quality (Walne, 1970; Enright *et al.*, 1986a). The amino acids essential for growth have been defined for *Mytilus californianus* (Harrison, 1975³), although the optimum levels are unknown. However, Brown (1991) found that the levels of essential amino acids in 16 species of micro-algae used in mariculture were similar or greater than the levels in oyster larvae and thus concluded that amino-acid composition may not be critical in determining the nutritional value of these algae.

carbohydrate

Various authors have emphasized the importance of dietary carbohydrate throughout the bivalve's life cycle. The nutritional condition in terms of energy content for larvae of *Crassadoma gigantea* and *Patinopecten yessoensis* was correlated with the content of dietary carbohydrate rather than dietary lipid or protein (Whyte *et al.*, 1989, 1990). Comparing growth of juvenile *O. edulis* fed *C. gracilis* of varied chemical composition, Enright *et al.* (1986b) found indications that higher growth rates are possible with additional carbohydrate, provided that adequate protein and essential fatty acids are supplied. High dietary

³ threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine, arginine, tryptophan, proline

carbohydrate levels (e.g. 60%) resulted in greater glycogen production in adult oysters, although the latter is not necessarily a good criterion for nutritional value (Castell & Trider, 1974).

The sugar composition of the polysaccharides from microalgae showed major differences among species and classes (Brown, 1991). In this way, the quality of carbohydrate may be of nutritional significance, since the efficiency with which animals digest carbohydrate depends on the polysaccharide type.

lipid, fatty acid composition, and sterols

Lipid, in particular triacylglycerol, content has been recognized as an index for growth potential and viability in larvae as well as juveniles of various bivalve species (Gallager et al., 1986; Laing & Millican, 1986), although few authors report a positive correlation between total algal lipid content and bivalve growth (Wikfors et al., 1984).

By contrast, several experiments have demonstrated the importance of certain lipid classes or fatty acids. Waldock & Nascimento (1979) found that the growth rate of *C. gigas* larvae was correlated with the neutral lipid content of the algal food. The requirement of *C. gigas* for (n-3) HUFA was shown by Langdon & Waldock (1981) who enhanced growth of spat fed *Dunaliella tertiolecta* (lacking (n-3) HUFAs of chain length greater than C18) by supplementing micro-encapsulated C22:6n3. Since growth was satisfactory with *Tetraselmis suecica*, which is deficient in C22:6n3 but contains C20:5n3, either of these HUFAs appeared to be adequate. Nevertheless, Waldock & Holland (1984) showed that juvenile oysters are capable of elongation and desaturation of dietary precursors, be it at too low a level to sustain growth.

Conflicting data can be found in the literature with regard to the HUFA requirements of bivalve larvae, which may be explained by interspecific differences. Numaguchi & Nell (1991) improved growth of larval *S. commercialis* fed algae by supplementing encapsulated cod liver oil, while lower growth was obtained by using vegetable oils or squid oils containing,

respectively, lower and higher HUFA levels. Larvae of the Manila clam were grown through to metamorphosis on dried *Nannochloris* sp., which is deficient in long-chain HUFAs (Laing *et al.*, 1990). Since the level of C22:6n3 increased during larval development, it was hypothesized that the larvae could synthesize long-chain HUFA from dietary C18 fatty acids. Larvae of *Tridacna gigas*, fed solely on microcapsules, did not show a HUFA requirement, which might be due to their relatively short larval life and large maternally-derived lipid reserves (Southgate *et al.*, in press).

It is generally accepted that bivalves have a limited capacity for *de novo* biosynthesis of sterols, and require dietary sources for growth and survival (Teshima, 1983).

vitamins

Information on the vitamin requirements of bivalve molluscs is restricted to some feeding experiments with artificial diets of known chemical composition which sustained larval development through metamorphosis (Chu *et al.*, 1987) and limited growth of juveniles (Langdon & Siegfried, 1984). These diets contained lipid-walled microcapsules delivering fat-soluble⁴ and water-soluble⁵ vitamins through, respectively, the matrix and the inclusion of the capsules. However, the absolute requirement and the optimal level of each individual vitamin has not been documented.

III.3.3. The role of micro-organisms

For an extensive review of possible interactions between bivalves and bacteria in the marine environment, we refer to the recent work of Prieur *et al.* (1990). That bivalves can utilize

⁴ fat-soluble vitamins: vit A, D, E, K, and lipoic acid

⁵ water-soluble vitamins: p-aminobenzoic acid, biotin, pantothenic acid, choline, folic acid, inositol, nicotinic acid, pyridoxine, cyanocobalamin, riboflavin, thiamine, ascorbic acid (artificial diet of Langdon & Siegfried, 1984).

bacteria as a food source was shown in the late 30's by Zobell & Feltham (1937) who demonstrated growth of *Mytilus californianus* fed an exclusive diet of bacteria and detected a bacteriolytic action in extracts of the digestive tract. Ingestion of bacteria was furthermore demonstrated for adult (Prieur, 1981; Birkbeck & McHenery, 1982; Wright et al., 1982; Bernard, 1989; Langdon & Newell, 1990) as well as larval bivalves (Douillet, 1990). On the other hand, studies of the relation between particle size (see III.2.2.) and retention efficiency suggest that particles of less than 1 μm are generally not, or at extremely low efficiencies, retained in either adult bivalves or veliger larvae. This would preclude the use of bacteria as food unless they were aggregated or attached to detrital particles. In this way, Prieur (1981) observed that bacteria present in the stomach of *Mytilus edulis* were agglomerated by mucus secreted by the animal or formed microcolonies 4-6 μm in diameter. However, some bivalve species (e.g. *Geukensia demissa*, see III.2.2.) are capable of efficiently retaining bacterioplankton with filtering dimensions smaller than 1 μm . Nannociliates and flagellates, which feed primarily on bacteria, are 2 to 20 μm in size and may be an important link in the transfer of bacterial nutrients to bivalves (Sherr et al., 1986).

The digestive system of bivalves, exhibiting high concentrations of the bacteriolytic enzyme lysozyme, appears to be well equipped for the degradation of bacteria (McHenery et al., 1979). Birkbeck & McHenery (1982) demonstrated the degradation of radioactively labelled bacteria by *M. edulis* *in vivo* as well as *in vitro* by extracts of the digestive gland. However, degradation was correlated with lysozyme sensitivity of the bacteria since lysozyme resistant strains, although cleared from suspension at similar rates, were excreted intact. Scanning electronic microscopic observations of the digestive tract contents of *M. edulis* revealed digested cells in the stomach while the hindgut contained intact bacteria, particularly when the mussel ingested *Vibrio* sp. (Prieur, 1981). The latter author hypothesized that the bivalve gut may be a suitable microbiotope for certain bacteria that resist digestion, explaining the

accumulation of, for example, coliforms in mussels. Crosby *et al.* (1990) reported that cellulolytic bacteria were utilized by the oyster *C. virginica* with a ^{14}C assimilation efficiency of 52%.

In environments with sufficiently high bacterial concentrations, bacteria can significantly contribute to the nutrition of suspension-feeding bivalves and function as mediators for the transfer of inorganic nitrogen from the seawater and cellulosic carbon from detrital material to the bivalve (Langdon & Newell, 1990). The latter authors estimated for example that bacteria in salt marches during summer could provide 31% and 71% of the metabolic requirements for carbon and nitrogen, respectively, of intertidal ribbed mussels (*G. demissa*). The contribution of bacteria to the requirements of bivalves for vitamins and other essential nutrients has not been studied.

In aquaculture systems, attempts are normally made to limit the development of bacteria because they may produce toxic metabolites (Zobell & Feltham, 1937), degrade and clump food particles (Masson, 1977; Langdon & Bolton, 1984) and cause diseases (Brown, 1973). However, some studies showed that controlled microbial presence may be beneficial for bivalve cultures. Martin & Mengus (1977) found that the growth of *Mytilus galloprovincialis* larvae could be improved by supplementing an algal diet with various bacterial strains. Recently, Douillet (1990) reported that larval growth, the proportion of larvae that settled successfully, and the size of the resulting spat could be increased in *C. gigas* by adding a selected bacterial strain at concentrations of 10^4 or 10^5 ml^{-1} . Castagna (1990) suggested that a commercially available bacteria concentrate might be useful in increasing survival of clam seed (*M. mercenaria*) during the nursery phase.

III.3.4. The role of dissolved organic compounds

The hypothesis that dissolved organic material (DOM) may play a significant role in the nutrition of aquatic organisms was introduced by Pütter in 1909. Only recent studies, using advanced

analytical procedures to quantify extremely low concentrations of organic molecules under bacteria-free experimental conditions, have evidenced that marine bivalves can acquire and utilize small organic molecules, such as free amino acids (reviewed by Stephens, 1988; Manahan, 1989) and various monosaccharides as well as complex sugars (Welborn & Manahan, 1990). The uptake of DOM occurs primarily by transport directly into the epidermis rather than via the gut and appears to be restricted to marine, soft-bodied invertebrates (Stephens, 1988). The uptake of simple sugars and free amino acids by adult *M. edulis* contributed, respectively, 13% and 10% of the mussel's energy and nitrogen requirements (Gorham, 1988). A similar contribution of glucose uptake in the total energy cost of *M. edulis* veliger larvae was reported by Melaouah (1989).

Further proof for the utilization of DOM is supplied by Langdon (1983) who showed a limited, though significant growth of axenic oyster larvae on the dissolved organic fraction of a biphasic artificial diet. Also, a micro-encapsulated diet supported greater growth of *Tridacna gigas* larvae when supplemented with dissolved yeast extract and inorganic fertilizer (Southgate *et al.*, in press).

In conclusion, marine bivalves appear to feed on a biphasic diet throughout their life cycle, consuming particulate food through the gut and acquiring organic compounds from solution via the gills in adults or the velum in larvae (Stephens & Manahan, 1984).

III.4. REPLACEMENT DIETS FOR LIVE ALGAE IN THE HATCHERY AND NURSERY REARING OF BIVALVE MOLLUSCS

III.4.1. Introduction

Intensive rearing of bivalves has so far relied on the production of live algae, which may account for 15 to 85% (Bolton, 1982) of the operating costs in a bivalve hatchery. The relative algal requirements of the various stages of the bivalve culture process depend on whether the operation aims at the mass-production of larvae for remote setting (*e.g.* Donaldson, 1991) or growing millions of seed till planting size (*e.g.* Pomeroy *et al.*, in prep.). In the latter case, the juveniles, representing the largest biomass in the hatchery and demanding the highest weight-specific rations, consume the largest volumes of algal culture (Helm, 1990a; Table 7).

Table 7: Algal demand in a bivalve hatchery for the maintenance of broodstock, larval culture and postset.

Source	Ref. 1 (% of total)	Ref. 2 (% of total)	Ref. 3 (liters day ⁻¹)
broodstock	30	5	50/100 ind ^a
larvae	10	42	15/10 ⁶ ind ^a
postset	60	53	1200/10 ⁶ ind ^a

References: 1: Pomeroy *et al.* (in prep), 2: Donaldson (1991), 3: Helm (1990a)

Estimates of the algal production cost range from US \$ 4 to 300 per kg dry biomass (Table 8). Algal production in outdoor ponds is relatively cheap, but is only suitable for a few, fast-growing species and is characterized by a poor batch-to-batch consistency and unpredictable culture crashes due to contaminations and/or fluctuating climatological conditions (Gladue, 1991). Indoor algal production offers a better control of the culture conditions and the algal species being grown, but is more expensive than outdoor culture due to space, energy, and skilled labor requirements.

In order to overcome or reduce the problems and limitations

associated with algal cultures, several investigators have attempted to replace the algae by using artificial diets either as supplements or as the main food source for larval, juvenile and adult bivalves (Table 9). Studies on non-algal feeds for bivalves have largely ignored economic considerations by selecting diets that minimize food costs or maximize revenues (Urban & Pruder, in press). These authors emphasized that experimental feeds or supplements will only be used for commercial production if there is an expectation of increased profit, which not only depends on the difference in cost between the live algae and the experimental diet, but also on their relative performance in terms of growth rate, growth efficiency, and mortality.

Table 8: Production cost of marine micro-algae

production cost (US \$/kg dry weight)	remarks	source
300	<i>Tetraselmis suecica</i> 200 l batch culture	calculated from Helm et al. (1979)
77		Pruder (1981; in Urban & Langdon, 1984)
167	various diatoms continuous flow cultures (240 m ³)*	calculated from Walsh et al. (1987)
4-20 160-200	outdoor culture indoor culture	De Pauw & Persoone (1988)
23-115	summer-winter production continuous flow cultures in bags (8 m ³) and tanks (150 m ³)*	Dravers (pers. comm., 1990)
50	tank culture (450 m ³)*	Donaldson (1991)
50 - 400	international survey	Chapter X

*: total volume available for algal production

III.4.2. Centrifuged and dried algae

As algae are the natural food of bivalves, an alternative to on-site algal culture would be the distribution of preserved

algae that are produced at relatively low cost in a large facility under optimal climatological conditions and using the most cost-effective production systems (Anonymous, 1991). Centrifugation of algae into a paste form and subsequent refrigeration until required may facilitate hatchery management and was believed to have an impact on the oyster industry in North America of the same significance as the remote setting of eyed larvae (Watson, 1986). However, the limited shelf-life and/or the high prices of the presently available algal pastes (US \$ 200 and more per kg DW) have discouraged many growers from using them. Recently, the development of preservation techniques extended the shelf-life of *Thalassiosira pseudonana* concentrates from about 10 days to more than one year, which makes it possible to valorize excess and off-season algal production (Donaldson, 1991).

Outdoor pond production on a large scale has lead to the bulk availability of a limited number of "algal meals", such as spray-dried *Spirulina* (Earthrise Farms, CA, USA) and a spray-dried extract of *Dunaliella salina* (Betatene Ltd., Australia). Recent work showed that the latter algal meal improved the growth of Sydney rock oyster larvae when it was supplemented to live algae (Numaguchi & Nell, 1991).

Recently, techniques have been developed for the large scale production of dried marine micro-algae under heterotrophic growth conditions, i.e. utilizing an organic carbon instead of light as an energy source. Heterotrophic algal cultures can attain up to 1,000 times higher densities than photoautotrophic cultures and are preserved by spray-drying (Gladue, 1991). The latter author projected costs of producing algae in industrial fermentors between US \$ 5 and 25 per kg. This cost estimate conflicts with the price of a comparable product which has been marketed by a British company ("Algal 161", Celsys, UK: US \$ 170/kg). Recent trials have demonstrated that growth of bivalve larvae and juveniles fed spray-dried *Tetraselmis suecica* is comparable to that obtained for live, light-grown *T. suecica*. Although the performance of dried algae was always inferior to that of controls fed an algal mixture, they offer interesting

possibilities as a partial substitute for live algae (Laing et al., 1990; Laing & Verdugo, 1991; Table 9). Also, the mixture of spray-dried algae (*T. suecica*/*Cyclotella cryptica*, 70/30%) supported better growth than a sole diet of *T. suecica* (Laing, 1991). Unfortunately, heterotrophic mass-production has been realized for very few algal species, and most of the species that are known to be of high nutritional value for bivalves (e.g. *Chaetoceros*, *Isochrysis*, *Skeletonema*, *Thalassiosira*, *Monochrysis*) are not capable of growing in the dark (Gladue, 1991). Furthermore, heterotrophic conditions resulted in a drastic change in the gross composition as compared to light-grown *T. suecica* (Laing et al., 1990) and reduced (n-3) HUFA content (Pohl & Zurheide, 1979). Nevertheless, further developments in this rather new technology may improve the biochemical composition and the range of dried algae available.

III.4.3. Microparticulate diets

Multi-component diets consisting of both particulate and dissolved nutrients have been used to study the nutritional requirements in bivalves (Castell & Trider, 1974; Trider & Castell, 1980; Langdon, 1983) or as fattening feeds for oysters (Nell, 1985; Nell & Wiseley, 1983). Furthermore, several authors have reported the use of starch supplementation to increase the condition index and glycogen content in oysters (Haven, 1965; Turgeon & Haven, 1978; Wisely & Reid, 1978). These powder diets, apart from being nutritionally incomplete, caused water quality problems and subsequently promoted bacterial proliferation in the culture systems. Through micro-encapsulation techniques dietary ingredients can be encapsulated within digestible capsules and delivered to suspension-feeders without losses of nutrients to the aqueous medium (reviewed by Jones et al., 1984; Langdon et al., 1985). Juvenile bivalves fed microcapsules or microgel particles showed growth up to 60-70 % of that obtained for controls fed live algae (Laing, 1987; Langdon & Siegfried, 1984). Furthermore, feeding microcapsules high in (n-3) HUFA as a supplement to live algae can improve growth of oyster seed fed

algae that are deficient in these essential fatty acids (Langdon & Waldock, 1981) and increase the reproductive output of oyster broodstock (Lane, 1989). Very recently, promising results are reported by Southgate *et al.* (in press) using protein-walled microcapsules for larval feeding of the giant clam and the Sydney rock oyster (Table 9). The procedure to manufacture the microcapsules (AUS \$ 200/kg DW) on a laboratory scale is not detailed by the authors, but the microcapsules may be stored for several months as a sterilized slurry. In this way, detrimental effects of drying on the capsule wall stability and buoyancy is avoided (Southgate, 1991).

The main problems arising from the use of microparticulate feeds are settling, clumping and bacterial degradation of the particles, leaching of nutrients, and low digestibility of the wall material (Langdon & Bolton, 1984; Chu *et al.*, 1987; Langdon, 1989). In this regard, low susceptibility to bacterial attack and high digestibility for the bivalve may be conflicting requirements for a capsule wall, as demonstrated by Langdon & DeBevoise (1990) for two types of protein-walled microcapsules ingested by the Pacific oyster. So far, micro-encapsulated diets for bivalves mostly remain at the research level due to cost and difficulty in producing capsules of the correct small size on a large scale.

III.4.4. Non-algal single-cell proteins (SCP)

Dried food yeast (*Candida utilis*) can substitute up to 50% of the algal diet of certain bivalve species without a significant drop in growth rate (Epifanio, 1979a; Alatalo, 1980; Table 9). The extremely low nutritional value of some non-algal SCP could not be explained by differences in dietary composition and has been attributed to the low digestibility of the microbial cell walls (*Candida utilis* for the American oyster, Epifanio, 1979a; *S. cerevisiae* and *Methylomonas clara* for the Sydney rock oyster, Nell, 1985). However, carbon and nitrogen assimilation efficiencies of *C. virginica* fed algae were only slightly greater than the efficiencies with which oysters utilized yeast

(Alatalo, 1980; Table 9). By contrast, Portères (1988) observed by means of transmission electron microscopy that multiplying yeasts are preferentially lysed in the digestive system of the Manila clam and found an assimilation efficiency for yeast of only 30% using radiotracer techniques. Alternatively, a deficiency or imbalance of nutrients in yeast may explain its poor nutritional value. Urban & Langdon (1984) thus greatly improved the growth of *C. virginica* on a 50/50 algae/*C. utilis* diet by supplementing rice starch and kaolinite.

Table 9: Literature review on the evaluation of various algal replacement diets for larval, juvenile, and adult bivalves

SPECIES	REPLACEMENT DIET	RESULTS	SOURCE
LARVAE			
<i>Mercenaria mercenaria</i>	freeze-dried <i>Dunaliella euchlora</i> , <i>Isochrysis galbana</i> , heat-dried <i>Scenedesmus obliquus</i>	culture through metamorphosis	Hidu & Ukeles (1964)
<i>Mytilus galloprovincialis</i>	(1) various detritic foods (ground <i>Spirulina</i> , <i>Ulva</i> , plant and animal tissue) (2) frozen and lyophilized algae (<i>Monochrysis lutheri</i> , <i>Tetraselmis suecica</i> , <i>Chlorella</i> sp.)	(1) problems: clumping of food particles, bacterial blooming (2) growth rates equivalent to those for live algae	Masson (1977)
<i>Crassostrea virginica</i>	gelatin-accacia (GA) and nylon-protein (NP) microcapsules	GA more digestible than NP GA encapsulated cod liver oil results in similar growth as live algae	Chu <i>et al.</i> (1982)
<i>Crassostrea gigas</i>	biphasic diet of dissolved nutrients and co-precipitated particles	umbo stage reached after 6 days, after which little further growth occurred (axenic growth experiment)	Langdon (1983)
<i>Mimachlamys nobilis</i>	carrageenan micro-binding diet (MBD) and nylon-protein microcapsules (NP)	higher growth and survival for MBD: <i>Chlorella</i> (1:1) than for <i>Chaetoceros:Chlorella</i> (1:1) no supplementary effect for NP	Teshima <i>et al.</i> (1982)
<i>Crassostrea virginica</i>	mixture of gelatin-accacia and lipid-walled microcapsules	growth through metamorphosis, though with survival of less than 5%, lipid algal extract promotes growth	Chu <i>et al.</i> (1987)
<i>Tridacna gigas</i>	gelatin-accacia and protein-walled microcapsules	growth through metamorphosis, though inferior to live algae	Southgate (1988)
<i>Tapes philippinarum</i>	spray-dried, heterotrophically grown <i>Nannochloris</i> sp. and <i>Tetraselmis suecica</i>	growth at least equal to the live, light-grown algae, but inferior to algal mixture (<i>C. calcitrans</i> + <i>I. galbana</i>)	Laing <i>et al.</i> (1990)
<i>Saccostrea commercialis</i>	gelatin-accacia microcapsules and dried extract of <i>Dunaliella salina</i>	useful as a supplement for live algae (<i>I. galbana</i> + <i>Pavlova lutheri</i>), but unsuitable as a total replacement	Numaguchi & Nell (1991)
(1) <i>Tridacna gigas</i> (2) <i>Saccostrea commercialis</i>	protein-walled microcapsules supplemented with dissolved yeast extract (YE) + inorganic fertilizer (IF)	(1) superior growth compared to algal control only if supplemented with YE and IF (2) growth of more than 80% that of algae-fed larvae after 8 days of culture, no effect of YE supplementation	Southgate <i>et al.</i> (in press)

JUVENILES

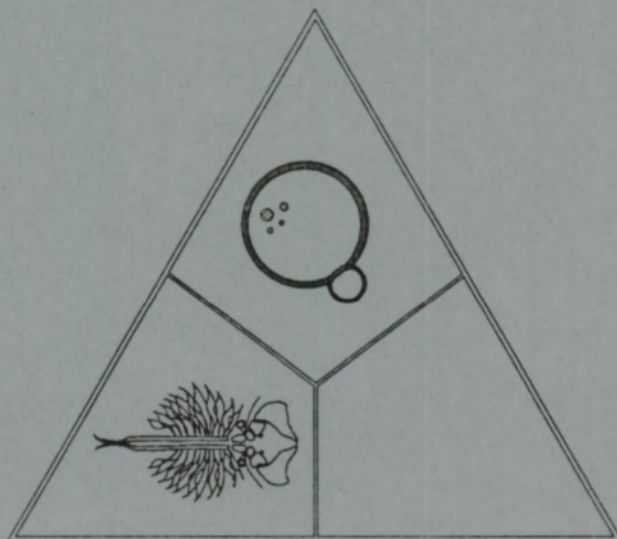
(1) <i>Crassostrea gigas</i> (2) <i>Mytilus edulis</i>	nylon-protein and glycopeptides microcapsules	(1) growth after 3 weeks 30-50% of that for live algal mixture (2) determination of suitable particle size range for ingestion	Gabbott et al. (1976)									
<i>Crassostrea gigas</i>	nylon-protein microcapsules consisting of haemoglobin, starch, and cholesterol	-growth less than 50% of growth on live algae (<i>T. suecica</i>) -low value as supplement to suboptimal algal rations	Langdon (1977)									
<i>Argopecten irradians</i> <i>Crassostrea virginica</i> <i>Mercenaria mercenaria</i> <i>Mytilus edulis</i>	spray-dried food yeast (<i>Candida utilis</i>)	- <i>A. irradians</i> , <i>M. mercenaria</i> , <i>M. edulis</i> : growth as fast or faster than controls fed <i>T. pseudonana</i> for algal substitution level up to 50% - <i>C. virginica</i> : growth decreased with amount of yeast in the diet	Epifanio (1979a)									
<i>Mercenaria mercenaria</i> <i>Crassostrea virginica</i>	spray-dried food yeast (<i>Candida utilis</i>)	-growth of oysters and clams fed 50/50 mixture of <i>I. galbana</i> and yeast similar to 100% algal control -growth of clams fed a sole diet of yeast is about 50% that of control, while oysters exhibited no or negative growth -C and N assimilation efficiency (%) for <i>C. virginica</i> fed yeast and algae: <table><tr><td></td><td>carbon</td><td>nitrogen</td></tr><tr><td>yeast</td><td>64</td><td>70</td></tr><tr><td>algae</td><td>74</td><td>78</td></tr></table>		carbon	nitrogen	yeast	64	70	algae	74	78	Alatalo (1980)
	carbon	nitrogen										
yeast	64	70										
algae	74	78										
<i>Crassostrea gigas</i>	gelatin-accacia microcapsules	demonstration of requirement for C22:6n3 through supplementation of GA encapsulated C22:6n3 to algae that are deficient in C20:5n3 and C22:6n3	Langdon & Waldock (1981)									
<i>Crassostrea virginica</i>	mixed diet of carboxymethyl cellulose microgel particles, lipid-walled microcapsules, kaolin, and dissolved trace metal mix	addition of dispersant/antibiotic mixture reduced variation among experiments for the growth of oysters fed the artificial diets from 19-89% to 41-64% of the growth of the algal-fed controls -> variation due to qualitative differences in the composition of bacterial populations	Langdon & Bolton (1984)									
<i>Crassostrea virginica</i>	mixed diet of carboxymethyl cellulose microgel particles, lipid-walled microcapsules, kaolin, and dissolved trace metal mix	-continuous feeding in beakers: growth up to 73% that of algal-fed controls (<i>T. pseudonana</i>) -flow-through culture: growth less than 25% of algal-fed control -> bacteria play role in utilization and/or supplementation	Langdon & Siegfried (1984)									
<i>Crassostrea virginica</i>	spray-dried food yeast (<i>Candida utilis</i>) supplemented with lipid-walled vitamin microcapsules, kaolinite, and various non-algal feeds (e.g. rice starch, cheese way, blood meal)	-growth on algae/yeast diets mainly dependent on proportion of algae (<i>T. pseudonana</i> + <i>I. galbana</i>) -limited growth improvement by supplementation of vitamin capsules -best growth occurred when 50% of algal ration was replaced by a mixture of yeast, rice starch, and kaolinite	Urban & Langdon (1984)									
<i>Crassostrea gigas</i>	liposomes	demonstration of ingestion and metabolism of radioactively labeled cholesterol and phosphatidylcholine (in liposomal wall), and glucose (encapsulated)	Parker & Selivonchick (1986)									

<i>Tapes semidecussata</i> [*] <i>Mercenaria mercenaria</i> <i>Ostrea edulis</i> <i>Crassostrea virginica</i> <i>Crassostrea gigas</i>	freeze-dried microcapsules (Frippak Feeds)	-growth 54-64% of that obtained for live algae (<i>C. calcitrans</i>) -substituting microcapsules for 60-85% of the algae resulted in similar growth compared to the algal controls	Laing (1987)
<i>Placopecten magellanicus</i>	calcium alginate microparticles	demonstration of ingestion	Kean-Howie <i>et al.</i> (1989)
<i>Crassostrea gigas</i>	protein-walled microcapsules containing ¹⁴ C-labelled protein	¹⁴ C-labelled protein was absorbed with an efficiency of 40%	Langdon (1989)
<i>Crassostrea gigas</i>	protein-walled (P) and glyceride-coated, nylon-protein-walled (GNP) microcapsules containing ¹⁴ C-labelled protein	GNP less susceptible to bacterial degradation than P, but giving lower protein assimilation efficiency (GNP and P, respectively, 29% and 39%)	Langdon & DeBevoise (1990)
<i>Tapes philippinarum</i> <i>Tapes decussata</i> <i>Mercenaria mercenaria</i> <i>Ostrea edulis</i>	spray-dried heterotrophically grown <i>Tetraselmis suecica</i>	growth similar to live <i>Tetraselmis</i> , but less than on <i>C. calcitrans</i> or mixed live diet (<i>T. suecica</i> + <i>C. calcitrans</i>)	Laing & Verdugo (1991)
ADULTS			
<i>Crassostrea virginica</i>	cornstarch, wheat flour, dextrose	reduced meat growth due to suboptimal food supply compensated by supplementing starch (5-32 mg/l) or high concentrations of dextrose (34 mg/l), no detectable effect on shell growth	Haven (1965)
<i>Crassostrea virginica</i>	multi-component diet consisting of cornstarch, (corn or cod liver) oil, casein, cellulose, vitamin and mineral mix, choline chloride, and vit E	-10-fold slower growth compared to oysters fed natural phytoplankton -high levels of dietary carbohydrate resulted in greater oyster glycogen production -dietary cod liver oil produced oysters with higher glycogen content than corn oil -increasing the dietary lipid from 5 to 18% of the DW resulted in greater meat production	Castell & Trider (1974)
<i>Crassostrea virginica</i>	cornstarch, dextrose	-glycogen level and wet tissue weight increased in oysters fed cornstarch supplement, while feeding dextrose influenced only glycogen level -no influence on shell dimensions	Turgeon & Haven (1978)
<i>Saccostrea commercialis</i>	multi-component diet consisting of starch, cod liver oil, vitamin premix, cholesterol, "Pruteen" (<i>Methylophilus methylotrophus</i>), lysine, and methionine	condition index increases slower for oysters fed artificial diet than oysters held in a fertilized pond	Nell & Wisely (1983)
<i>Saccostrea commercialis</i>	multi-component diet consisting of starch, cod liver oil, vitamin premix, cholesterol, and one of four different types of SCP	-"Pruteen" (<i>Methylophilus methylotrophus</i>) and <i>Candida utilis</i> are more suitable as protein source for oyster fattening than baker's yeast (<i>S. cerevisiae</i>) and "Probion" (<i>Methylophilus clara</i>)	Nell (1985)
<i>Ostrea edulis</i>	dried microcapsules (Frippack Feeds)	more production of larvae in oysters conditioned on a 50/50 mixture of algae and capsules than on either diet alone	Lane (1989)

* = *Tapes philippinarum*

EXPERIMENTAL PART 1:
STUDY OF FEEDING AND GROWTH IN *ARTEMIA* USING
BAKER'S YEAST AS A FOOD SOURCE

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Chapter IV

THE NUTRITIONAL VALUE OF BAKER'S YEAST AS FOOD FOR *ARTEMIA*

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Chapter IV

THE NUTRITIONAL VALUE OF BAKER'S YEAST AS FOOD FOR *ARTEMIA*

IV.1. INTRODUCTION

Because of their small particle size, high protein content and relatively low production cost, yeasts have been considered as a potential algal substitute for several species of filter-feeders, e.g. rotifers (Hirayama & Watanabe, 1973; Fukusho, 1980; Lemilinaire, 1984), *Artemia* (Shimaya *et al.*, 1967; Talloen, 1978; Johnson, 1980; Nimmannit & Assawamunkong, 1985) and bivalve molluscs (Epifanio, 1979a; Urban & Langdon, 1984). However, yeasts have not proven to be of consistently high nutritional value. Problems that arise when feeding a yeast monodiet have often been assigned to nutritional deficiencies of the yeast diet (Urban & Langdon, 1984; Douillet, 1987; Hirayama, 1987). However, the nutritional value of any diet depends first on its degree of digestibility and second on its content of essential elements. Because yeast cells are known to have a complex and thick cell envelope, poor digestibility may be an important constraint in the use of this Single-Cell Protein (SCP) as a food source in aquaculture in particular and in animal husbandry in general. Two decades ago it was proven that efficient utilization of the protein content of *Torula* yeast (Mitsuda *et al.*, 1969) and *Saccharomyces cerevisiae* (Omstedt *et al.*, 1975) by rats was prevented by the rigid yeast cell wall. More recently, Johnson *et al.* (1980) reported that the cell wall of *Phaffia rhodozyma* was a barrier to the uptake of the yeast's astaxanthin by rainbow trout.

Several methods have been developed to improve the digestibility of SCP products; mechanical disruption, autolysis, and enzymatic treatment (Kihlberg, 1972; Hedenskog & Mogren,

1973). However, as a result of these drastic treatments soluble cytoplasmic contents in the yeast cells are released in the water. As a consequence, essential yeast nutrients for the filter-feeder may be lost and moreover, culture conditions deteriorate due to reduced water quality. In this chapter we document the low digestibility of fresh baker's yeast fed to *Artemia*, and propose a chemical treatment which makes the yeast digestible while maintaining cell integrity (IV.2.). Furthermore, various chemical treatments were screened for their efficiency to improve the digestibility of baker's yeast for the brine shrimp (IV.3.). On the basis of the latter study, a simple chemical treatment was adopted for the routine preparation of baker's yeast in the study of feeding and growth of *Artemia* (IV.4.). Finally, the potential application of two types of cell wall defective mutants of *S. cerevisiae* was evaluated (IV.5.).

IV.2. LIMITED DIGESTIBILITY OF BAKER'S YEAST AS A FOOD SOURCE FOR ARTEMIA

IV.2.1. Rationale

In preliminary experiments, it was shown that growth and survival of *Artemia* fed a sole diet of baker's yeast was very limited, in spite of its suitable physical and chemical characteristics as a food particle. The observation of large quantities of undigested yeast cells in the faeces indicated that *Artemia* efficiently ingested the yeast cells, but was not capable of utilizing their cell contents. By means of microbiological techniques, which are widely used to digest the cell wall of whole yeast cells *in vitro* as a preparatory step to protoplast fusion, the present study attempted to demonstrate that the yeast cell wall forms a barrier to the digestive enzymes of the brine shrimp.

IV.2.2. Materials and methods

IV.2.2.1. *Artemia* culture conditions

Artemia franciscana (Great Salt Lake, Utah, USA; Sanders Brine Shrimp Co., lot 185-0) were hatched in 0.2- μ m filtered artificial seawater (35 ppt salinity) at 25 °C (Sorgeloos *et al.*, 1986). All experiments were performed with artificial seawater which was prepared according to the formula of Dietrich & Kalle (in Kinne, 1971). After 24 h (*i.e.* on day 1 of the culture test) freshly-hatched nauplii were transferred to cylindro-conical Falcon tubes (Falcon Blue Max 2070) containing 50 ml of artificial seawater which was gently aerated by means of a Pasteur pipet. On the fourth day of culture, the initial density of 25 larvae/50 ml medium was reduced by transferring *ad random* 15 of the surviving nauplii to 50 ml of fresh seawater. In this way, interference from decreased water quality, which may occur in the *Artemia* cultures exhibiting slow growth due to the accumulation of food, was reduced. Cultures were kept under continuous darkness at 25 \pm 1 °C.

Table 10: Feeding regime for rearing *Artemia* on *Dunaliella tertiolecta* during the first week after hatching.

	daily ration (10 ³ cells individual ⁻¹)	number of animals per culture	volume of algal stock suspension [18 10 ⁶ cells ml ⁻¹] (μ l day ⁻¹)
1	150	25	210
2	300	25	420
3	300	25	420
4	300	15	250
5	450	15	380
6	450	15	380
7	600	15	500

The live alga *Dunaliella tertiolecta* Butch, cultured according to Liao *et al.* (1983) and known to be an excellent food for *Artemia*, was used as a reference diet. This algal diet was

fed once a day according to the feeding schedule given in Table 10, which is based on the feeding rate of Vanhaecke & Sorgeloos (1980) in 10-ml test tubes. Dosage of the yeast diets was derived from the previous schedule by substituting three or more yeast cells for one algal cell (experiment 1 and 2, respectively 3). All cell counts were performed with a Bürker haemocytometer.

IV.2.2.2. Yeast culture and treatment

An industrial strain of *Saccharomyces cerevisiae* (R5, obtained from Algist Bruggeman N.V., Belgium) was grown at 30 °C under continuous shaking in a liquid YPG medium containing yeast extract (Oxoid L21, 1% weight/volume), peptone (Oxoid L37, 2% w/v) and D-glucose (2% w/v). Yeast was harvested either in the exponential or stationary phase by centrifugation after 12 hours or 3 days of culture, respectively. Fresh caked baker's yeast was obtained from the same company.

Yeasts were converted into protoplasts by means of a two-step procedure (Machtelinckx, 1987). First, yeasts were suspended at a concentration of 200 mg wet weight/ml in a sterilized medium containing Na₂EDTA (0.05 M) and Tris-buffer (0.2 M; pH 8). After addition of 2-mercaptoethanol (2% volume/volume) the yeast was incubated for 30 minutes at 30 °C. Pretreated yeast was collected and washed with protoplasting medium comprising a phosphate-citrate buffer (KH₂PO₄ 0.08 M; Na₃-citrate 0.016 M; pH 5.8) and KCl (0.6 M). A yeast pellet of 4 g wet weight was resuspended in 16 ml protoplasting medium to which 3 ml of an enzyme solution (Novozym 234, Novo; 40 mg/ml) was added. Conversion into protoplasts was completed after approximately 30 minutes of incubation at 30 °C, whereupon the cells were separated from the enzyme mixture by centrifugation at 3,000 rpm for 5 min.

Chemical treatment consisted of the first step in the previous procedure followed by washing the yeast cells three times with filtered seawater. In this way we tried to improve digestibility of the yeast without removing the cell wall, but by making the latter permeable for the digestive enzymes of *Artemia*. Finally, autoclaved yeast was prepared by autoclaving

a suspension of fresh baker's yeast in a steam-boiler at 122 °C and 1 atm for 45 minutes.

Yeast protoplasts and thiol-treated yeast could be stored in seawater at 4 °C for at least one week without cell lysis. The autoclaved yeast was stored for maximal three days at 4 °C.

IV.2.2.3. Parameters followed and data analysis

Artemia survival and mean length were determined on day 4 and 8 for each test tube. Animals were fixed with lugol solution and their length from top of head to the base of the caudal furca was measured, using a dissecting microscope equipped with a drawing mirror (Sorgeloos et al., 1986). Average body length was estimated on day 4 by measuring 20 of the culled larvae per treatment, except for one experiment (DIG 1), where additional cultures were terminated for this purpose.

The data were analyzed statistically with a one-way analysis of variance (ANOVA). Tukey's honestly significant difference (HSD) method was used to detect significant differences between the means at the significance level of $P \leq 0.05$. Prior to analysis, the data were checked for homoscedasticity and normality using Hartley's test and the Kolmogorov-Smirnov test, respectively. In some cases departure from the assumptions of analysis of variance were rectified by transformation of the original data (Sokal & Rohlf, 1981).

IV.2.2.4. Preparation of samples for electron microscopy

The effect of the various treatments on the appearance of the yeast cell was evaluated by means of transmission electron microscopy, which was performed at the Laboratory of Anatomy (University of Ghent). The yeast was treated immediately prior to fixation. Washed yeast cells were prefixed in formaldehyde-glutaraldehyde fixative (Karnovski, 1965) at 4 °C for 4 h. The cells were washed by centrifugation, embedded in 8% agar and fixed in 2% osmium tetroxide in 0.2 M cacodylate buffer according to Hayat (1986). The agar blocks were dehydrated and embedded in

Epon. Photographs were enlarged 3x and 2.5x for the thin sections examined at a magnification of 10,000x and 20,000x, respectively.

IV.2.2.5. Experimental design

The design of the various experiments is detailed in Table 11. In the first experiment (DIG 1), protoplasts and chemically treated yeast cells were compared to untreated yeast in order to evaluate the effect on digestibility of enzymatic and chemical treatment. In the second set of experiments (DIG 2) we evaluated the effectiveness of the chemical treatment with the commercially available baker's yeast. We also examined the effect of autoclaving on the digestibility of the yeast. We used laboratory-cultured *S. cerevisiae* to evaluate the effect of culture age on the susceptibility to digestion by *Artemia*, and on the efficiency of the chemical treatment. Finally, tests were run to determine the optimal feeding schedule for chemically treated Bruggeman's yeast under our culture conditions (DIG 3). Each treatment was run in six replicates.

IV.2.3. Results

For all experiments, mean data and corresponding standard deviations for survival and length on day 4 and 8 are given in Table 11. The multiple range test for the first experiment (DIG 1) indicated that the untreated yeast diet yielded significantly lower growth and survival of the *Artemia* cultures compared to the other three diets. Among these latter treatments no differences could be detected except for higher survival of brine shrimp fed *Dunaliella*, and poor growth of *Artemia* fed yeast protoplasts compared to those fed chemically treated yeast. Microscopic examination of the faecal material produced by the larvae fed untreated baker's yeast revealed intact yeast cells. Staining with methylene blue showed that most of these cells still contained their cytoplasmic contents after passage through the digestive system (Photo 1). By contrast, faecal pellets of *Artemia* grown on treated diets were acellular and fine-granular (Photo 2), revealing efficient digestion.

Photo 1: Faecal pellet of *Artemia* fed untreated caked yeast. Staining with methylene blue reveals that the majority of the yeast cells are metabolic inactive, but still contain their cytoplasm (colored cells). A fraction of the yeast cells still exhibits a viable cytoplasmic content (uncolored cells) (x 400).

Photo 2: Faecal pellet of *Artemia* fed thiol-treated yeast. Staining with methylene blue reveals that the bulk of the faecal material consists of yeast cell wall debris (same color as background), while only few whole yeast cells are present (colored cells) (x 400)

Table 11: Growth and survival in *Artemia* fed various yeast preparations (DIG 1 and 2) and different rations of thiol-treated caked yeast (DIG 3). Yeast rations Y1, Y3, Y4, Y5 correspond with the replacement of one algal cell by 1, 3, 4, and 5 yeast cells, respectively. Data represent means and standard deviations from 6 replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD, $P \leq 0.05$).

feeding regime	DAY 4		DAY 8	
	survival (%) [†]	body length (mm) [§]	survival (%) [‡]	body length (mm)
experiment DIG 1 (1-3: Y3)				
1 untreated EP yeast	65 ± 12 ^b	1.31 ± 0.06 ^b	29 ± 8 ^c	2.91 ± 0.10 ^c
2 EP yeast protoplasts	87 ± 7 ^a	1.77 ± 0.10 ^a	67 ± 10 ^b	3.98 ± 0.31 ^b
3 2-ME-treated EP yeast	85 ± 7 ^a	1.63 ± 0.17 ^a	68 ± 13 ^b	4.63 ± 0.26 ^a
4 <i>Dunaliella tertiolecta</i> (reference diet)	94 ± 7 ^a	1.80 ± 0.15 ^a	95 ± 6 ^a	4.24 ± 0.18 ^{ab}
experiment DIG 2 (1-7: Y3)				
1 untreated caked yeast	77 ± 10 ^c	1.10	26 ± 20 ^{cd}	1.56 ± 0.08 ^d
2 untreated EP yeast	81 ± 9 ^{bc}	1.43	52 ± 18 ^{bc}	2.93 ± 0.54 ^b
3 untreated SP yeast	76 ± 10 ^c	1.17	13 ± 11 ^d	2.11 ± 0.13 ^{cd}
4 2-ME-treated caked yeast	96 ± 4 ^a	1.67	53 ± 6 ^{bc}	4.52 ± 0.28 ^a
5 2-ME-treated EP yeast	83 ± 5 ^{abc}	1.88	61 ± 8 ^b	5.00 ± 0.25 ^a
6 2-ME-treated SP yeast	85 ± 6 ^{abc}	1.42	10 ± 3 ^d	2.63 ± 0.74 ^{bc}
7 autoclaved caked yeast	91 ± 4 ^{ab}	1.52	36 ± 20 ^b	2.51 ± 0.09 ^{bc}
8 <i>Dunaliella tertiolecta</i> (reference diet)	95 ± 6 ^a	1.72	94 ± 8 ^a	4.22 ± 0.08 ^a
experiment DIG 3 (1-5: 2-ME-treated caked yeast)				
1 Y1	98 ± 5 ^a	1.36	19 ± 9 ^c	2.89 ± 0.25 ^b
2 Y3	95 ± 6 ^a	1.84	49 ± 13 ^b	4.56 ± 0.37 ^a
3 Y4	98 ± 2 ^a	1.85	58 ± 17 ^b	4.91 ± 0.42 ^a
4 day 1-3: Y3, day 4-7: Y4	97 ± 3 ^a	1.82	66 ± 15 ^b	4.76 ± 0.25 ^a
5 day 1-3: Y3, day 4-7: Y5	97 ± 6 ^a	1.73	63 ± 12 ^b	4.58 ± 0.22 ^a
6 <i>Dunaliella tertiolecta</i> (reference diet)	98 ± 2 ^a	2.04	93 ± 9 ^a	4.37 ± 0.30 ^a

†: survival day 1 = 100%

‡: survival day 4 = 100%

§: standard deviations are only indicated when based on length measurements from additional cultures that were terminated on day 4 (CHEM 1).

abbreviations: EP and SP: exponential and stationary phase yeast, cultivated at laboratory scale
2-ME: 2-mercaptoethanol treatment (see IV.2.2.2.)

ANOVA for data obtained in the second experiment (DIG 2, Table 11) showed a significant effect of yeast culture age on digestibility. On day 8 length and survival of *Artemia* fed exponential-phase yeast were significantly higher in comparison to those fed the stationary-phase yeast. Chemical treatment significantly improved *Artemia* growth when applied to log-phase yeast and caked yeast, but was ineffective in promoting digestion of the stationary-phase yeast. A diet of autoclaved caked yeast resulted in significantly larger *Artemia* and higher survival rates.

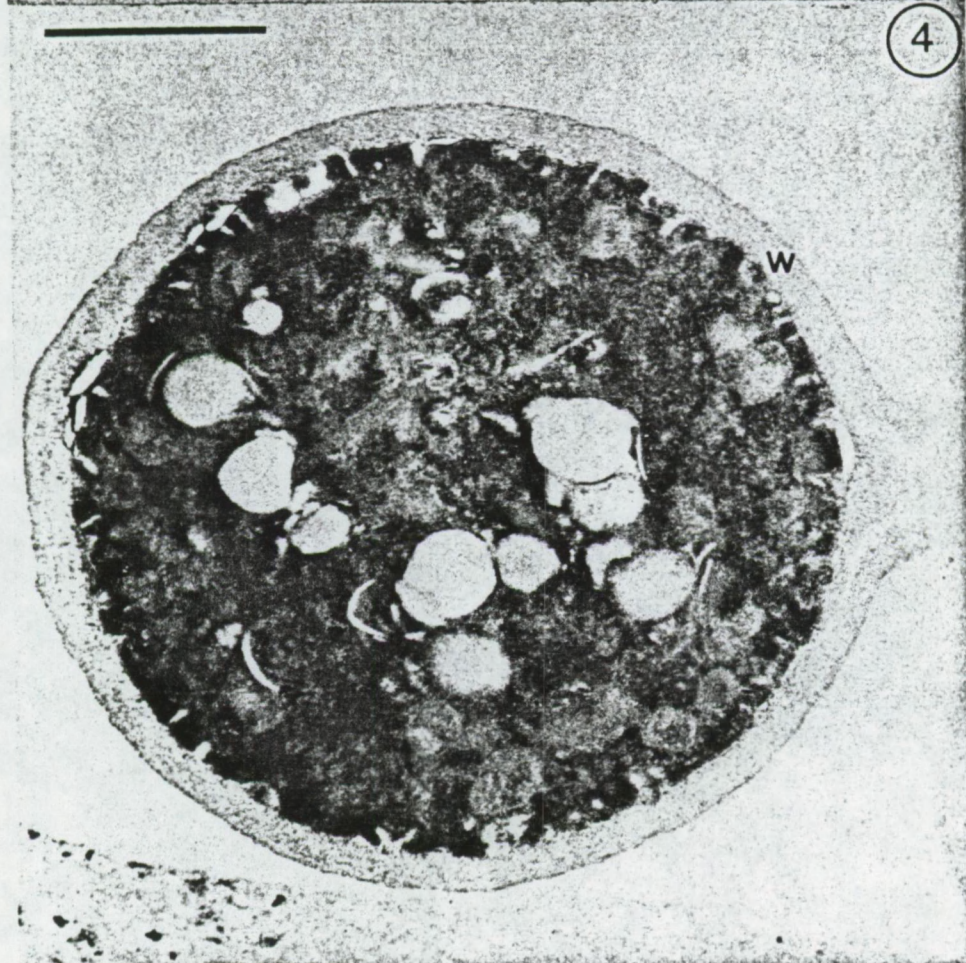
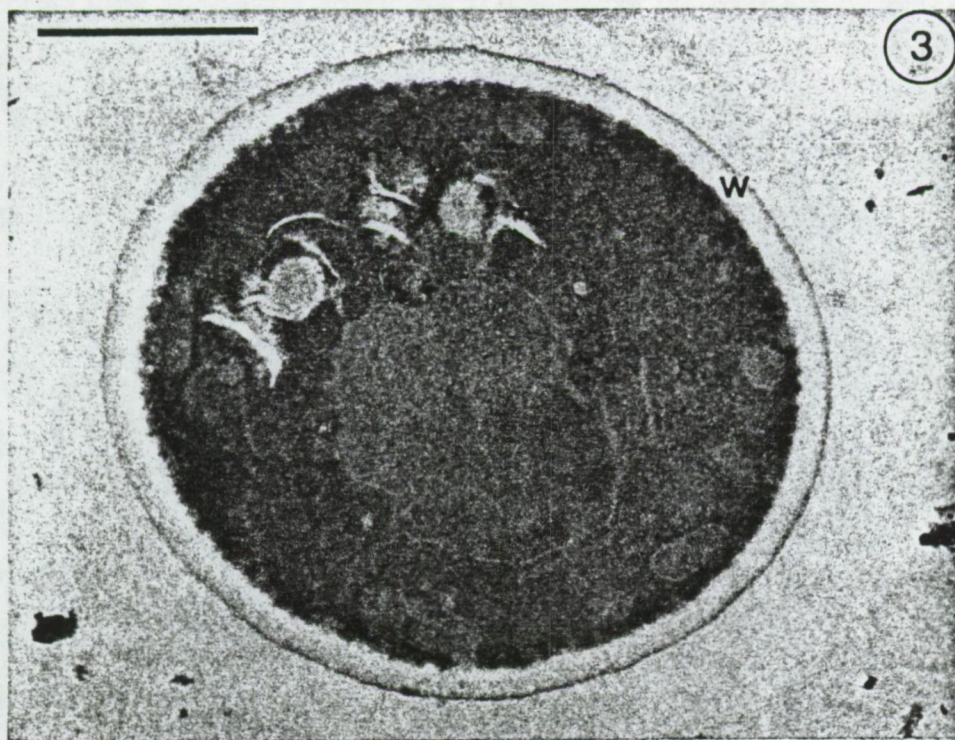
In the third set of experiments (DIG 3, Table 11) we could not detect any significant difference between the results obtained by substituting one *Dunaliella* cell by 3, 4 or 5 yeast cells, respectively. Only the lowest food dosage, where one algal cell was replaced by one yeast cell, resulted in significant differences in growth and survival on day 8.

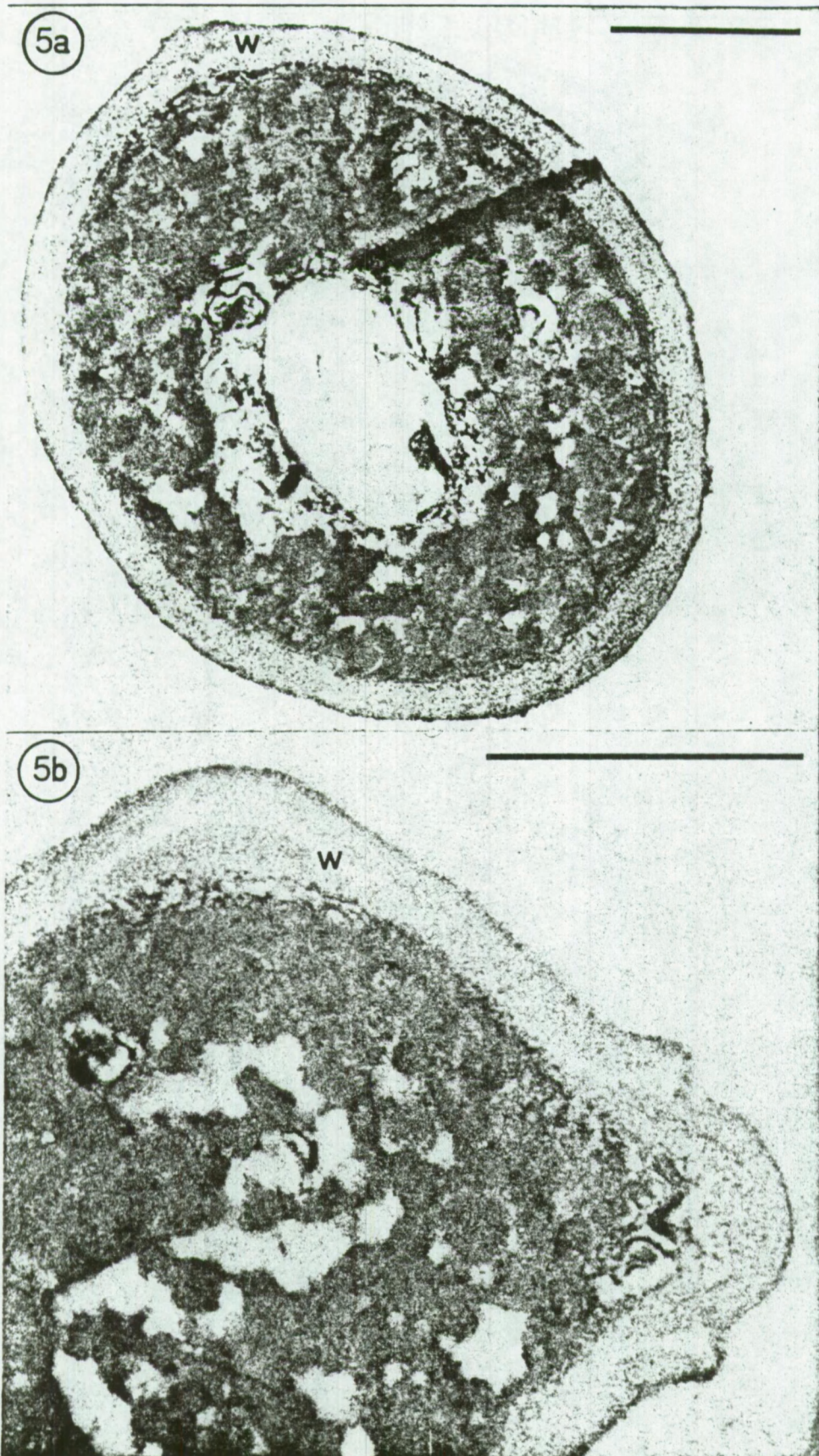
The *Dunaliella* diet proved to be a stable internal reference during the present series of tests; no significant (ANOVA, $P > 0.10$) deviations in *Artemia* growth and survival were noted among the three experiments.

Photographs 3, 4, and 5 show thin sections of an untreated, a thiol-treated, and an autoclaved yeast cell, respectively. The major effect of the thiol treatment is that the cell wall of treated yeast is completely stained and exhibits a loose and fibrillar structure, whereas only the surface of the cell wall takes up the stain in untreated yeast. Furthermore, the treatment did not affect the cytoplasmic contents, which had a similar appearance in the untreated and the thiol-treated yeast cells. By contrast, no intact cellular structures could be detected in the granular cytoplasm of autoclaved yeast. As opposed to the stability of the yeast protoplasts and thiol-treated yeast during storage at 4 °C, the autoclaved yeast did not contain any intact cells after one week.

→ Photo 3: Thin section of untreated yeast cell. Only the surface of the cell wall (w) takes up the stain (x 30,000). Bar indicates 1 μm .

→ Photo 4: Thin section of thiol-treated yeast cell. The texture of the cell wall (w) is more loose and fibrillar, and the stainability of the wall material is increased (x 30,000). Bar indicates 1 μm .





← Photo 5: Thin section of autoclaved yeast cell. The cell wall (5A: w), including the bud scar regions (5B), shows a very loose texture and releases material from its outer surface. The texture of the cytoplasm is granular (A: x 30,000, B: x 50,000). Bar indicates 1 μ m.

IV.2.4. Discussion

Extremely poor survival and growth of *Artemia* cultured on fresh baker's yeast clearly show that this diet is an inadequate food for brine shrimp. The observation of intact yeast cells in the faecal material of *Artemia* fed fresh baker's yeast reveal a problem with digestibility of this SCP. Gibor (1956) also observed viable *Stichococcus* sp. in faecal pellets of *Artemia* and attributed the low nutritional value of this alga to its low digestibility. Because the nutritional value of the yeast improved after removal of the cell wall by enzymatic digestion, it is apparent that the primary limiting factor for using baker's yeast as a food for brine shrimp is the lack of digestion of the cell envelope.

Complete breakdown of the cell wall does not seem to be necessary for rendering the cytoplasmic contents of the yeast cell accessible to the digestive system of *Artemia*. In fact, we obtained better growth performance after 8 days of culture when feeding chemically treated yeast than when feeding protoplasts. Thiol treatment is often applied as a preparatory step for the isolation of yeast protoplasts for use in biochemical and microbiological studies (reviewed by Arnold, 1981; Davis, 1985; Ferenczy, 1985). It is generally believed that thiol compounds cleave the disulfide linkages in the cell wall, making it more permeable and susceptible to enzymatic degradation (Kidby & Davies, 1970; Bacon, 1973; Villaneuva *et al.*, 1973; Davis, 1985). A hypothetical model of the cell wall structure of *S. cerevisiae* (Fig. 11) indicates that two layers of wall material can be recognized. The outer layer consists of a mannoprotein complex in which the individual molecules are cross-linked by disulfide bridges made between their protein moieties. Farkas (1985) hypothesized that this cross-linking forms a barrier to penetration of extracellular glucanases into the internal glucan layer, which is the main structural constituent of the cell wall. For this reason, a mycolytic enzyme mixture should contain at least two components: first an agent which increases the permeability of the mannoprotein layer and second an agent which

dissolves the internal glucan microfibrils. The first component can be a mannanase (McLellan & Lampen, 1968) or a specific proteolytic enzyme and may be substituted by treatment with 2-mercaptoethanol (Scott & Schekman, 1980).

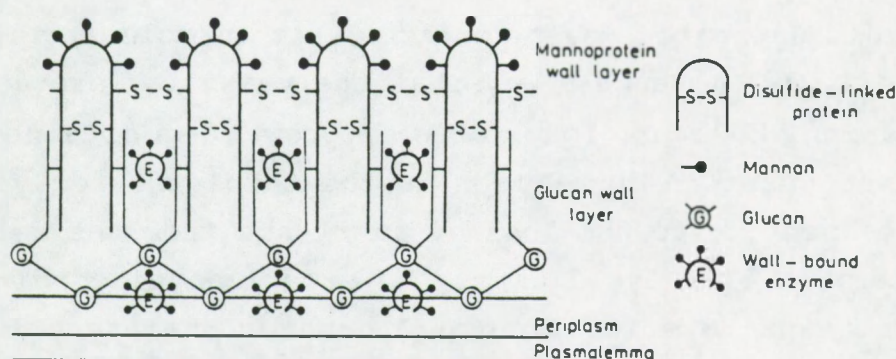


Fig. 11: Hypothetical model of the cell wall structure of *Saccharomyces cerevisiae* (Farkas, 1985).

The electron-microscopic observations demonstrated that the thiol treatment only affected the cell wall, whereas the cytoplasmic contents of the yeast cell remained intact. In addition, although the fibrillar structure of the cell wall appeared to be more loose after thiol treatment, the stability of the treated yeast during storage showed that the cell envelope maintained its function as a rigid, mechanical barrier. On the contrary, the structural organization of the yeast cell was destroyed by autoclaving, which caused cell lysis within one week of storage at 4 °C. The dark stained outer region in the cell wall of untreated *S. cerevisiae* has also been observed by other authors (Phaff, 1971; McLellan *et al.*, 1970) and claimed to contain mainly mannoprotein, while the inner region would consist of the non-stainable glucan (Phaff, 1971). In this regard, it is interesting to note that McLellan *et al.* (1970) observed an increase of the stainability of the cell wall by treating baker's yeast with phosphomannanase. The similar effect of the treatment

with 2-mercaptoethanol further supports the hypothesis that the thiol reagent acts primarily upon the outer mannoprotein layer of the yeast cell wall.

Bacon *et al.* (1965) demonstrated that autoclaving facilitates digestion of whole yeast cells by microbial enzymes. Significantly better growth of *Artemia* fed autoclaved yeast compared to untreated yeast confirms that autoclaving affects the yeast cell wall in such a way that the yeast is more susceptible to digestion. However, lower *Artemia* growth was obtained with the autoclaved yeast in comparison to the thiol-treated yeast. This may have been due to the loss of nutrients from the cells, which may have partially lysed under the experimental conditions, and the subsequent deterioration of the water quality.

In vitro experiments have shown that *Saccharomyces cerevisiae*, during its transition from exponential to stationary growth phase, builds up a resistance to enzymatic breakdown (Shahin, 1972; Deutch & Parry, 1974). Moreover, Schwencke *et al.* (1977) revealed that culture age of yeast influenced effectiveness of the thiol pretreatment on protoplast isolation. Our paper is the first confirmation of this effect for *in vivo* digestion.

As mandibular grinding of captured food particles is unknown for *Artemia*, we may suppose that requirements for the digestion of yeast *in vitro* are similar to those for the enzymatic breakdown in the digestive tube. Little is known about the spectrum of enzyme activities in the digestive tract of the brine shrimp. Telford (1970) found β -glucanase activity in a whole-body homogenate of *Artemia* but could not detect any mannanase activity. We have confirmed his findings in a qualitative assay on these two enzymes (Coutteau, 1987). The inability of *Artemia* to grow on baker's yeast strongly suggests that the digestive enzymes of *Artemia* cannot penetrate the outer mannoprotein layer of the yeast cell wall. Absence of mannanase activity and the efficient digestion of yeast after thiol treatment or autoclaving confirm this hypothesis.

Reports on the more or less successful culture of brine shrimp on yeasts (Bond, 1937; Weisz, 1946; Bowen, 1962; Shimaya

et al., 1967; Kawano *et al.*, 1976; Bowen *et al.*, 1985; Blanco Rubio, 1987; James *et al.*, 1987a) are not necessarily in contradiction with our findings. In fact, during preliminary experiments in which we substituted up to 75% of *Dunaliella* cells with fresh baker's yeast, we obtained culture results which were sometimes comparable to those of the 100% algal diet. Many of the results reported in the literature could be explained by a contamination of the culture with algae or other micro-organisms. Moreover, supplying yeast to the medium induces development of a microflora which may play an essential role in the nutritional properties of the diet for *Artemia* (Douillet, 1987). This effect may be the result of supplementation of nutrients and/or the improvement of digestibility characteristics. In addition, it has been demonstrated that the structural organization, composition and digestibility of the yeast cell wall differ considerably with species (Bartnicki-Garcia, 1968; Kreger-Van Rij & Veenhuis, 1971) and strain (Rost & Venner, 1965; Kaneko *et al.*, 1973). Finally, susceptibility to enzymatic degradation may also be influenced by age of the culture as well as by medium composition (Killick, 1971; Kratky *et al.*, 1975) and various other culture conditions (Kaneko *et al.*, 1973).

In conclusion, the ineffectiveness of baker's yeast as a diet for *Artemia* appears to be mainly due to poor digestibility rather than its nutritional composition. Moreover, this problem can be solved without affecting the structural backbone of the yeast cell wall. In this way, the suitable characteristics of yeast (*i.e.* individual cells that do not lose nutrients into the culture medium) can be retained and yeast might eventually be used as a diet for intensively cultured brine shrimp and aquatic filter-feeders, *e.g.* bivalves and penaeid shrimp larvae.

IV.3. EFFECT OF VARIOUS CHEMICAL TREATMENTS ON THE DIGESTIBILITY OF BAKER'S YEAST

IV.3.1. Rationale

Previous work (IV.2.) yielded a chemical treatment which was effective to improve the digestibility of baker's yeast for *Artemia*. However, the relative complexity and in particular the requirement for high concentrations of the toxic 2-mercapto-ethanol largely restricted the application of this technique. In the present study, we tried to simplify the treatment medium by omission of superfluous compounds, reduction of the quantity and eventually replacement of the toxic reagents, without affecting its efficiency to render baker's yeast digestible for the brine shrimp. In this regard, valuable information could be obtained from the extensive literature on the chemical treatment of yeast prior to *in vitro* enzymatic digestion of the cell wall (reviewed by Peberdy, 1979; Arnold, 1981; Davis, 1985; Ferenczy, 1985). On the basis of the present study, a simple chemical treatment was adopted (IV.4.) to prepare baker's yeast for use as a food particle in the studies of feeding and growth in *Artemia* (Chapter V, VI, VII).

IV.3.2. Materials and methods

IV.3.2.1. *Artemia* culture conditions

The experimental conditions of the growth test were identical to those described in IV.2.2.1., except that the culture system consisted of culture flasks (Nunclon, 270 ml capacity), which were filled with 50 ml of seawater and mounted to a shaking apparatus (GFL 3016, shaking amplitude and frequency, 3 cm and 30 min⁻¹, respectively), instead of aerated Falcon tubes. In the latter, due to changes in the pressure of the compressed air supply and crystallization of salts in the pasteur pipet, a frequent adjustment was required to maintain a constant aeration intensity in all replicates. The

standardization of agitation and aeration could be improved by the use of shaking cultures.

The feeding regime corresponded with that specified in Table 10 (IV.2.2.1.), with one algal cell replaced by four yeast cells.

IV.3.2.2. Yeast treatment[†]

The yeast, consisting of caked baker's yeast (provided by Algist Bruggeman N.V., Belgium), was chemically treated in various ways, rinsed with filtered seawater, and stored as a standard stock suspension at 4 °C during the one week culture period. The different treatments and the ranges of the parameters examined are described below. The pH values represent initial values, *i.e.* measured prior to the start of the incubation of the yeast suspension.

Treatment with acid

The yeast was suspended in deionized water (25% wet weight/volume, WW/v) and the suspension was set to pH 0.4 or 1.0 with HCl prior to incubation for 2-24 h at 4-35 °C.

Treatment with 2-mercaptoethanol (2-ME)

The thiol treatment medium, which was applied in the previous study (IV.2.), consisted of a solution of ethylenediaminetetraacetate (EDTA) and 2-mercaptoethanol in Tris-buffer. Preliminary experiments showed that the Tris-buffer, which was found to inhibit growth of *Artemia* (Provasoli & D'Agostino, 1969), could be omitted from the treatment medium without reducing its effectiveness. Further experimentation with the thiol medium consisted of suspending the yeast (25-75% WW/v) either in a solution of Na₂EDTA (0.005-0.05 M) or in deionized water. The yeast suspension was set to pH 9-12 with NaOH prior to the addition of 2-ME (2-0.002% v/v) and subsequent incubation for 30 min at 30 °C.

[†]: protected by International Patents PCT/BE 89/00009 and EP-89870040.6 (old 09.03.89) "Feed for Aquaculture" filed in Europe, USA, Japan, Canada, Australia (various file numbers; pending; owned by Artemia Systems N.V./S.A., Baasrode, Belgium).

Treatment with cysteine (CYS)

The yeast was suspended (50% WW/v) in a solution of L-cysteine hydrochloride (0.001-0.3 M), previously set to pH 6-12 with NaOH. The pH of the yeast suspension was restored to pH 6-12 prior to incubation for 30 min at 30 °C.

Treatment with methionine (MET)

The yeast was suspended (50% WW/v) in a solution of DL-methionine (0.1 M), previously set to pH 8 with NaOH, and incubated for 30 min at 30 °C.

IV.3.2.3. Parameters followed and data analysis

Artemia survival and mean length were determined on day 4 and 8 of the culture as described in IV.2.2.3. The length estimate on day 4, based on the measurement of 20 of the culled animals per treatment, provided an intermediate indication of growth. Statistical analysis (ANOVA, Tukey HSD; t-test) was restricted to the data obtained after 7 days of culture.

IV.3.2.4. Experimental design

The present experiments constituted only a part of a more extensive investigation evaluating the effect of yeast mutations (IV.5.), strain characteristics, fermentation conditions, and various drying techniques on the nutritional value of yeast for *Artemia*. Due to the intensive use of the culture system, the number of treatments per experiment, allocated to the screening of various chemical treatments, was often limited. As a result, various aspects of one chemical treatment were investigated in different experiments. The design of the various experiments is detailed in the Tables 12 and 13.

A first series of experiments (STAN 1-4) compared the growth and survival of *Artemia* in the shaking culture system with that obtained in the aerated Falcon tubes, which was used in the previous study (IV.2.). The standardization experiments were performed with *Artemia* fed either *Dunaliella tertiolecta* (STAN

1-4) or 2-mercaptoethanol-treated baker's yeast (STAN 4).

In a second series of growth tests (CHEM 1-7), various chemical treatments were screened for their effectiveness to improve the digestibility of baker's yeast for *Artemia*. The conditions of the different treatments are detailed in Table 13. Various treatments of yeast with acid, differing with regard to initial pH, temperature, and duration of the exposure, were tried in experiment CHEM 1. A number of experiments were run to determine the role of the various components of the 2-mercaptoethanol treatment medium, i.e. concentration of 2-mercaptoethanol (CHEM 2, 3, 5), the density of the yeast suspension (CHEM 2), the initial pH (CHEM 4), and the concentration of EDTA (CHEM 5). The effect of a treatment with the amino acids methionine and cysteine was evaluated in experiment CHEM 4. Finally, the role of the concentration and pH of the cysteine solution was examined in experiments CHEM 6 and CHEM 7, respectively.

IV.3.3. Results

The preliminary experiments demonstrated that survival of *Artemia* fed *D. tertiolecta* in the shaking system was not significantly different from that observed in the aerated Falcon tubes (STAN 1-4, Table 12). However, in three out of four experiments, final body length significantly differed between *Artemia* reared in either of the systems. It is interesting to note that while significant differences in growth according to the culture system were observed in brine shrimp fed algae, similar growth was found for the animals fed treated yeast in the same experiment (STAN 4, Table 12). Contrary to the previous series of experiments (IV.2.) significant differences were measured in *Artemia* growth among experiments in the Falcon tube system (ANOVA, $F_s = 33.6$, $P < 0.001$) as well as the shaking system (ANOVA, $F_s = 32.4$, $P < 0.001$).

Table 12: Comparison of growth and survival in *Artemia* fed either *Dunaliella tertiolecta* or chemically treated yeast in the aerated Falcon tubes and the shaking system. Data represent means and standard deviations from 6 replicates. Unlike superscripts denote significant differences among means (F_5 : ANOVA, Tukey HSD; t_5 : t-test).

feeding regime	DAY 4		DAY 8	
	survival (%) [†]	body length (mm) [‡]	survival (%) [‡]	body length (mm)
experiment STAN 1			$t_5 = 1.96$ ns	$t_5 = 3.16^{**}$
1 shaking, <i>Dunaliella</i>	95 ± 4	1.74	97 ± 4	4.56 ± 0.19
2 Falcon, <i>Dunaliella</i>	94 ± 8	1.88	87 ± 12	3.91 ± 0.47
experiment STAN 2			$t_5 = 0.46$ ns	$t_5 = 0.32$ ns
1 shaking, <i>Dunaliella</i>	99 ± 2	-	88 ± 8	5.46 ± 0.34
2 Falcon, <i>Dunaliella</i>	97 ± 6	-	86 ± 17	5.52 ± 0.29
experiment STAN 3			$t_5 = 0$ ns	$t_5 = 4.61^{***}$
1 shaking, <i>Dunaliella</i>	96 ± 4	1.87	96 ± 11	4.15 ± 0.18
2 Falcon, <i>Dunaliella</i>	95 ± 2	1.79	96 ± 4	4.59 ± 0.15
experiment STAN 4			$F_5 = 0.98$ ns	$F_5 = 7.10^{**}$
1 shaking, <i>Dunaliella</i>	87 ± 14	1.80	84 ± 19	4.83 ± 0.20 ^a
2 Falcon, <i>Dunaliella</i>	89 ± 7	1.35	93 ± 7	4.30 ± 0.09 ^b
3 shaking, 2-ME-yeast ¹	72 ± 12	1.33	77 ± 9	4.72 ± 0.30 ^a
4 Falcon, 2-ME-yeast	92 ± 8	1.71	82 ± 22	4.85 ± 0.31 ^a

significance levels: ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$

†: survival day 1 = 100%

‡: survival day 4 = 100%

§: intermediate length was measured on the culled animals per treatment on day 4

1: caked baker's yeast treated with 2-mercaptoethanol (2% v/v) in EDTA (0.05 M, pH 9) for 30 min at 30 °C.

The results of the experiments evaluating various chemical treatments of baker's yeast (CHEM 1-7) are presented in Table 13. Growth and survival of *Artemia* fed baker's yeast significantly improved after treating the yeast with acid for 24 h at 4° C (CHEM 1). However, the growth improvement was limited and no significant effect was obtained by varying the pH, temperature, and duration of the treatment. Increasing the density of the yeast suspension from 25% to 75% (WW/v) in the 2-mercaptoethanol treatment medium at pH 8 significantly affected growth (CHEM 2). For practical reasons, a standard yeast density of 50% was adopted (CHEM 4-7).

Table 13: Growth and survival in *Artemia* fed various preparations of caked baker's yeast. For each experiment, the treatment conditions that were varied follow the treatment's number, whereas those that were not the object of experimentation follow the number of the test. Data represent means and standard deviations from 6 replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD).

treatment	DAY 4		DAY 8	
	survival (%) [†]	body length (mm) [‡]	survival (%) [‡]	body length (mm) [‡]
experiment CHEM 1 (25% WW/v)			$F_s = 3.19^{**}$	$F_s = 2.77^*$
1 untreated	91 ± 14	1.06	13 ± 13 ^b	1.40 ± 0.16 ^c
2 pH 0.4, 24 h, 4 °C	95 ± 8	1.22	56 ± 24 ^a	1.89 ± 0.18 ^a
3 pH 0.4, 4 h, 25 °C	89 ± 11	1.10	26 ± 22 ^{ab}	1.73 ± 0.32 ^{abc}
4 pH 1.0, 24 h, 4 °C	83 ± 9	1.07	38 ± 30 ^{ab}	1.59 ± 0.22 ^{abc}
5 pH 1.0, 4 h, 25 °C	81 ± 6	1.17	37 ± 11 ^{ab}	1.84 ± 0.22 ^{ab}
6 pH 1.0, 8 h, 25 °C	85 ± 13	1.07	51 ± 12 ^a	1.49 ± 0.16 ^{bc}
7 pH 1.0, 24 h, 25 °C	95 ± 5	1.23	52 ± 11 ^a	1.73 ± 0.21 ^{abc}
8 pH 1.0, 2 h, 35 °C	84 ± 7	1.13	32 ± 24 ^{ab}	1.65 ± 0.30 ^{abc}
experiment CHEM 2 (EDTA 0.05 M, pH 9, 30 min at 30 °C)			$F_s = 1.92$ ns	$F_s = 6.95^{**}$
1 2-ME 2%, 25% WW/v	99 ± 2	1.71	80 ± 9	4.92 ± 0.25 ^a
2 2-ME 1%, 25% WW/v	97 ± 2	1.59	71 ± 12	4.65 ± 0.31 ^{ab}
3 2-ME 0.2%, 25% WW/v	98 ± 2	1.63	73 ± 15	4.31 ± 0.19 ^b
4 2-ME 2%, 75% WW/v	99 ± 2	1.71	87 ± 12	4.42 ± 0.24 ^b
experiment CHEM 3 (EDTA 0.05 M, pH 9, 25% WW/v, 30 min at 30 °C)			$F_s = 5.62^{**}$	$F_s = 113.9^{***}$
1 2-ME 0.2%	99 ± 2	1.46	68 ± 14 ^a	4.07 ± 0.27 ^a
2 2-ME 0.02%	95 ± 5	1.50	36 ± 30 ^{ab}	3.64 ± 0.63 ^a
3 2-ME 0.002%	94 ± 6	1.20	22 ± 18 ^b	1.79 ± 0.07 ^b
4 2-ME 0%	92 ± 6	1.17	28 ± 11 ^b	1.47 ± 0.17 ^b
experiment CHEM 4 (50% WW/v, 30 min at 30 °C)			$F_s = 32.37^{***}$	$F_s = 87.82^{***}$
1 2-ME 2%, EDTA 0.05 M, pH 9	93 ± 5	1.49	74 ± 8 ^a	4.34 ± 0.16 ^b
2 2-ME 0.02%, EDTA 0.05 M, pH 12	99 ± 2	1.49	67 ± 12 ^a	4.68 ± 0.13 ^{ab}
3 EDTA 0.05 M, pH 12	64 ± 4	1.11	1 ± 3 ^d	(1.50)
4 aq. des., pH 12	76 ± 7	1.15	20 ± 20 ^{cd}	2.19 ± 0.23 ^d
5 CYS 0.1 M, pH 8	91 ± 5	1.76	41 ± 14 ^b	4.61 ± 0.27 ^{ab}
6 CYS 0.3 M, pH 8	91 ± 9	1.76	43 ± 8 ^b	4.80 ± 0.46 ^a
7 CYS 0.3 M, pH 2	89 ± 9	1.67	32 ± 14 ^{bc}	3.22 ± 0.16 ^c
8 MET 0.1 M, pH 8	79 ± 8	1.28	3 ± 6 ^d	(2.00)
experiment CHEM 5 (pH 12, 50% WW/v, 30 min at 30 °C)			$F_s = 6.08^{**}$	$F_s = 0.96$ ns
1 2-ME 0.02%, EDTA 0.05 M	96 ± 3	1.78	71 ± 17 ^a	5.19 ± 0.58
2 2-ME 0.02%, EDTA 0.005 M	89 ± 4	1.83	66 ± 11 ^{ab}	4.87 ± 0.30
3 2-ME 0.02%, EDTA 0 M	95 ± 5	1.67	48 ± 15 ^{bc}	4.68 ± 0.69
4 2-ME 0.01%, EDTA 0.05 M	95 ± 3	1.70	53 ± 12 ^{abc}	5.01 ± 0.39
5 2-ME 0.002%, EDTA 0.05 M	94 ± 9	1.71	38 ± 10 ^c	5.01 ± 0.25

Table 13 (continued)

treatment	DAY 4		DAY 8	
	survival (%) [†]	body length (mm) [§]	survival (%) [‡]	body length (mm) [‡]
experiment CHEM 6 (pH 8, 50% WW/v, 30 min at 30 °C)			$F_0 = 0.30$ ns	$F_0 = 25.83^{***}$
1 CYS 0.1 M	97 ± 4	1.27	26 ± 17	3.72 ± 0.38 ^a
2 CYS 0.01 M	89 ± 7	0.95	24 ± 16	1.93 ± 0.78 ^b
3 CYS 0.001 M	82 ± 7	0.90	31 ± 16	1.50 ± 0.26 ^b
experiment CHEM 7 (50% WW/v, 30 min at 30 °C)			$F_0 = 11.06^{***}$	$F_0 = 3.11$ ns
1 CYS 0.01 M, pH 6	56 ± 10	1.30	4 ± 7 ^b	(3.50)
2 CYS 0.01 M, pH 8	79 ± 5	1.79	9 ± 11 ^b	3.83 ± 0.58
3 CYS 0.01 M, pH 10	94 ± 6	1.79	22 ± 19 ^b	4.30 ± 0.45
4 CYS 0.01 M, pH 12	94 ± 9	1.95	52 ± 20 ^a	4.61 ± 0.38

significance levels: ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$

†: survival day 1 = 100%

‡: survival day 4 = 100%

§: intermediate length was measured on the culled animals per treatment on day 4

^a: values between brackets are based on a limited number of replicates due to the collapse of several cultures

A reduction of the 2-mercaptoethanol concentration in the treatment medium from 2 to 0.2% (v/v) resulted in a significant decrease of *Artemia* growth (CHEM 2). Furthermore, although *Artemia* fed yeast which was treated with 2-mercaptoethanol at a concentration as low as 0.02% still grew significantly faster than animals fed untreated yeast, survival was low and variable (CHEM 3). An increase of the initial pH of the treatment medium from pH 9 to pH 12 allowed to decrease the 2-mercaptoethanol concentration from 2 to 0.02% without a significant decrease of *Artemia* survival and growth (CHEM 4). In the absence of the thiol compound, the treatment with alkali whether or not in combination with EDTA did not improve the digestibility of the yeast (CHEM 4). A further reduction of the concentration of the sulfhydryl compound in the treatment medium at pH 12 resulted in a significant decrease of survival, but did not affect growth of *Artemia* (CHEM 5). Omitting the EDTA from the thiol medium did not significantly affect *Artemia* growth, but resulted in a significant decrease of survival (CHEM 5).

Treating yeast with methionine was not effective to make baker's yeast digestible for *Artemia*, whereas the treatment with cysteine under the same conditions (pH, concentration, temperature) significantly improved growth and survival (CHEM 4). The cysteine treatment at pH 2 was significantly less effective than that at pH 8 (CHEM 4). At pH 8, a concentration of 0.1 M cysteine appeared to be optimal since higher and lower concentrations resulted in, respectively, similar (CHEM 4) and lower growth (CHEM 6). The efficiency of a treatment using lower concentrations of cysteine could be enhanced by increasing the initial pH (CHEM 7). Although growth of *Artemia* fed the cysteine-treated yeast was similar to that of animals fed the 2-mercapto-ethanol-treated yeast, consistently higher survival was attained by feeding the latter (CHEM 4, 6, 7).

IV.3.4. Discussion

The preliminary experiments revealed that growth of *Artemia* fed *D. tertiolecta* may vary according to the culture system used as well as between experiments performed with the same culture method. Deviations of growth between experiments may be due to the inevitable variation of some experimental conditions, such as microbial development and algal quality. Although it was tried to control the latter in the present study by harvesting the algae at approximately the same growth phase, some factors may have been beyond the control of the experimenter (e.g. the amount of nutrients provided to the algal culture through the natural seawater). In this regard, the biochemical composition of *D. tertiolecta* has been shown to vary greatly with the algal culture conditions (Fabregas et al., 1986). Differences in growth rate according to the culture system may be ascribed to a differential effect of physical conditions, such as the intensity of the agitation, on the availability of the food, which in turn depends on the food quality. In this way, *Artemia* fed algae grew at a different rate in the two culture systems in experiment STAN 4, whereas a similar growth was observed when feeding treated yeast. Growth rate of *Artemia* fed algae in the shaking system was found

to be similar, higher, as well as lower compared to animals reared in the Falcon tubes. This indicates that the behaviour of the algal cells (e.g. growth, mobility, flocculation) under the different culture conditions may also vary according to the batch of the algal culture.

The growth tests showed that a gradually decreasing treatment efficiency, e.g. when decreasing thiol concentrations or less alkaline pH were applied, affected primarily *Artemia* survival rate. By contrast, the growth rate was only significantly depressed when *Artemia* was feeding on yeast which was subjected to a very mild treatment. The impact of yeast digestibility on survival may be explained by the lower rate with which the latter is cleared from suspension by *Artemia* (see Chapter V). This resulted in a build-up of the yeast concentration and a reduction of the water quality in the course of the experiment. Alternatively, individual variation in the capability to digest yeast of an intermediate digestibility, may result in a low and variable number of surviving larvae that are capable of growing at an acceptable rate. Increased survival with increasing efficiency of the treatment would then be the result of an enlargement of the fraction of larvae which can digest the yeast.

The findings of the present study are in agreement with earlier studies showing that the enzymatic removal of the cell wall *in vitro*, and the subsequent liberation of yeast protoplasts, could be greatly facilitated for many yeast species by the pretreatment with thiol compounds. Although most authors have applied 2-mercaptoethanol or dithiothreitol in combination with EDTA and Tris to increase the yield of protoplast formation, the optimal conditions of the pretreatment (concentration of the sulfhydryl compound, pH, duration, temperature) depend on the yeast species and strain, the age and growth conditions of the yeast culture, and the enzyme mixture used to digest the cell wall (reviewed by Davis, 1985; Ferenczy, 1985). In this way, Schwencke *et al.* (1977) overcame the higher resistance of stationary phase cells of *S. cerevisiae* to *in vitro* digestion by snail gut enzymes through pretreating the yeast at a more

alkaline pH and higher dithiothreitol concentrations. Similarly, the efficiency of the treatment with 2-mercaptoethanol and cysteine for rendering baker's yeast digestible for the brine shrimp increased with increasing pH of the treatment medium. Also, the complementary effect of EDTA in the sulfhydryl pretreatment is supported by *in vitro* experiments and has been attributed to the prevention of cell clumping (Duell *et al.*, 1964). Although not widely applied, the pretreatment with cysteine has been shown to increase the rate of protoplast formation in several yeast species, including baker's yeast (Kaneko *et al.*, 1973; Dowhanick *et al.*, 1984). Furthermore, the latter authors concluded from the ineffectiveness of cystine to enhance protoplast formation that sulfur containing compounds should be present in the sulfhydryl form to make the yeast cell wall more susceptible to enzymatic degradation. This may explain the low efficiency of the methionine treatment and further substantiates the hypothesis that sulfhydryl compounds act as reducing agents in modifying the outer cell wall layer (see IV.2.4.). The most effective thiol treatments for rendering baker's yeast digestible for *Artemia* are summarized in Table 14.

Table 14: Effective treatment media for improving the digestibility of baker's yeast for *Artemia*. All treatments imply the incubation of yeast in the treatment medium at a density of 50% (WW/v) for 30 min at 30 °C.

sulfhydryl reagent	other compounds	initial pH
2-mercaptoethanol, 2% (v/v)	EDTA 0.05 M	9
2-mercaptoethanol, 0.02% (v/v)	-	12
L-cysteine.HCl, 0.01 M	-	12

IV.4. CHARACTERIZATION OF THE STANDARD CYSTEINE-TREATED BAKER'S YEAST ("C-YEAST")

IV.4.1. Rationale

The previous study identified a number of simple chemical treatments which improve the digestibility of baker's yeast for *Artemia* (IV.3.4., Table 14). The cysteine treatment was selected for the routine preparation of yeast for feeding experiments, because the toxicity of 2-mercaptoethanol hindered its application for preparing relatively large amounts of yeast. In the present section, the effect of the standard cysteine treatment on the dry weight and particle size of yeast cells is documented. Furthermore, preservation of the treated yeast as a frozen pellet is proposed. Finally, the nutritional value of the preserved "C-yeast" is demonstrated in a short term *Artemia* culture test.

IV.4.2. Materials and methods

IV.4.2.1. Standard cysteine treatment[†] and preservation of the "C-yeast"

The standard cysteine treatment consisted of suspending caked baker's yeast at a density of 50% (wet weight/volume) in a solution of L-cysteine hydrochloride (0.05 M), previously adjusted to pH 12 with NaOH. The latter treatment medium was derived from the cysteine treatment presented earlier (Table 14) by increasing the cysteine concentration in order to avoid limiting treatment conditions. After mixing for 1 min in a kitchen blender, the yeast suspension was again adjusted to pH 12 and incubated for 30 min at 30 °C. In the course of the incubation, pH normally declined to a value of 10.5-10.8 and was finally neutralized with HCl. The treated yeast was harvested by centrifugation (5 min at 3,000 rpm) and rinsed twice with filtered seawater. The thus prepared "C-yeast" was preserved as a pellet at -22 °C or under a dried form (provided by Artemia Systems N.V.-S.A., Belgium).

[†]: protected by International Patents PCT/BE 89/00009 and EP-89870040.6 (old 09.03.89) "Feed for Aquaculture" filed in Europe, USA, Japan, Canada, Australia (various file numbers; pending: owned by Artemia Systems N.V./S.A., Baasrode, Belgium).

IV.4.2.2. Particle size distribution

The particle size spectrum of various yeast preparations was determined by means of a Coulter counter, model Zf, equipped with a 70 μm aperture tube. Yeast cells were suspended in seawater by mixing in a kitchen blender for 1 (fresh yeast) or 5 min (dried yeast) in order to disintegrate the cell clumps. The particle size analysis was performed using the single threshold technique (Sheldon & Parsons, 1967; Anonymous, 1972). The scale of the lower threshold for counting was calibrated in terms of spherical equivalent diameter (SED) by the half-count technique using latex particles (5.02 μm modal size, Coulter Electronics Ltd.) (Anonymous, 1972).

IV.4.2.3. Dry weight analysis

Dry weight of yeast cells was determined by filtering aliquots from yeast suspensions of which the concentration was determined by means of a haemocytometer. Yeast was suspended in deionized water and retained on tared, glass-fiber filters (1 μm pore size). Filters were dried at 60 °C for 24 h and weighed on an analytical balance.

IV.4.2.4. Standard *Artemia* culture test

The present culture test consisted of a simple technique to grow *Artemia* for one week without water renewal. The latter required a minimal sedimentation of the food, which may eventually result in a gradual decrease of the water quality in the course of the 1-week experimental period. Preliminary experiments had shown that the C-yeast was available for a longer period of time in rotating test tubes than in the small scale culture systems which were previously used (aerated Falcon tubes, IV.2.2; shaking culture flasks, IV.3.2.; Fig. 12). The test tubes were mounted to a horizontal rod which rotated over its longitudinal axis at 5 rpm (see V.2.3., Fig. 14A).

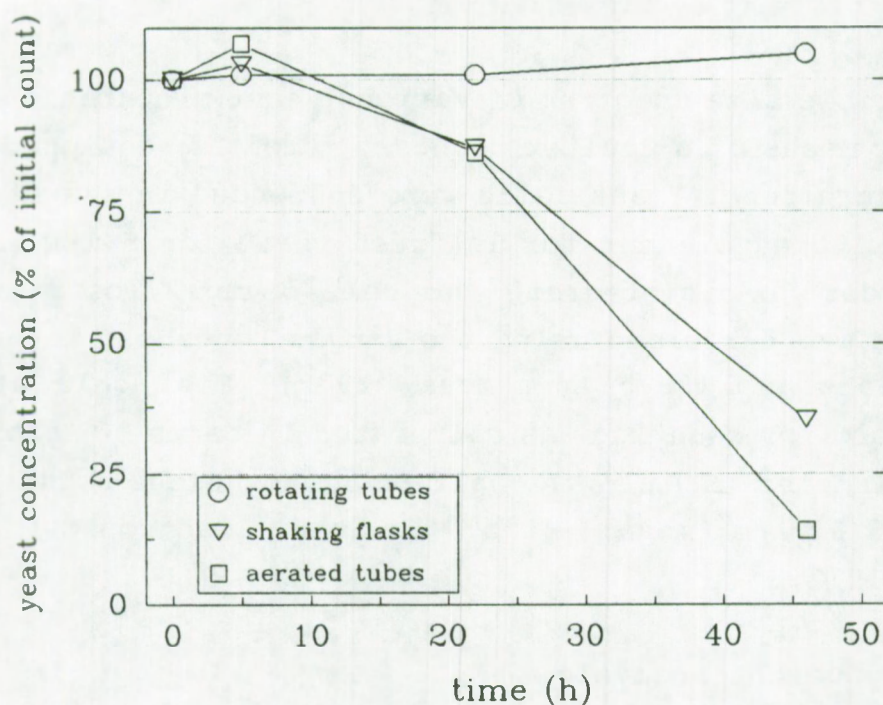


Fig. 12. Change of C-yeast concentration in rotating test tubes, shaking culture flasks, and aerated Falcon tubes under the *Artemia* culture conditions (25 °C, 35 salinity). Initial concentration was approximately 800 cells μl^{-1} . Data represent means from three replicates (CV < 5%).

Artemia franciscana (Great Salt Lake, Utah, USA; Sanders Brine Shrimp Co., lot 185-0) were hatched in 0.2- μm filtered artificial seawater (35 ppt salinity; prepared according to the formula of Dietrich & Kalle, in Kinne 1971) at 25 °C (Sorgeloos *et al.*, 1986). The freshly-hatched nauplii were transferred to capped test tubes (120 ml capacity) containing 90 ml of filtered seawater. The latter stocking density and culture volume resulted in a non-limiting oxygen concentration throughout the experiment, and minimized the mechanical disturbance of the animals due to the toppling movements of the water. The feeding regime for the C-yeast (stored at -22 °C) was derived from that for rearing *Artemia* with *Dunaliella* (see IV.2.2.1.) by replacing one algal cell by 4 (ration Y4, Table 15; experiments ROTOR 1.1-6), 5, 6 or 7 yeast cells (experiment ROTOR 2). After 7 days of culture, survival and mean body length were determined as described in IV.2.2.1.

Table 15: Feeding regime for rearing *Artemia* on C-yeast in the rotating tubes during the first week after hatching (initial density: 40 nauplii (90 ml)⁻¹, ration Y4).

day	daily ration (10 ³ cells individual ⁻¹)	volume of C-yeast stock suspension [72 10 ⁸ cells ml ⁻¹] (μl day ⁻¹)
1	600	330
2, 3, 4	1,200	660
5, 6	1,800	990
7	2,400	1,320
total	10,200	5,610

A first series of experiments (ROTOR 1.1-6) evaluated the consistency of growth and survival of *Artemia* fed the C-yeast in the rotating tubes. Three different batches of C-yeast were tested in six independent growth tests. In a subsequent experiment (ROTOR 2), the effect of the feeding regime on the results of the *Artemia* growth test was investigated by feeding various rations in the course of the experiment ranging from Y4 to Y7. The latter were selected on the basis of a preliminary test which showed decreased growth when feeding lower rations than Y4. Additional treatments were run to estimate survival and growth on the fourth day of culture.

IV.4.3. Results and discussion

The analysis of the particle size distribution showed that the median SED of the dried yeast was approximately 1 μm smaller than that of the fresh treated and untreated yeast, which exhibited both a SED of about 5 μm (Fig 13). This may indicate that the dried yeast did not fully rehydrate upon suspension in seawater. Dry weight per yeast cell did not change due to the standard cysteine treatment nor subsequent storage at -22 °C and thawing at room temperature (Table 16). Furthermore, no cell lysis could be observed in either of the yeast preparations.

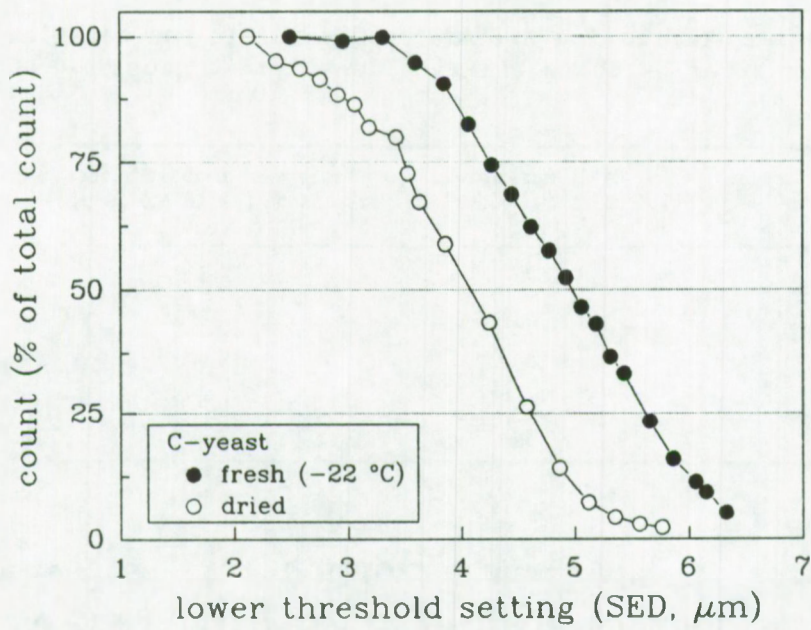


Fig. 13: Cumulative frequency distribution of dried and fresh C-yeast. The particle size distribution of the fresh untreated baker's yeast was similar to the latter and omitted for clarity. Lower threshold setting of the Coulter counter is expressed as spherical equivalent diameter.

Table 16: Dry weight of untreated baker's yeast and C-yeast.

yeast	dry weight (pg cell ⁻¹)
untreated baker's yeast	36.2
C-yeast, freshly-prepared	36.5
C-yeast, stored at -22 °C	36.7

Survival of *Artemia* reared in the rotating tubes averaged 73% after 7 days of culture and was not significantly different between experiments (Table 17). By contrast, average body length varied significantly according to the experiment and ranged between 3.68 and 4.78 mm. The significant differences between growth of *Artemia* fed the same C-yeast suggests that some experimental factors are not fully controlled, despite the high standardization of the experimental set-up and diet. Possibly, minor differences in the timing of the experiment (*e.g.* hatching of the nauplii, feeding, termination) may have contributed to the variation between experiments.

Table 17: Survival and growth of *Artemia* fed C-yeast in the rotating tubes after one week of culture. Data represent means and standard deviations for 6 independent experiments. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD, $P \leq 0.05$).

experiment	n	survival (%)	body length (mm)
1	4	72 \pm 13	3.77 \pm 0.25 ^a
2	6	68 \pm 16	4.26 \pm 0.29 ^b
3	4	78 \pm 3	4.11 \pm 0.25 ^{bc}
4	4	74 \pm 11	4.78 \pm 0.21 ^a
5	4	58 \pm 11	3.68 \pm 0.16 ^c
6	4	85 \pm 7	4.12 \pm 0.06 ^{bc}
ANOVA, $F_{5,20}$		2.48 ns	11.77***
overall mean \pm SD (n=6)		73 \pm 9	4.12 \pm 0.39

The feeding regime did not significantly affect the survival rate of *Artemia* in the standard growth test, although slightly higher survival was observed in the cultures fed the lowest ration (Table 18). Growth was depressed significantly in the initial three days of the experiment in *Artemia* fed the Y6 ration compared to those fed Y4 or Y5. However, final length did not differ significantly according to the feeding regime. These results confirm that the feeding regime based on the replacement of one *Dunaliella* cell by 4 C-yeast cells is satisfying the requirements of *Artemia* during the first week after hatching.

Short term growth tests using *D. tertiolecta* as a food source have been proposed for the characterization of *Artemia* strains (Vanhaecke & Sorgeloos, 1980; Tackaert *et al.*, 1987) and for the study of toxicity, accumulation, and elimination of chemical compounds in the brine shrimp (Canton *et al.*, 1978; Le Roy, 1988). In this regard, the growth test using preserved C-yeast offers an interesting alternative to the latter, as it excludes the necessity for maintaining algal cultures and allows a better control of the food quality.

Table 18: Growth and survival of *Artemia* fed C-yeast according to various feeding regimes. Data represent means and standard deviations from 4 replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD, $P \leq 0.05$).

	DAY 4		DAY 8	
	survival (%)	body length (mm)	survival (%)	body length (mm)
1 day 1-3: Y4	98 ± 3	2.14 ± 0.05 ^a		
2 day 1-7: Y4			78 ± 2	4.11 ± 0.25
3 day 1-3: Y4, day 4-7: Y5			79 ± 9	4.55 ± 0.31
4 day 1-3: Y5	99 ± 1	2.09 ± 0.03 ^a		
5 day 1-7: Y5			71 ± 13	4.39 ± 0.45
6 day 1-3: Y5, day 4-7: Y6			68 ± 11	4.29 ± 0.53
7 day 1-3: Y6	91 ± 9	1.84 ± 0.08 ^b		
8 day 1-7: Y6			73 ± 7	4.41 ± 0.16
9 day 1-3: Y6, day 4-7: Y7			68 ± 20	4.41 ± 0.20
ANOVA, F_{α}	1.14 ns	22.7**	0.67 ns	0.75 ns

significance levels: ns: $P > 0.05$, **: $P \leq 0.01$

IV.5. EVALUATION OF THE DIGESTIBILITY OF CELL WALL DEFECTIVE MUTANTS OF *SACCHAROMYCES CEREVISIAE*

IV.5.1. Introduction and rationale

The rigid cell wall is commonly considered as a barrier to the efficient utilization of yeasts as single-cell protein in human food and animal feed (Tannenbaum, 1968; Kihlberg, 1972; Peppler, 1970). This digestibility problem has been solved by the extraction of proteins from yeast by various methods, such as autolysis, chemical treatment, enzymatic lysis and mechanical disruption (Dunnill & Lilly, 1975). However, these techniques have several disadvantages, including partial hydrolysis of the proteins, large energy requirements and low efficiency. For this reason, microbiologists have attempted to overcome the barrier imposed by the cell wall by the isolation of mutants with defective cell walls. In this regard, cell wall mutants that are more susceptible to enzymatic degradation offer interesting possibilities to improve the digestibility of whole yeast cells as food for filter-feeders.

Mehta & Gregory (1981) reported the isolation of *Candida utilis* and *Saccharomyces cerevisiae* mutants with an increased *in vitro* susceptibility to the digestive enzymes of the snail *Helix pomatia*, an enzyme preparation which is routinely applied for protoplast isolation. Although these authors demonstrated an increased sensitivity of the *S. cerevisiae* mutants to pepsin *in vitro*, the yeast protein of the mutant strains was not utilized more efficiently by rats than that of the parental culture. This lack of correlation between *in vitro* and *in vivo* susceptibility was ascribed by the authors to the relatively high digestibility of the parental culture itself. Nevertheless, the higher glucan/mannan ratio in the cell wall and the increased sensitivity to digestion by snail enzymes without the necessity of a sulfhydryl pretreatment compared to the parental strain, suggest that the mutants of Mehta & Gregory (1981) may have interesting cell wall characteristics for digestion by *Artemia*. The digestive system of the latter is believed to have the same

barrier as the snail enzyme preparation for an efficient digestion of the yeast cell wall, i.e. the permeabilization of the outer mannoprotein layer (see IV.2.4., IV.3.4.).

An alternative approach to the exploitation of yeast protein has been proposed by Venkov and co-workers, who reported the potential application of osmotic-dependent fragile mutants (Venkov & Stateva, 1980; Stateva *et al.*, 1988, Stateva *et al.*, 1990). The latter mutants, which can be grown only in media supplemented with an osmotic stabilizer, lyse and release a large fraction of their cellular proteins (e.g. up to 60%; Stateva *et al.*, 1990) spontaneously upon suspension in water. The cell wall of the fragile mutant VY1160 exhibits an increased permeability due to shortening of the mannan side chains (Venkov & Stateva, 1980) and a reduction of the alkali-soluble glucan (Blagoeva & Venkov, 1990). In this regard, the defective cell wall of VY1160 may be more susceptible to degradation by the digestive enzymes of the brine shrimp. The high osmotic value of seawater, which was found to stabilize yeast protoplasts (see IV.2.), excludes the possible lysis of the fragile mutants in the *Artemia* culture medium. The cultivation of the fragile strain VY1160, which is polyauxotrophic and growing slowly, is only possible in expensive laboratory growth media. However, the construction of polyploid fragile strains which can be propagated in industrial growth media (Stateva *et al.*, 1988, 1990), allows mass-production of fragile yeast.

The present study evaluated the use of the cell wall mutants of Mehta & Gregory (1981) and Venkov & co-workers as a food source for rearing *Artemia*. Besides the evaluation of various yeast strains grown at a small scale on laboratory media, it was attempted to document the effect of industrial culture conditions on the nutritional value of the mutant yeast strains for *Artemia*.

IV.5.2. Materials and methods

IV.5.2.1. *Artemia* culture conditions

The experimental conditions of the growth tests were

identical to those described in IV.3.2.1.

IV.5.2.2. Yeast strains and their cultivation

The *S. cerevisiae* strains, which were kindly provided by Prof. Dr. K.F. Gregory (University of Guelph, Ontario, Canada) and Prof. Dr. P. Venkov (Academy of Sciences, Sofia, Bulgaria), are presented in Table 19. In addition, the industrial strain B1 (provided by Algist Bruggeman, N.V., Belgium) was cultivated as a reference strain to evaluate the yield of the other strains under the same culture conditions.

Table 19: Parental and cell wall defective strains of *S. cerevisiae* evaluated in the present study as food for *Artemia*.

strain	characteristics	reference
OSMOTIC-DEPENDENT FRAGILE MUTANTS (obtained from P. Venkov)		
S288C	wild type parental strain	Venkov <i>et al.</i> (1974)
VY1160	laboratory osmotic-dependent fragile mutant (ATCC 44427) genotype: <i>srbl ts1</i>	"
V174	polyploid osmotic-dependent fragile mutant (triploid) genotype: <i>srbl</i>	Stateva <i>et al.</i> (1988)
V178	polyploid osmotic-dependent fragile mutant (tetraploid) genotype: <i>srbl</i>	"
HELICASE[†]-SENSITIVE MUTANTS (obtained from K.F. Gregory)		
Y5	wild type parental strain (diploid)	Mehta & Gregory (1981)
Y5 α	alpha mating type of Y5	"
RM-2	helicase-sensitive (<i>hs</i>) mutant, haploid	"
RM-2d	diploid for <i>hs</i> mutation, derived from RM-2	"
RM-4	helicase-sensitive mutant, haploid	"
RM-4d	diploid for <i>hs</i> mutation, derived from RM-4	"

†: helicase = enzyme preparation from the gut of the snail *Helix pomatia*

The various yeast strains were cultivated by the company Algist Bruggeman N.V., Belgium. The laboratory cultures were grown either on YPD medium, containing yeast extract (Oxoid L21, 2% w/v), peptone (Oxoid L37, 2% w/v) and sucrose (6% w/v), or the industrial growth medium (MOL), which has molasses as its principal carbon source. For the culture of osmotic-dependent fragile mutants, the osmotic value of the media was increased by the supplementation of 10% sorbitol (YPDS) or 1.6% NaCl (MOLN) to the laboratory and industrial medium, respectively. The cultivation occurred in Erlenmeyer flasks under continuous shaking at 30 °C. The culture of the strains obtained from Prof. Gregory and Prof. Venkov were started with, respectively, a 1% and a 0.03% inoculum, which had been prepared by, respectively, 24-h and 48-h incubation of a loop of an agar-slant culture in 50 ml of the appropriate medium. A limited number of strains was cultivated under industrial culture conditions in a 20 l Chemap fermentor on the molasses medium (MOL or MOLN). The yeast cells were harvested at stationary growth phase by centrifugation and rinsed three times with filtered seawater prior to use. The yield was determined as the total wet weight of yeast harvested per culture.

IV.5.2.3. Experimental design

The design of the various experiments evaluating the mutant strains of *S. cerevisiae* are detailed in Table 21.

A first experiment (GREG 1) compared growth and survival of *Artemia* fed the various helicase-sensitive mutants, derived from stationary phase cultures on YPD medium, with that of animals fed the corresponding parental strains or C-yeast (see IV.4.). The experiment was repeated for RM-4 and Y5 α , harvested at the late-stationary phase (GREG 2). Furthermore, the effect of the industrial culture conditions on the nutritional value of RM-4 was evaluated.

The effect of osmotic-dependent fragility on the digestibility of yeast for *Artemia* was investigated by comparing the nutritional value of the parental strain S288C and the mutant

strain VY1160, which were both grown under the same conditions (YPDS, shaking culture; VENK 1). *C*-yeast or *D. tertiolecta* was used as a reference diet (VENK 1 and 2, respectively). The nutritional value of the polyploid fragile strains, grown at a laboratory scale on either the YPDS or the MOLN medium, was compared with that of the laboratory fragile mutant in experiment VENK 3. Finally, growth and survival of *Artemia* fed the polyploid strains, which were propagated in the Chemap fermentor, was compared with that of animals fed the algal control diet (VENK 4).

IV.5.3. Results

The strains isolated by Mehta & Gregory (1981) exhibited poor yields in the shaking cultures, i.e. maximally 50% of the yield obtained for the industrial baker's yeast strain B1 under the same culture conditions (Table 20). An extremely poor growth was observed for the diploid helicase-sensitive strains, which may have been degenerated due to the long storage (freeze-dried since 1974; Gregory, pers. comm., 1988). Furthermore, the yield of the RM-4 strain in the Chemap fermentor was only 25% of that routinely obtained for the B1 strain (Saveyn, pers. comm.). All helicase-sensitive strains formed buds which were delayed in separating from the parental cells. This was also observed by Mehta & Gregory (1981).

By contrast, the fragile mutant VY1160 and its parental strain attained up to 70 and 87%, respectively, of the yield of the B1 strain (Table 20). The yield of the polyploid fragile strains in the Chemap fermentor (VENK 4) amounted to 70-75% of that of the B1 strain (Saveyn, pers. comm.). For experiment VENK 3, the yeast cultures were harvested after 16 h of incubation, which was appropriate for the faster growing polyploid strains to attain stationary phase. The low yield found for VY1160 indicated that the latter was still in the early exponential growth phase.

Table 20: Yield of the shaking cultures for the different *S. cerevisiae* strains.

experiment	strain	medium (incubation period)	yield (g WW (300 ml growth medium) ⁻¹)
GREG 1	Y5α	YPD (26 h)	3.3
	Y5	"	3.2
	RM-2	"	2.5
	RM-2d	"	0.7
	RM-4	"	3.9
	RM-4d	"	1.1
GREG 2	Y5α	YPD (48 h)	3.6
	RM4	"	4.1
VENK 1	VY1160	YPDS (43 h)	4.7
	S288C	"	6.9
VENK 2	VY1160	"	5.6
VENK 3	VY1160	YPDS (16 h)	0.7
	V174	YPDS (16 h)	3.0
	V174	MOLN (16 h)	4.7
	V178	YPDS (16 h)	3.4
	V178	MOLN (16h)	4.7
reference	B1	YPD (43 h)	7.8
	B1	MOL (43 h)	7.9

Growth of *Artemia* fed either of the helicase-sensitive yeast strains was significantly lower compared to those fed the C-yeast (GREG 1, Table 21). However, the RM-4 strain yielded significantly higher growth and survival than the corresponding parental haploid strain Y5α. The low performance of the RM-2 strain was due to its very limited availability for *Artemia*, caused by its fast flocculation and subsequent settling in the culture vessels. The diploid wild type strain Y5 resulted in a significantly higher growth and survival of *Artemia* than the haploid analogue, but still yielded inferior growth compared to the helicase-sensitive counterpart (RM-4d). The difference in nutritional value between Y5α and RM-4 was confirmed in experiment GREG 2, although the late-stationary phase yeast resulted in lower survival compared to the previous test. By contrast, RM-4 which was produced in the Chemap fermentor gave *Artemia* culture results similar to those found in test GREG 1 for the same strain derived from shaking cultures (GREG 2).

Table 21: Growth and survival in *Artemia* fed various cell wall mutants of *S. cerevisiae*, cultivated either on laboratory media (YPD, YPDS) or on industrial molasses media (MOL, MOLN). Data represent means and standard deviations from 6 replicates. Unlike superscripts denote significant differences among means (F_s : ANOVA, Tukey HSD; t_s : t-test).

treatment	DAY 4		DAY 8	
	survival (%) [†]	body length (mm) [‡]	survival (%) [‡]	body length (mm)
experiment GREG 1 (YPD: 26 h shaking culture) (n=6)			$F_s = 11.68^{***}$	$F_s = 51.63^{***}$
1 C-yeast ¹	97 ± 3	1.98	79 ± 8 ^a	3.78 ± 0.32 ^a
2 Y5 α	86 ± 4	1.48	34 ± 25 ^b	2.01 ± 0.15 ^e
3 Y5	91 ± 4	1.46	68 ± 19 ^a	2.55 ± 0.21 ^d
4 RM-2	85 ± 9	1.19	23 ± 10 ^b	1.32 ± 0.09 ^f
5 RM-2d	97 ± 3	1.51	68 ± 7 ^a	2.71 ± 0.22 ^{cd}
6 RM-4	91 ± 7	1.63	64 ± 11 ^a	2.95 ± 0.25 ^{bc}
7 RM-4d	93 ± 5	1.65	70 ± 15 ^a	3.19 ± 0.14 ^b
experiment GREG 2 (YPD: 48 h shaking culture; MOL: Chemap fermentor) (n=6)			$t_s = 3.82^{**}$	$t_s = 3.68^{**}$
1 Y5 α (YPD)	87 ± 6	1.33	0	-
2 RM-4 (YPD)	83 ± 10	1.68	32 ± 14	3.68 ± 0.33
3 RM-4 (MOL)	95 ± 2	1.47	69 ± 19	3.12 ± 0.18
experiment VENK 1 (YPDS: 43 h shaking culture) (n=4)			$F_s = 3.25$ ns	$F_s = 170.0^{***}$
1 C-yeast ¹	-	-	85 ± 7	4.12 ± 0.06 ^a
2 VY1160 (YPDS)	-	-	68 ± 13	3.52 ± 0.15 ^b
3 S288C (YPDS)	-	-	66 ± 10	2.53 ± 0.10 ^c
experiment VENK 2 (YPDS: 43 h shaking culture) (n=4)			$t_s = 1.06$ ns	$t_s = 7.70^{***}$
1 <i>Dunaliella tertiolecta</i>	90 ± 4	1.80	95 ± 10	5.00 ± 0.22
2 VY1160 (YPDS)	90 ± 8	1.77	87 ± 10	4.02 ± 0.14
experiment VENK 3 (YPDS and MOLN: 16 h shaking culture) (n=5)			$F_s = 22.79^{***}$	$F_s = 200.9^{***}$
1 <i>Dunaliella tertiolecta</i>	94 ± 6	1.82	91 ± 11 ^a	4.05 ± 0.12 ^a
2 VY1160 (YPDS)	89 ± 6	1.86	79 ± 12 ^{ab}	3.01 ± 0.16 ^c
3 V174 (YPDS)	80 ± 8	1.42	37 ± 9 ^c	1.67 ± 0.15 ^e
4 V174 (MOLN)	95 ± 3	1.95	92 ± 7 ^a	3.44 ± 0.16 ^b
5 V178 (YPDS)	83 ± 3	1.49	69 ± 15 ^b	1.97 ± 0.15 ^d
6 V178 (MOLN)	91 ± 10	1.61	84 ± 5 ^{ab}	3.13 ± 0.14 ^c
experiment VENK 4 (MOLN: Chemap fermentor) (n=5)			$F_s = 73.69^{***}$	$F_s = 7.86^{**}$
1 <i>Dunaliella tertiolecta</i>	90 ± 8	1.39	83 ± 17 ^a	3.99 ± 0.32 ^a
2 V174 (MOLN)	82 ± 12	1.44	48 ± 6 ^b	2.17 ± 0.18 ^b
3 V178 (MOLN)	85 ± 15	1.55	52 ± 18 ^b	2.54 ± 0.20 ^b

significance levels: ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$

†: survival day 1 = 100%

‡: survival day 4 = 100%

§: intermediate length was measured on the culled animals per treatment on day 4

1: caked baker's yeast treated according to the standard cysteine treatment (see IV.4.)

Brine shrimp fed the fragile yeast strain VY1160 grew significantly better than those fed the wild type strain (VENK 1), although growth remained inferior to that obtained when feeding C-yeast (VENK 1) or *D. tertiolecta* (VENK 2). Survival in the *Artemia* cultures fed VY1160 did not differ significantly from that observed in the control treatments (VENK 1, 2, 3). The relative performance of VY1160 compared to that of the algal control diet was independent of its growth phase (VENK 2 and 3: stationary and log phase, respectively). The polyploid fragile strains yielded significantly lower *Artemia* survival (V174) and growth (V174, V178) than VY1160 when grown in the YPDS medium (VENK 3). However, the *Artemia* culture results significantly improved when the polyploid strains were grown on the molasses medium. In the latter case, feeding V174 yielded similar survival and growth of 85% of that observed in the algae-fed controls. Surprisingly, this result could not be confirmed for the polyploid strains cultivated in the Chemap fermentor (VENK 4). Although the same molasses medium was applied, survival and growth of *Artemia* fed V174 or V178 amounted to only 60% of that observed in the algae-fed animals.

Faecal material of *Artemia* feeding on the mutant yeast strains consisted for a large fraction of empty cell walls. As opposed to the granular appearance of faeces produced by brine shrimp fed the C-yeast, these cell walls still retained the shape of the yeast cell.

IV.5.4. Discussion

The present study demonstrated that the nutritional value of helicase-sensitive and osmotic-dependent fragile yeast mutants was higher for *Artemia* than the corresponding wild type parental strains. The nutritional value of any diet for a filter-feeding organism depends on various factors, such as availability in the water column, particle size, digestibility, and the biochemical composition. In this way, survival and growth of *Artemia* fed the RM-2 strain was strongly limited because the yeast cells could not be efficiently ingested. However, if the physical

characteristics allow an efficient uptake, the nutritional value of baker's yeast is primarily restricted by its low digestibility (see IV.2.). Furthermore, it is improbable that the nutritional composition of a cell wall mutant and its parental strain, cultivated under the same conditions, deviate strongly with regard to nutrients that are limiting the growth of *Artemia*. For this reasons, the increased sensitivity to helicase and the osmotic-dependent fragility of the examined mutants is most likely associated with an increased digestibility of the cell wall for the brine shrimp. The modified cell wall structure reported for the mutants, i.e. increased permeability due to shortening of the mannan side chains (Venkov & Stateva, 1980), a reduction of the alkali-soluble glucan (Blagoeva & Venkov, 1990), and the higher glucan/mannan ratio (Mehta & Gregory, 1981), is in agreement with the belief that the outer mannoprotein layer is the main barrier for an efficient digestion of baker's yeast by *Artemia*.

The presence of apparently intact, though empty cell walls in the faeces of *Artemia* feeding on mutant yeast strains, suggests a similarity between the digestion mechanism for these strains and that of untreated yeast. The latter was observed to lose its cell contents during gut passage in a similar way, although at a much lower frequency. The intact cell walls of digested yeast cells may indicate that the digestive enzymes are capable of penetrating the cell wall at weak sites, but are unable to degrade its skeletal structures consisting of glucan fibrils. In the same way, *in vitro* studies have shown that yeast protoplasts normally emerge through a pore in the cell wall, which is produced by the action of a lytic enzyme, whereas only prolonged incubations result in a total wall digestion (Davis, 1985). The acellular appearance of the faecal material of *Artemia* fed thiol-treated yeast (see IV.2.) further substantiates the efficient digestion of the cell wall due to the increased exposure of the glucan layer to enzymatic action.

The evaluation of cell wall defective mutants to overcome the low digestibility of baker's yeast for the brine shrimp may be regarded as a test case, which documents some of the potential

problems encountered when applying mutant single-cell proteins. Despite the significant effect of the cell wall mutations on the yeast digestibility, the culture results remained inferior to those obtained by feeding the thiol-treated baker's yeast. Furthermore, the nutritional value of the cell wall mutants was affected by the composition of the growth medium and the yeast culture conditions. In this way, the promising results obtained with the polyploid fragile strains, which were derived from shaking cultures, could not be confirmed when the same strains were cultivated in the Chemap fermentor. An additional problem of mutant yeasts was demonstrated for the helicase-sensitive mutants, which exhibited unsuitable growth characteristics for industrial propagation.

Nevertheless, cell wall mutants offer the possibility to study the genetic basis for the low digestibility of the yeast cell wall. Venkov and co-workers associated the osmotic-dependent fragility of the VY1160 mutant with two mutations, *srbl* and *tsl*, responsible for the modified mannan structure and reduced alkali-soluble glucan, respectively (Venkov & Stateva, 1980; Blagoeva & Venkov, 1990). Similar sets of mutations have been identified in two other fragile strains (Venkov & Stateva, 1980). Further research is needed to identify which of these mutations is responsible for the improved digestibility of VY1160 for the brine shrimp. Possibly, the single mutation (*srbl*), present in the polyploid strains V174 and V178, was not sufficient for enhancing digestion when the latter were grown on the same medium as VY1160. In this regard, polyploids containing multiple mutations, such as the "superfragile" strain 211 (*srbl srb2 tsl*, Stateva *et al.*, 1990), may synthesize a cell wall which is digestible for the brine shrimp.

Chapter V

STUDY OF FEEDING AND GROWTH IN *ARTEMIA* FED BAKER'S YEAST USING THE ELECTRONIC CELL COUNT METHOD

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Chapter V

STUDY OF FEEDING AND GROWTH IN *ARTEMIA* FED BAKER'S YEAST USING THE ELECTRONIC CELL COUNT METHOD

V.1. INTRODUCTION

Feeding represents the greatest of all interactions between an animal and its environment in terms of energy or mass flow (Spomer, 1973) and is thus a key factor in production in nature as well as in artificial rearing systems. The crucial role of secondary production in aquatic ecosystems has prompted many researchers to describe and measure the feeding process of zooplankton. In order to obtain measurements relevant to the field, aquatic ecologists have attempted to study feeding and grazing under laboratory conditions which are approximating those encountered in nature as good as possible. Consequently, the extent to which artificial conditions which prevail in most aquaculture systems, e.g. high animal densities and elevated levels of non-algal food particles, affect feeding of zooplankton is poorly documented. In this way, the use of yeasts to study feeding kinetics in zooplankton has been mainly limited to some pioneering studies applying baker's yeast (Rigler, 1961; Sushchenya, 1962), and field experiments in which radioactively labelled *Rhodotorula* is added to natural lake water (Burns & Rigler, 1967; Haney, 1971). This gap in the knowledge of zooplankton feeding behavior is further illustrated by a citation from Peters (1984): "Although most of us are not interested in the behavior of starving animals feeding on unpalatable and monotonous foods, crowded into small beakers, etc., these effects can bias our results. We should put more efforts into the evaluation of such effects."

The present study attempted to contribute to the knowledge required to apply yeast diets as a food source for intensive production of *Artemia*. Furthermore, it aimed at providing

information which may explain and possibly improve the currently applied culture conditions in systems for intensive rearing of the brine shrimp, which have been adopted based as a result of trial and error culture experiments. For this, the effect of food concentration on feeding was studied for various developmental stages of *Artemia*. The effect of food concentration on growth was verified in an 8-days culture experiment during which *Artemia* was reared at various constant food levels. In addition, the extent to which various environmental parameters, i.e. animal density, water quality, mechanical disturbance, and light intensity, affect feeding of brine shrimp was investigated by simulating a range of these factors in small scale grazing experiments. Finally, the differential digestibility of treated and untreated baker's yeast was exploited to evaluate the effect of food digestibility on the feeding kinetics of a filter-feeding organism.

V.2. MATERIALS AND METHODS

V.2.1. *Artemia*

The experiments were performed with two strains of *Artemia franciscana*, one originating from the Great Salt Lake, Utah, USA (GSL; Sanders Brine Shrimp Co., lot 31627) and one from San Francisco Bay, California, USA (SFB; San Francisco Bay Brand, lot 1090). The animals were grown in the laboratory as described in Chapter VII on a 25/75% mixed diet of *Dunaliella tertiolecta* Butch and dried yeast. In addition, adult *Artemia* (7-8 mm body length) were obtained from 300 l intensive cultures (Lavens, 1989) and acclimated to the experimental food for at least two days prior to the experiment.

V.2.2. Yeast diets

The yeast diet generally consisted of commercially available baker's yeast which was provided under a caked form by the company Algist Bruggeman N.V., Belgium. The yeast was chemically

treated according to the standard cysteine treatment (C-yeast, see IV.4.) and preserved under a dried form (provided by Artemia Systems, N.V.-S.A., Belgium) during the first series of experiments (V.2.4.1.). This dried C-yeast was resuspended by mixing with seawater in a kitchen blender for 5 min. The stability of the yeast under the experimental conditions could be improved by storing the freshly-treated yeast as a pellet at -22 °C (see V.2.3., Fig. 15A). After thawing, the yeast pellet was mixed in seawater for 30 sec to break cell clusters. A new suspension of the same batch of C-yeast was thus prepared daily. Experiments evaluating the ingestion rates of *Artemia* feeding on treated and untreated yeast were performed with freshly-prepared treated yeast to avoid different effects of storage on both types of yeast.

In addition, the differential ingestion by *Artemia* of treated and untreated yeast was verified for two industrial strains of *S. cerevisiae* (B1, R5; provided by Algist Bruggeman, N.V., Belgium). The yeasts were grown on YNBG medium in shaking cultures and harvested in stationary phase, as described in VI.2.2.

V.2.3. General methodology of the grazing experiment

All experiments were performed at 25 ± 1 °C with 0.2- μ m filtered artificial seawater, prepared according to the formula of Dietrich & Kalle (1963, in Kinne, 1971). The *Artemia* were harvested from the culture, rinsed with filtered seawater and transferred to a standard concentration of the C-yeast for 24 h. Prior to the experiment, animals of approximately the same size and viability were distributed into the grazing containers and allowed to feed at the experimental conditions for at least one hour. Since preliminary experiments revealed significantly higher feeding rates in females than in males, the sex ratio was kept constant in all replicates of the experiments using adult brine shrimp. Depending on the requirements of the tests, *Artemia* were allowed to graze in the experimental containers for periods ranging from 1 to 6 h.

Depending on the nature of the parameter that was investigated, various experimental containers were used. The effects of light, water quality, age, and animal density were evaluated in 170 ml test tubes, which contained 120 ml of yeast suspension. The tubes were mounted on a horizontal rod which rotated over its longitudinal axis at 5 rpm (Fig. 14A). At low animal densities (e.g. ≤ 0.5 adults ml^{-1}), the oxygen concentration remained above 90% of saturation during the course of the experiment. The effect of mechanical disturbance was simulated in 800 ml cylindro-conical tubes by applying different aeration intensities (Fig. 14B). In order to measure the effect of crowding on feeding rate without interference from changes of water quality and oxygen concentration, a 1,000 ml recirculation system was designed in which the animal density could be varied independently of the volume of food suspension available per animal. The animals were transferred to a grazing chamber which was mounted in a cylindro-conical tube filled with 1,000 ml of yeast suspension (Fig. 14C). An aeration point and an air-lift pump at the bottom of the tube maintained the food in suspension and provided a constant supply of food and oxygen to the grazing chamber. The latter consisted of a hard PVC tube which was cut at an angle of about 30° to prevent the accumulation of air bubbles at the bottom consisting of 200 μm nylon mesh. The volume of the grazing chamber could be varied between 30 and 175 ml by adjusting the depth of submersion in the food suspension (Fig. 14C). The recirculation system was furthermore used to evaluate the effect of yeast concentration and digestibility on the feeding rate in adult *Artemia*.

At 1-2 h time intervals duplicate samples were taken from each experimental container, diluted with filtered seawater to give approximately 60 cells μl^{-1} , and counted by means of a Coulter counter, model Zf. The latter was calibrated for counting baker's yeast using the plateau method. The raw counts were corrected for the background count in filtered seawater and coincidence (Sheldon & Parsons, 1967; Anonymous, 1972).

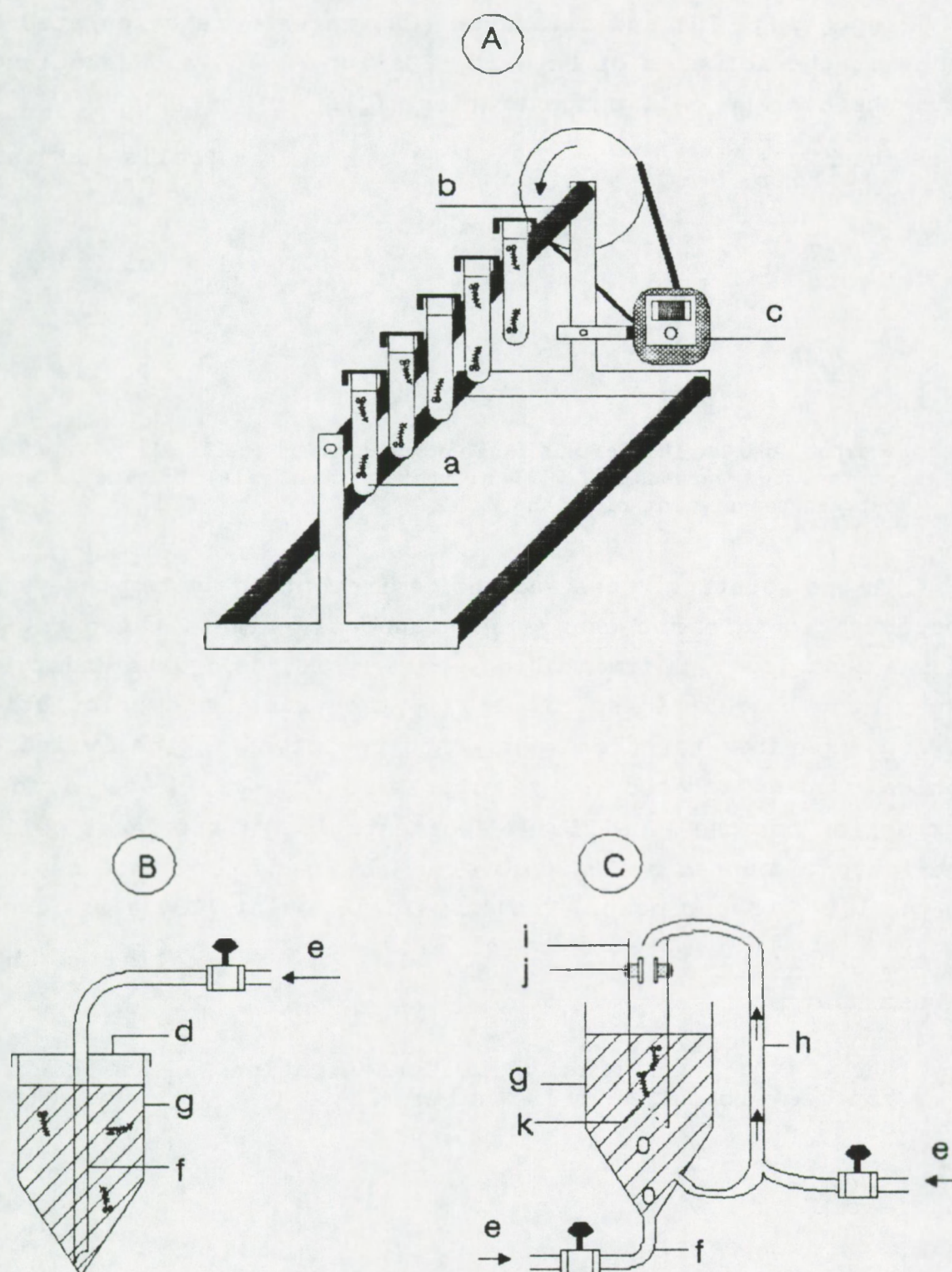


Fig. 14: Culture containers used for the grazing experiments.

A: rotating tube, B: aerated cylindro-conical tube, and C: recirculation system.

a: screw-capped tube, b: rotating rod, c: electrical motor, d: perforated petridish, e: adjustable air supply, f: aeration point, g: cylindro-conical glass tube (1,000 ml capacity), h: air-lift pump, i: grazing chamber (adjustable height), j: fixing clip, k: 200 μm mesh bottom.

Ingestion (IR) and clearance (CR) rates were calculated by means of the formulas of Hayward & Gallup (1976) and associated with the average cell concentration (C_m).

$$IR = \frac{(C_0 - C_t) V}{n t} \quad [\text{cells ind}^{-1} \text{ h}^{-1}]$$

$$CR = \frac{IR}{C_m} \quad [\text{ml ind}^{-1} \text{ h}^{-1}]$$

$$C_m = \frac{C_0 + C_t}{2} \quad [\text{cells ml}^{-1}]$$

where C_0 and C_t : initial and final concentration (cells ml^{-1}); V : volume of the food suspension (ml); n : number of animals; t : time interval between measurement of C_0 and C_t .

In the rotating tubes and the recirculating system the yeast concentration did not change spontaneously within the duration of the test due to sedimentation, cell lysis, or growth under the experimental conditions (Fig 15). By contrast, the calculation of feeding rates for the experiments performed in the cylindro-conical tubes aerated at a rate of 0.2 l min^{-1} , required a correction for the significant sedimentation of the yeast cells. The latter amounted to 10-20% over a 4 h period. For this reason, in the latter experiments, the formula of Poulet (1973) was used:

$$IR = \frac{(C'_t - C_t) V}{n t} \quad [\text{cells ind}^{-1} \text{ h}^{-1}]$$

where C'_t : mean final cell concentration in the control bottles without animals (cells ml^{-1}).

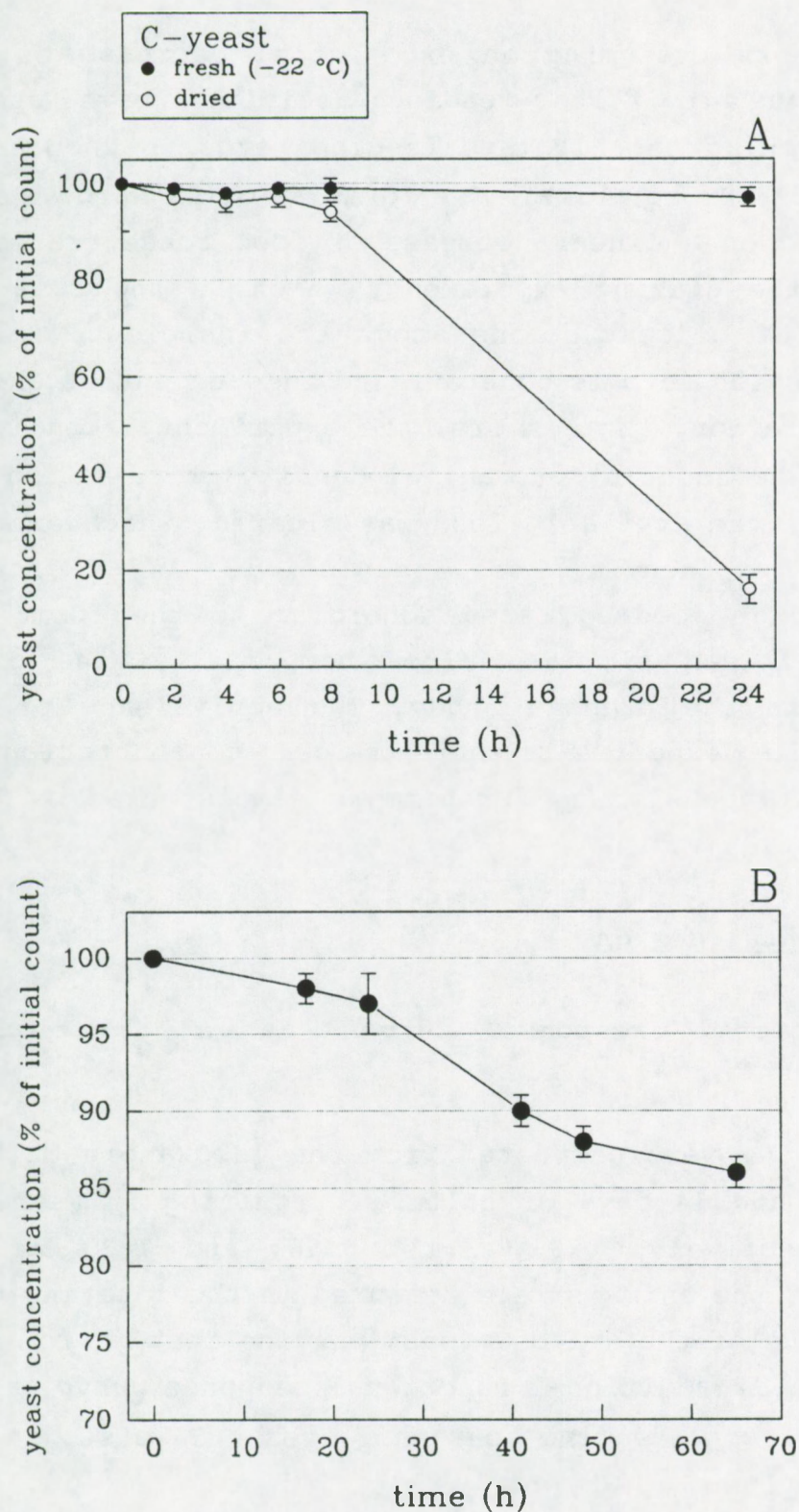


Fig. 15: Spontaneous change of cell concentration in the rotating tubes (A) and the recirculation system (B) as a function of time for either dried or fresh (after storage at -22°C) C-yeast. Experimental conditions were identical to those of the grazing tests (25°C , 35 ppt salinity) and the initial yeast concentration was approximately $800 \text{ cells } \mu\text{l}^{-1}$.

Other authors, assuming an exponential decrease of cell concentration due to filter-feeding activity, have applied logarithmic formulas to calculate feeding rates in zooplankton (e.g. Gauld, 1951; Frost, 1972). The formulas of Hayward & Gallup (1976) are based on a linear decrease of food concentration in the course of the grazing experiment, which is the case when feeding occurs at concentrations above the incipient limiting level when ingestion rate is constant and independent of changes in cell concentration. Furthermore, the experimental conditions (i.e. time, cell concentration, animal density) were set so that less than 25% of the available food was cleared, which excludes a significant exponential cell depletion. As a result, calculation of the feeding rates according to the formula of Frost (1972) yielded similar values compared to the direct formulas used in the present study. The equivalence of both formulas in feeding experiments where the cell concentration does not change spontaneously was furthermore demonstrated by Tackx & Van De Vrie (1985).

V.2.4. Experimental design

V.2.4.1. Functional response curves as a function of developmental stage

Artemia (GSL) were harvested from the laboratory cultures after 2, 5, 8, and 14 days of culture, resulting in mean body sizes of, respectively, 0.91, 2.43, 5.66, and 7.23 mm. The feeding rates of the animals were measured in the rotating tubes at concentrations of the dried C-yeast ranging mostly from 50 to 1,300 cells μl^{-1} . An additional functional response curve was run with adult *Artemia* feeding on the fresh C-yeast in the recirculation system.

V.2.4.2. Effect of culture conditions on feeding rate

The experiments evaluating the effect of various environmental parameters on the feeding rate were performed with

adult *Artemia* (GSL, unless otherwise stated) at food saturating conditions, *i.e.* yeast cell concentrations remained above 600 cells μl^{-1} during the experiment. The tests were mostly run with three replicates, and repeated in time with animals derived from independent cultures. The following parameters were altered in single variate experiments and kept at a standard level when not the object of experimentation.

animal density

A standard animal density of 0.08 adults ml^{-1} was adopted. A first attempt to study the effect of crowding on feeding rate was made by varying the number of animals in the rotating tubes to give densities between 0.08 and 3 adults ml^{-1} . The improved design of the recirculation system allowed to evaluate animal densities ranging from 1 to 8.3 adults ml^{-1} , which were attained by varying the volume of the grazing chamber containing between 200 and 250 animals.

water quality

The short term influence of reduced water quality on the feeding rate was studied by exposing *Artemia* (GSL or SFB) either to ionized ammonia, nitrite, or nitrate at concentrations ranging from 1 to 1,000 ppm. The nitrogen compounds were added to the filtered seawater through dissolving, respectively, NH_4Cl , NaNO_2 , and NaNO_3 . It was assumed that nitrification and ammonia excretion was negligible due to the short duration of the experiment and the relatively low animal densities applied. At the experimental conditions (25 °C, 35 ppt, pH 8.2), about 5% of the ammonia was present under the form of free ammonia (Huguenin & Colt, 1989).

mechanical disturbance

The gentle agitation of the culture medium in the rotating bottles and the recirculation system could only be altered to a limited degree by changing, respectively, the speed of rotation and the flow rate of the air-lift pump. By contrast, a weak, moderate, and strong mechanical disturbance of the animals was

attained by applying air flow rates of, respectively, 0.2, 2, and 7 l min⁻¹ in the cylindro-conical tubes.

light intensity

All experiments were run in the dark, except when light intensity was the parameter tested. In the latter case, the experiment was performed under fluorescent light with an intensity of approximately 3,000 lux. Feeding rate was measured for *Artemia* grazing in the transparent rotating tubes, whether or not obscured by means of black plastic foil.

V.2.4.3. Effect of yeast digestibility on feeding rate

The effect of the yeast treatment on the ingestion rate was determined by feeding adult *Artemia* (GSL) on various mixtures (0/100, 25/75, 50/50, 75/25, 100/0) of treated and untreated fresh baker's yeast at saturating food concentrations in the recirculation system. The experiment was partially repeated for the treated and untreated laboratory-grown yeast.

V.2.4.4. Effect of yeast concentration on growth

This experiment attempted to determine the optimal food concentration for maximal growth in the first 8 days of culture for *Artemia* (GSL) fed the C-yeast. Initially, 200 freshly-hatched nauplii were transferred to the recirculation system in 1,000 ml of yeast suspensions with a concentration ranging from 200 to 2,000 cells μl^{-1} . Each concentration was run with 5 replicates. During 8 days, the cell concentration was determined twice daily and replenished to the target concentration. To limit lysis and sedimentation of yeast cells that remained in the system, the total yeast suspension was renewed on day 3 and 6 of the culture. Furthermore, the deviation from the target concentration was restricted by reducing the number of animals per culture unit to 100 and 30 on day 3 and 6, respectively. Body length (see IV.2.2.3.) and survival were determined after 3, 6, and 8 days of the culture.

V.3. RESULTS

V.3.1. Functional response curves as a function of developmental stage

The functional response curves for four developmental stages of *Artemia* feeding on the dried yeast are presented in Fig. 16. The parameters of ingestion and clearance, derived by fitting the rectilinear model to the ingestion rate data, are given in Table 22.

Table 22: Parameters of the rectilinear models fitted by the least squares method to the ingestion rate data presented in Fig. 16 and 17. Y represents ingestion rate (10^3 cells $\text{ind}^{-1} \text{h}^{-1}$), X represents yeast concentration (cells μl^{-1}). The rectilinear model is given by:

$$X < \text{ilc}: Y = aX (+ a')$$

$$X > \text{ilc}: Y = b$$

Artemia age (days of culture)	body length (mm) (mean \pm SD, n=30)	parameters		ilc [†] (cells μ l ⁻¹)	df	r ² ‡	
		a (ml ind ⁻¹ h ⁻¹)	b (10 ³ cells ind ⁻¹ h ⁻¹)				
DRIED C-YEAST (rotating tubes)							
2	0.91 \pm 0.07	0.0213	11.6	543	14	0.90	
5	2.43 \pm 0.51	0.936	132	141	5	0.85	
8	5.66 \pm 0.49	5.72	442	77	6	0.90	
14	7.23 \pm 0.96	7.77	637	82	5	0.81	
FRESH C-YEAST (recirc. system)		a	a'				
14	-	0.597	133	428	495	30	0.93
15	-	0.639	122	437	437	22	0.79

†: incipient limiting concentration, calculated as the intercept of the two regression equations

‡: df and r² are specified for the regression equation $Y = aX (+ a')$

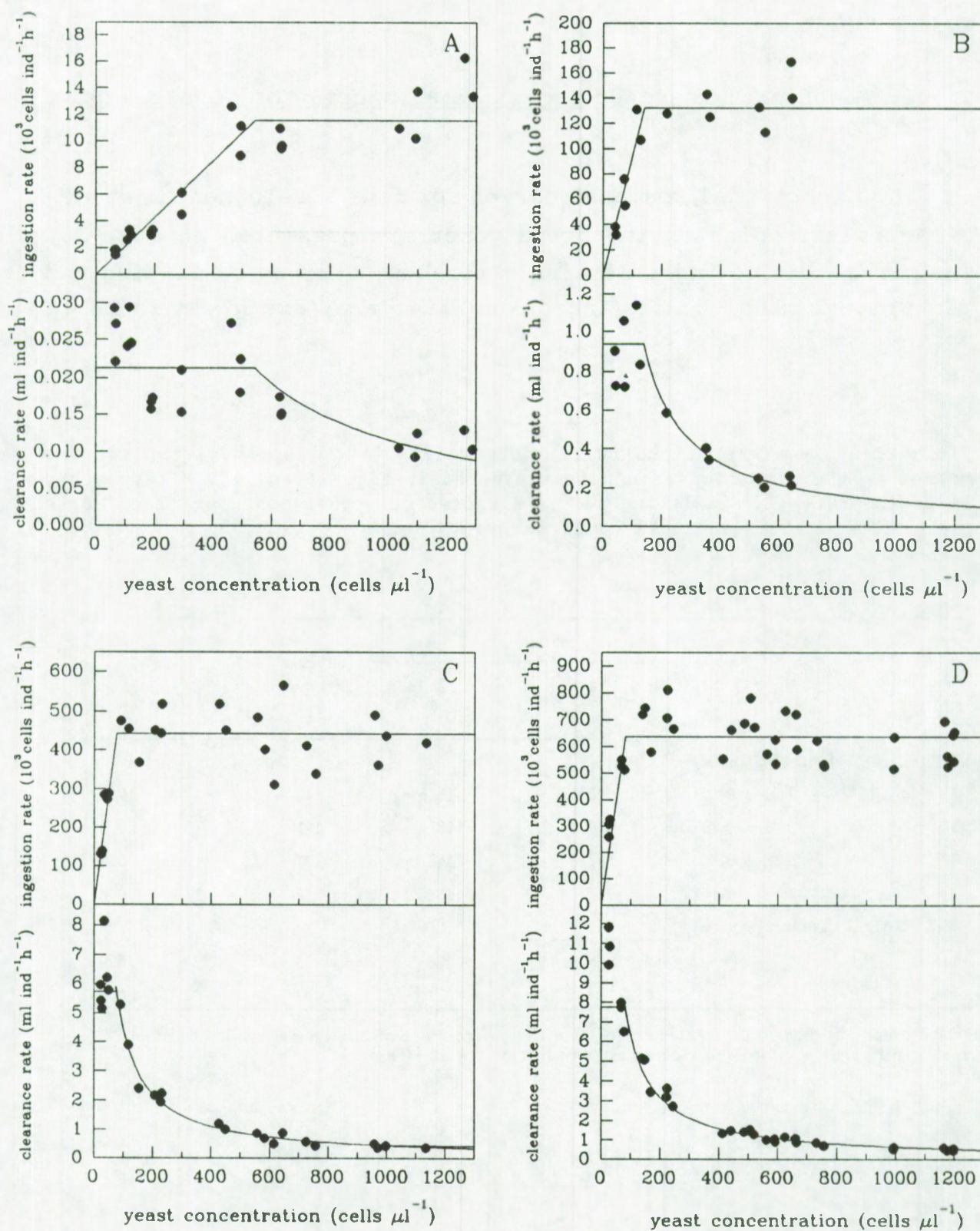


Fig. 16: Ingestion and clearance rate as a function of the concentration of dried C-yeast for *Artemia* at four different ages (A, B, C, D: day 2, 5, 8, 14, respectively). The parameters derived from the rectilinear model (—) are given in Table 22.

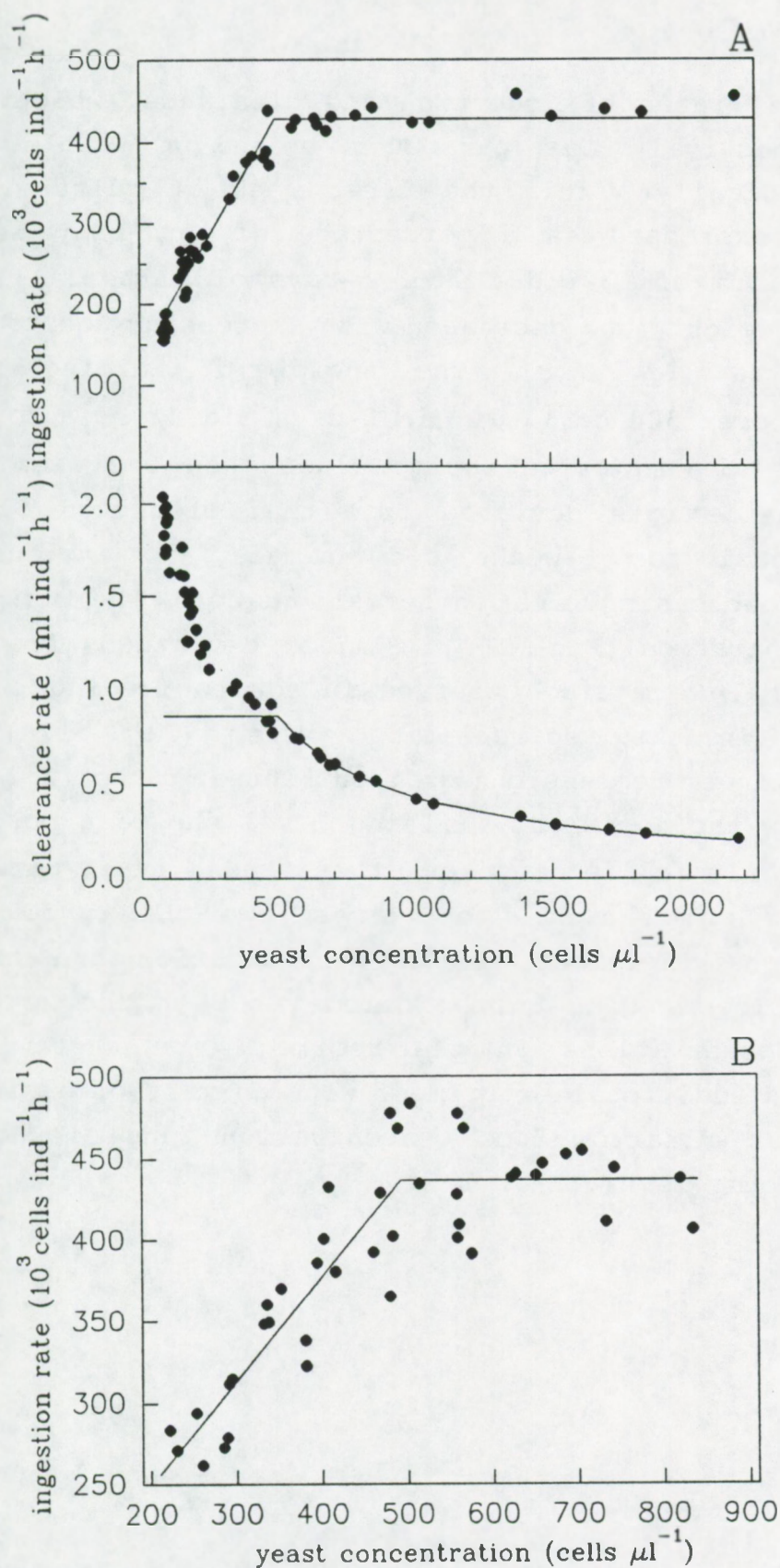


Fig. 17: Ingestion and clearance rate of adult *Artemia* in the recirculation system as a function of the concentration of fresh C-yeast. A and B represent data from two independent experiments. The parameters derived from the rectilinear model (—) are given in Table 22.

The maximal rates of ingestion and clearance, which were computed, respectively, as parameter b and a (Table 23), increased drastically within the first week of culture. The feeding rates expressed as a percentage of dry body weight attained a maximum of $630\% \text{ day}^{-1}$ for 5-days old *Artemia* ($17 \mu\text{g}$ individual dry weight) and decreased with increasing age to $170\% \text{ day}^{-1}$ for adults (Fig. 18). The incipient limiting level decreased from over $500 \text{ cells } \mu\text{l}^{-1}$ in 2-days old *Artemia* to less than $100 \text{ cells } \mu\text{l}^{-1}$ in brine shrimp older than one week. The measured clearance rate deviated from that predicted by the rectilinear model for 2-weeks old animals, as it further increased with decreasing cell concentrations below the incipient limiting concentration (Fig. 16D). Similar deviations from the rectilinear model were also observed in adults feeding on the fresh C-yeast in the recirculation system (Fig. 17A). In addition, the linear regression equation fitted to the ingestion rate data below the incipient limiting level showed a positive y-intercept. *Artemia* feeding on the fresh yeast in the recirculation system showed 2 to 3 times lower clearance rates and a 5-fold higher incipient limiting concentration than animals of a similar size feeding on the dried yeast in the rotating bottles. The observations in the recirculation system were confirmed in an additional experiment with animals of a similar size feeding at a narrow food concentration rang around the incipient limiting level (Fig. 17B).

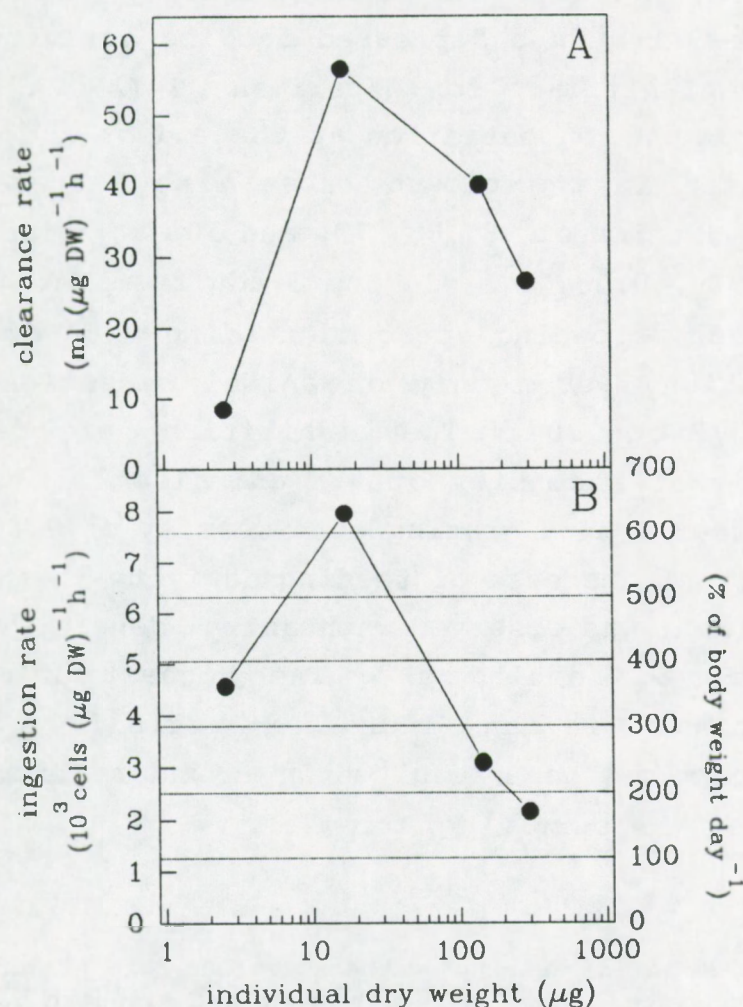


Fig. 18: Weight-specific maximal rates of clearance (A) and ingestion (B) in *Artemia* as a function of individual body weight. Dry body weight (DW, μg) was computed from body length (L, mm) by means of the regression equation of Abreu-Grobois *et al.* (1991):

$$DW = 10^{0.47 + 1.63 \log L + 0.81 (\log L)^2}$$

V.3.2. Effect of culture conditions on feeding rate

animal density

The effect of crowding largely depended on the experimental set-up. In the rotating tubes, no significant differences in feeding rate were observed in the range of 0.08 to 1 adult ml⁻¹

(Fig. 19, ANOVA, $P > 0.10$). The highest density examined, i.e. 3 adults ml^{-1} , resulted in a depressed feeding activity, although this was only significant for experiment 2 (ANOVA, Tukey HSD, $P \leq 0.05$). The oxygen concentration at the end of the 4-h feeding period decreased in the closed tubes with increasing animal density (O_2 saturation of 98, 88, 70, and 30% at animal densities of, respectively, 0.08, 0.5, 1, and 3 adults ml^{-1}).

By contrast, crowding affected feeding rate of *Artemia* in the recirculation system only at animal densities above 6.7 adults ml^{-1} (Fig. 20). The inhibition of feeding was instantaneously at a density of 8.3 animals ml^{-1} , but was only significant after 2 h of grazing at a density of 7.6 ml^{-1} (Table 23). A significant decrease of feeding activity in the course of the 6-h experiment was observed when animal density was equal to or larger than 6.7 adults ml^{-1} . The highest animal density examined resulted in an increased mortality during the experiment, which may have resulted in an underestimation of the feeding rate of the surviving *Artemia*.

Table 23: Effect of animal density on the ingestion rate of adult *Artemia* in the recirculation system. Data represent means and standard deviations from 3 replicates for experiment I. Unlike superscripts denote significant differences among means within periods (ANOVA, Tukey HSD, $P \leq 0.05$).

animal density (ind ml^{-1})	ingestion rate (10^3 cells ind $^{-1}$ h $^{-1}$)			F_0	survival (%) 0-6 h
	period 1 (0-2 h)	period 2 (2-4 h)	period 3 (4-6 h)		
4.0	570.6 \pm 17.2 ^a	557.6 \pm 62.7 ^a	520.5 \pm 63.5 ^a	0.74 ns	95.5 \pm 1.0
6.7	603.6 \pm 35.0 ^a	560.3 \pm 29.7 ^a	463.6 \pm 65.4 ^a	7.25*	91.2 \pm 3.5
7.6	525.2 \pm 59.3 ^{ab}	382.7 \pm 26.0 ^b	252.7 \pm 66.2 ^b	19.50**	89.9 \pm 2.9
8.3†	444.0 \pm 39.0 ^b	365.4 \pm 26.9 ^b	260.4 \pm 59.0 ^b	-	65.5 \pm 2.1
F_0	6.83*	18.33***	12.70**		

†: n=2, one replicate with only 16% survival excluded
significance levels: ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$

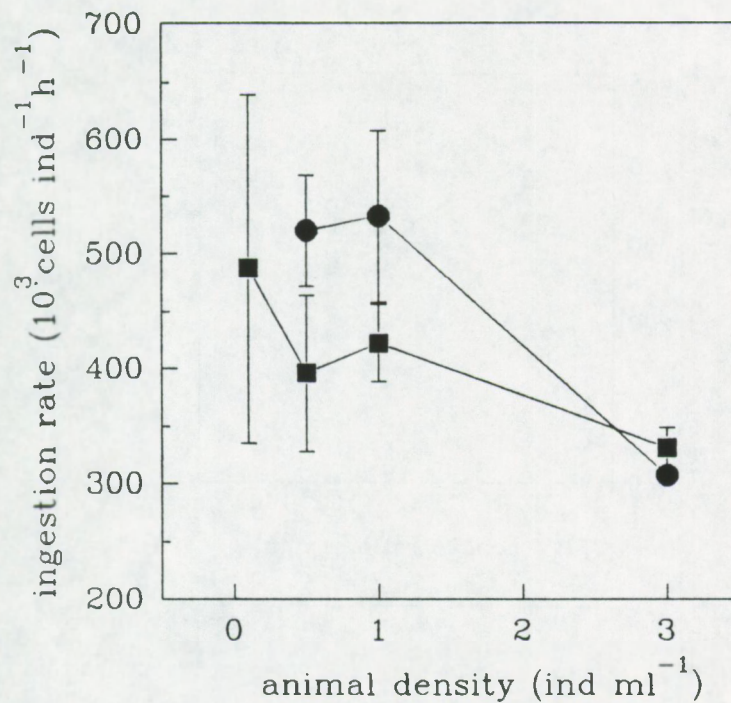


Fig. 19: Ingestion rate in *Artemia* as a function of animal density in the rotating tubes. Data represent means and standard deviations from 3 replicates for two independent experiments.

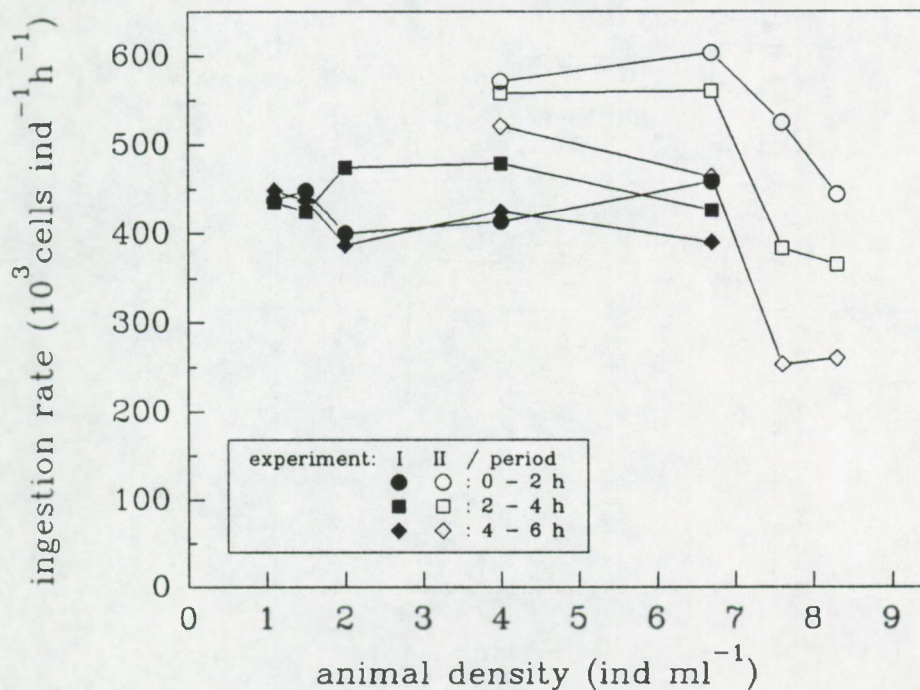


Fig. 20: Ingestion rate in *Artemia* as a function of animal density in the recirculation system. Data represent means and standard deviations from 2 (Exp. I) or 3 (Exp. II) replicates for three consecutive 2-h feeding periods.

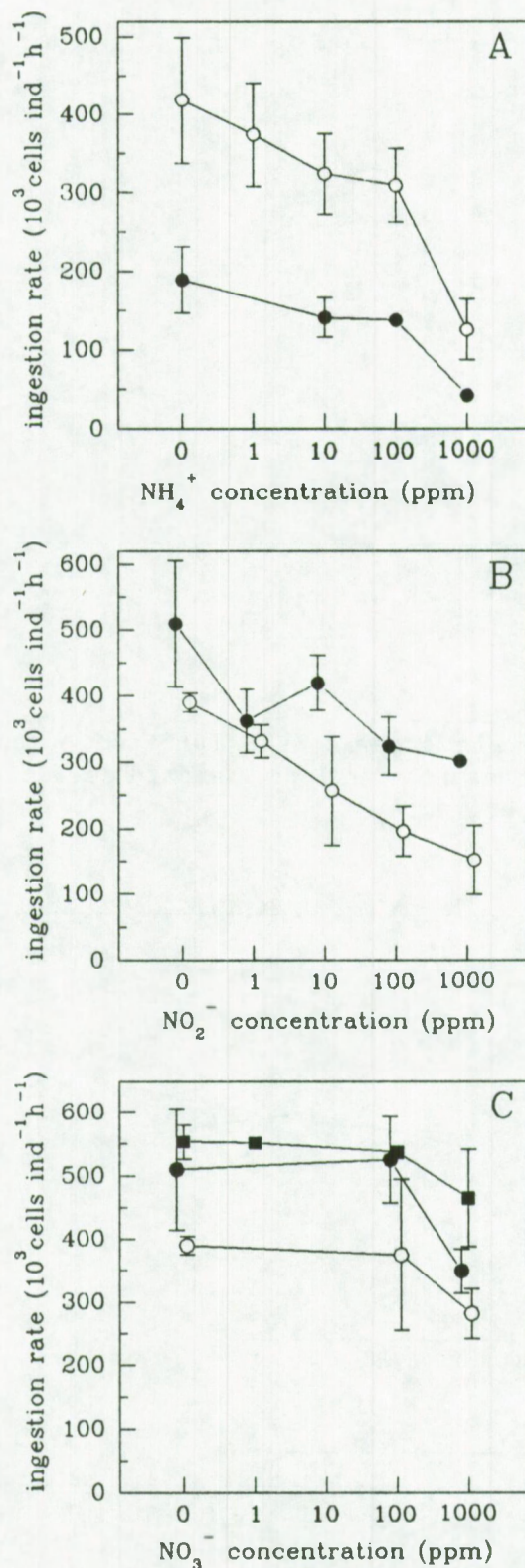


Fig. 21: Ingestion rate in adult *Artemia* (GSL and SFB: filled and hollow symbols, respectively) as a function of ionized ammonia (A), nitrite (B) and nitrate (C) concentration. Data represent means and standard deviations (indicated when larger than symbols) from 3 replicates for independent experiments.

water quality

Ionized ammonia affected feeding rate during the relatively short exposure periods of the grazing test significantly only at the highest concentration tested, *i.e.* 1,000 ppm (Fig. 21A; ANOVA, Tukey HSD, $P \leq 0.05$). Nitrite concentrations of 100 and 1,000 ppm resulted in significantly depressed ingestion rates compared to the controls (Fig. 21B; ANOVA, Tukey HSD, $P \leq 0.05$), whereas nitrate did not significantly influence feeding rate within the concentration range examined (Fig 21C, ANOVA, $P > 0.05$).

mechanical disturbance

Artemia fed at a maximal rate when the culture medium was aerated at the intermediate intensity (Fig. 22). Significantly lower feeding rates (*t*-test, $P \leq 0.05$) were found when applying lower (experiment 1 & 2) or higher (experiment 1) air flow rates during a 4-h feeding experiment. Survival after 24 h of aeration was higher than 93%, irrespective of the aeration intensity.

light intensity

No significant difference could be detected between the ingestion rate of *Artemia* grazing in the dark or at a light intensity of 3,000 lux (respectively, 413 ± 94 and $408 \pm 61 \cdot 10^3$ cells $\text{ind}^{-1} \text{h}^{-1}$; $t_s = 0.083$ ns).

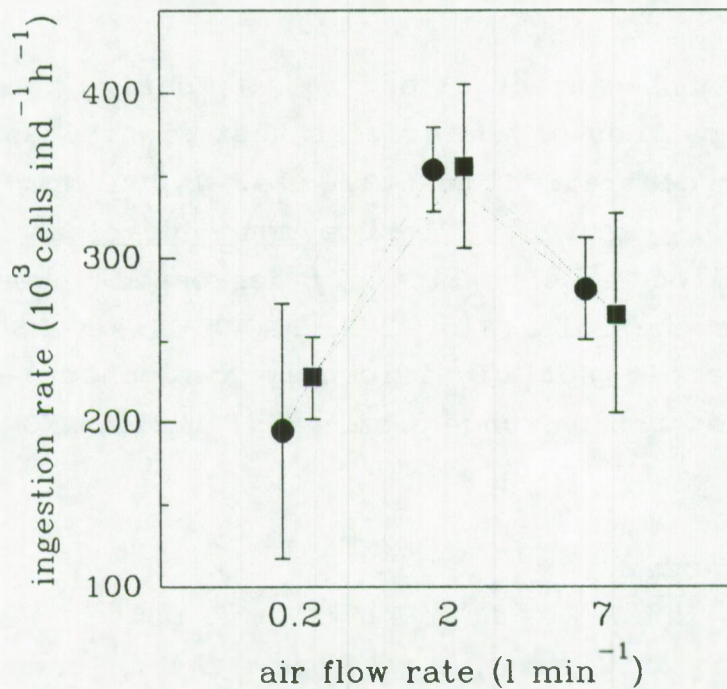


Fig. 22: Ingestion rate of adult *Artemia* as a function of aeration intensity in the cylindro-conical tubes. Data represent means and standard deviations from three replicates for two independent experiments (1:●, 2:■).

V.3.3. Effect of yeast digestibility on feeding rate

The C-yeast was cleared from suspension 40 to 60% more efficiently than the untreated baker's yeast, whereas the mixtures of both yeast types were ingested at intermediate rates (Fig. 23). Microscopic observations revealed the release of intact yeast cells from the faeces of *Artemia* fed the untreated yeast. Since these cells are returned to suspension, the feeding rate measured for the untreated yeast by the cell count method may have been underestimated. This was further demonstrated for the laboratory grown yeasts where no significant decrease in cell concentration could be observed during 6-h feeding experiments with untreated yeast (Table 24). In terms of cell numbers, the treated laboratory-grown yeasts were ingested at about 50% of the rate with which the C-yeast was consumed (Table 24).

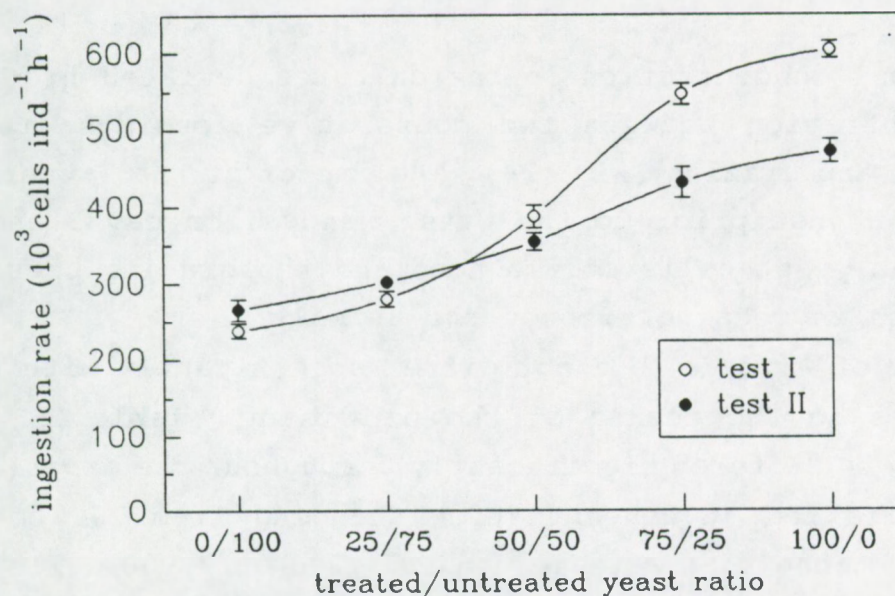


Fig. 23: Ingestion rate in adult *Artemia* fed various mixtures of C-yeast and untreated caked baker's yeast. Data represent means and standard deviations from 3 replicates.

Table 24: Ingestion rate of adult *Artemia* fed various treated and untreated yeast strains (FBY= commercially available caked baker's yeast; B1, R5= strains cultured at laboratory scale, "C-" denotes chemically treated yeasts). Data represent means and standard deviations from 3 replicates for three independent 6-h feeding experiments.

YEAST TYPE	ingestion rate (10 ³ cells ind ⁻¹ h ⁻¹)		
	TEST 1	TEST 2	TEST 3
FBY	-	-	143 ± 63
C-FBY	643 ± 31	514 ± 21	477 ± 22
B1	N.D.	-	N.D.
C-B1	301 ± 30	260 ± 7	231 ± 60
R5	N.D.	-	-
C-R5	384 ± 26	280 ± 16	-

N.D.= not detectable

V.3.4. Effect of yeast concentration on growth

The actual concentration in the cultures deviated from the target concentration between two consecutive feedings due to sedimentation and grazing (Fig. 24). The impact of the latter was more important just prior to the water renewal on day 3 and 6, as part of the yeast cells became aged and the grazing pressure increased with increasing size of the animals.

Survival of *Artemia* did not differ significantly with food concentrations in the course of the experiment (Table 25). By contrast, growth differed significantly throughout the experiment between *Artemia* fed at concentrations ranging from 200 to 800 cells μl^{-1} and those that were reared at 1,200 or 2,000 cells μl^{-1} (Fig. 25). No significant difference in final body length could be detected between *Artemia* feeding on either of the latter two yeast concentrations (Table 25).

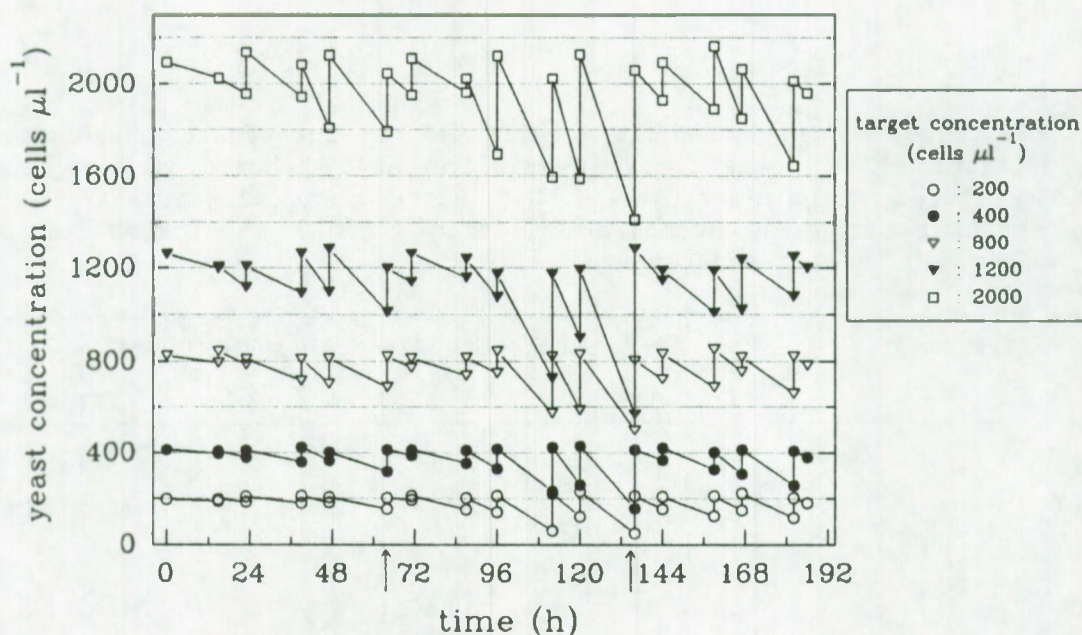


Fig. 24: Change of the actual cell concentration as a function of time in the *Artemia* cultures fed at various concentrations of the C-yeast. Data represent means and standard deviations from 5 replicates. Vertical arrows indicate the moment of water renewal and reduction of the number of animals per culture unit.

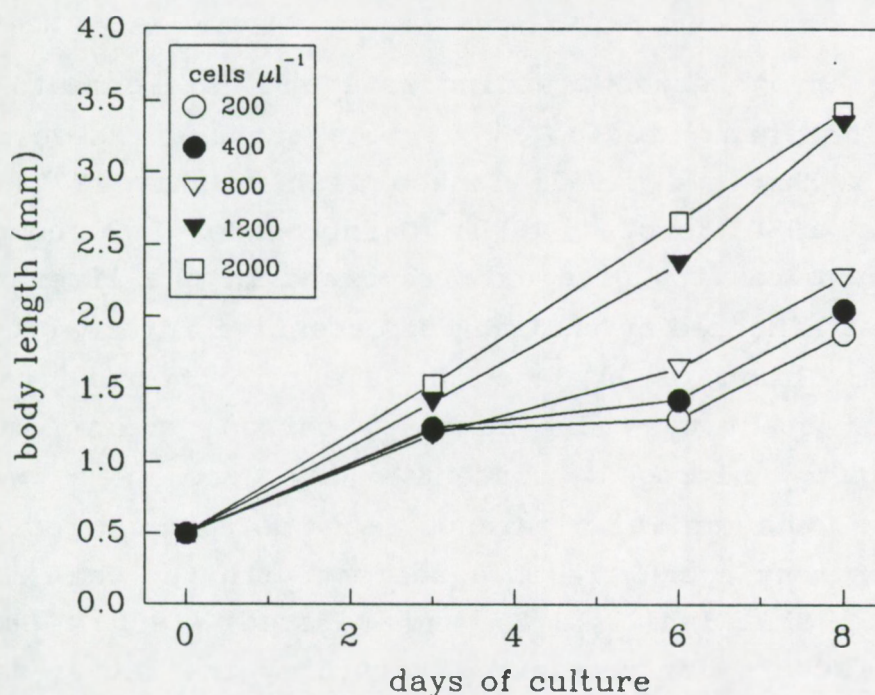


Fig. 25: Growth of *Artemia* fed at various constant concentrations of C-yeast in the recirculation system.

Table 25: Survival and growth of *Artemia* reared at various concentrations of C-yeast in the recirculation system. Data represent means and standard deviations from 5 replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD, $P \leq 0.05$).

	TARGET CONCENTRATION (cells μl^{-1})					ANOVA
	200	400	800	1,200	2,000	F_{α}
SURVIVAL (%)						
day 1-3	92.2 \pm 2.2	90.4 \pm 3.3	89.4 \pm 2.5	90.4 \pm 4.2	90.2 \pm 3.8	0.49 ns
day 3-6	92.0 \pm 2.7	87.0 \pm 6.9	91.0 \pm 4.1	89.8 \pm 4.4	87.6 \pm 3.4	1.13 ns
day 6-8	85.6 \pm 6.5	88.6 \pm 3.8	89.2 \pm 5.5	83.8 \pm 4.1	79.6 \pm 11.2	1.66 ns
BODY LENGTH (mm)						
day 3	1.22 \pm 0.06 ^c	1.23 \pm 0.07 ^{bc}	1.19 \pm 0.10 ^c	1.41 \pm 0.07 ^{ab}	1.54 \pm 0.14 ^a	12.74***
day 6	1.30 \pm 0.05 ^d	1.43 \pm 0.15 ^d	1.65 \pm 0.08 ^c	2.36 \pm 0.09 ^b	2.66 \pm 0.09 ^a	203.86***
day 8	1.89 \pm 0.10 ^c	2.05 \pm 0.15 ^{bc}	2.27 \pm 0.25 ^b	3.34 \pm 0.11 ^a	3.43 \pm 0.28 ^a	72.00***

significance levels: ns: $P > 0.05$, ***: $P \leq 0.001$

V.4. DISCUSSION

The relation between feeding rate and developmental stage of *Artemia* has been studied by various authors (Reeve, 1963a; Sushchenya & Khmeleva, 1967; Yanase & Shiraishi, 1972; Braun, 1980; Nimura, 1980; Korstad, 1990). Unfortunately, the comparison of the present results with data reported in the literature is in many cases hindered by the lack of essential information (e.g. animal size; Braun, 1980) and the use of different units to express food quantity (cell numbers, carbon) and animal size (body length, nitrogen content, dry and wet weight). Nevertheless, the maximal clearance rates determined for *Artemia* at four different ages were in agreement with the data reported by Yanase & Shiraishi (1972) and Korstad (1990). Based on conversion factors derived from literature, the latter data and the present results were used to compute an allometric relationship between maximal clearance rate and dry body weight in *Artemia* (Fig. 26). Similar equations have been proposed for the relationships between ration size and body size in various crustacean filter-feeders, including *Artemia* (Sushchenya & Khmeleva, 1967) and between the clearance rate and body size in bivalve molluscs (reviewed by Winter, 1978; Malouf & Bricelj, 1989; Bricelj & Shumway, 1991; see III.2.4.3.).

The variation of the weight-specific rates of filtration and feeding as a function of age, with a maximum observed for 5-days old *Artemia*, is corroborated by the findings of other authors. Yanase & Shiraishi (1972) found that the weight-specific feeding rates decreased with growth, attained a minimum at about instar III, reached a maximum at instar VII-VIII and subsequently decreased in the course of the further development. For *Artemia* ranging from 0.74 to 3.94 mm body length, maximal weight-specific ingestion rates were observed for 0.89 mm animals (Korstad, 1990). Also, the maximal daily rations ranging from 173 to over 600% of body weight for, respectively, 14 and 5-days old *Artemia*, are realistic in comparison with literature data. Nimura (1980) demonstrated that 3 and 10 mm brine shrimp could daily ingest, respectively, 794 and 102% of their body mass. A daily ration

between 255 and 55% was reported by Sushchenya & Khmeleva (1967) for *Artemia* in the range of 35-6,000 μg wet weight. Nauplii of *Calanus helgolandicus* consumed 292-481% of their body weight, whereas this was 23-85% for adult females (Paffenhöfer, 1971).

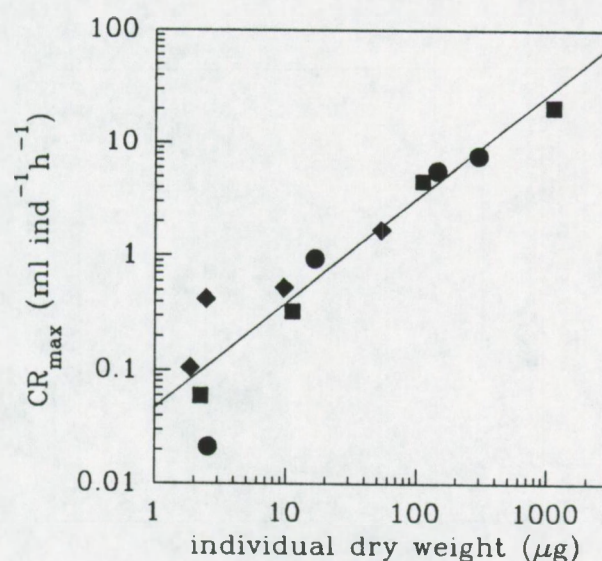


Fig. 26: Maximal clearance rate (CR_{max}) as a function of animal size in *Artemia*. Body length (L: present study, ■; Korstad, 1990, ◆) and nitrogen content (NW: Yanase & Shiraishi, 1972, ●) were converted to dry weight (DW) using, respectively, the equation of Abreu-Grobois *et al.* (1991, see Fig. 18) and the conversion factor $\text{NW/DW}=0.09$ (Peters & Downing, 1984). The allometric equation, computed by linear regression of the log transformed data, is given by:

$$\text{CR}_{\text{max}} = 0.047 \text{ DW}^{0.918} \quad (r^2=0.89).$$

The decrease of the incipient limiting level with increasing body size confirmed the findings of Reeve (1963a) and Nimura (1980) for brine shrimp feeding on *Phaeodactylum tricornutum* and *Chlamydomonas sp.*, respectively. In the present study, the critical concentration at which the ingestion rate reached a maximal value, corresponded with the concentration at which the clearance rate started to decline, except for the oldest stage examined. For adult *Artemia*, the critical concentration for the clearance rate may have been lower than the lowest food concentration tested. In the same way, Reeve (1963a) found an

extremely low critical concentration for clearance in 10 mm *Artemia* (Fig. 27). The lower incipient limiting concentration for younger stages implicates that the food levels in intensive *Artemia* cultures should be maintained at higher levels during the first week to maximize feed uptake.

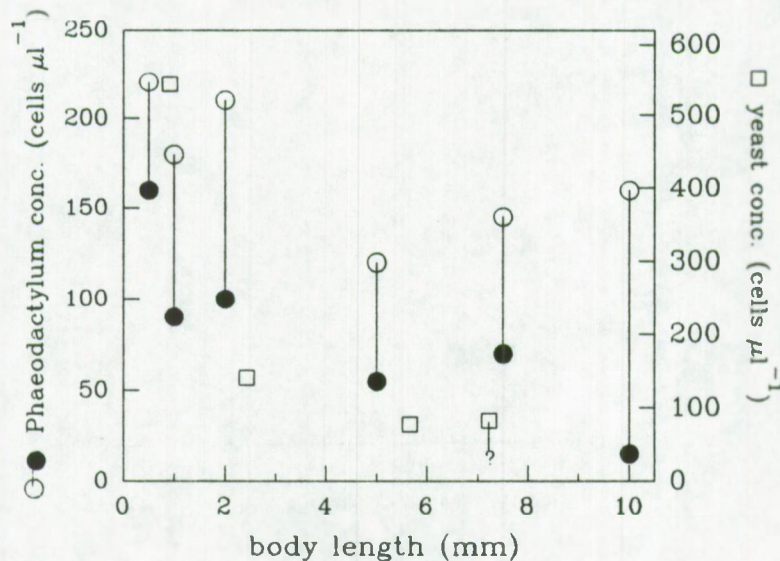


Fig. 27: Incipient limiting concentration as a function of body length in *Artemia* fed *Phaeodactylum tricornutum* (data from Reeve, 1963a) and dried C-yeast. Different critical concentrations for ingestion (O) and clearance (●) rate for the same animal size are connected with a vertical line. ? indicates an undefined critical concentration for clearance rate.

The differences between the functional response curves determined for *Artemia* of similar size feeding either in the rotating bottles on the dried yeast or in the recirculation system on the fresh C-yeast could have been due to the differences in the food type and/or environmental conditions. The smaller particle size of dried yeast cells (see IV.4.2.2.) compared to fresh yeast cells, may explain the relatively lower ingestion rates measured in *Artemia* feeding on the latter. However, the observation of a 6-fold higher incipient limiting concentration for the fresh yeast conflicts with the generally accepted inverse relationship between the size of algal cells and

the critical concentration for feeding in *Artemia* (Reeve, 1963b; Yanase & Shiraishi, 1972) as well as calanoid copepods (Frost, 1974). Possibly, the physical conditions in the recirculation system (e.g. water current created by the air-lift pump) may have caused a lower filtration efficiency and, as a result, a higher critical concentration compared to the animals grazing in the rotating tubes.

In the extensive literature on zooplankton feeding and filtering rates (reviewed by Omori & Ikeda, 1984; Peters, 1984), few authors have investigated the influence of animal density. Multiple regression analysis of published feeding rates showed that the total volume of water used in experiments and the volume allowed per animal was positively correlated with feeding rate in, respectively, calanoid copepods and cladocerans (Peters & Downing, 1984). The latter authors suggested that cladocerans interfere with each other when crowded, while calanoids may be inhibited by contact with the walls of the experimental vessel. The present study demonstrated the importance of the methodology applied to evaluate the effects of crowding on feeding rate. Whereas oxygen depletion and/or accumulation of excretory products resulted in depressed feeding rates in the closed rotating tubes at densities of 3 adult *Artemia* ml⁻¹, crowding did not affect feeding in the open recirculation system up to densities of 6.7 adults ml⁻¹. The depressed feeding activity and increased mortality, occurring respectively at 7.6 and 8.3 adults ml⁻¹, may be ascribed to the relatively small volume of the grazing chamber which resulted in a frequent contact of the animals with the walls and the mesh bottom. In this regard, it is interesting to note that the experimental evidence for feeding rate inhibition in crowded daphnids was generated just by increasing the number of animals in a certain volume of food suspension (Hayward & Gallup, 1976; Helgen, 1987), and thus does not exclude possible interference from increased concentrations of excretory products and oxygen stress. Furthermore, Helgen (1987) showed that water preconditioned with crowded *Daphnia* depressed feeding rates of uncrowded *Daphnia* after six hours, and attributed this to a possible "auto-allelopathic effect...", if

it is not the result of, for instance, the excretion of ammonia".

The high resistance of *Artemia* to crowding is further supported by the reports of animal densities of up to 18 adult brine shrimp ml^{-1} in large scale cultures fed *Chaetoceros curvisetus* (Tobias *et al.*, 1980). Recently, Abreu-Grobois *et al.* (1991) evaluated density effects on growth of *Artemia* fed *Dunaliella tertiolecta* in a culture system with a similar design as the present recirculation system, which allowed to exclude side-effects from changes in water quality and maintain constant densities throughout the culture test. The latter authors found maximal growth during a 2-weeks experiment irrespective of animal density ranging between 2 and 16 ml^{-1} . By contrast, intensive *Artemia* cultures fed micronized agricultural by-products showed an increased mortality and reduced growth rate during the second week of culture if the rearing densities exceeded 10 animals ml^{-1} (Lavens, 1989), which may again be attributed to the interference from suboptimal culture conditions.

Feeding rate of adult brine shrimp was not very sensitive to short term exposure to inorganic nitrogen compounds. Concentrations of up to 1,000 ppm of ionized ammonia, which was present for about 5% under the form of the toxic free ammonia, were required to inhibit feeding significantly after 4 hours. This agrees with the measurements of high ammonia levels (up to 50 ppm) in intensive *Artemia* cultures (Sorgeloos *et al.*, 1980). On the contrary, Hanaoka (1977) found a reduction of the feeding rate to 1/3-1/2 of the normal rate in *Artemia* exposed to more than 0.1 ppm $\text{NH}_3\text{-N}$. Also, Chen *et al.* (1988) reported 24 h LC50 values for freshly-hatched *Artemia* nauplii of 840 and 7.16 ppm for, respectively, $\text{NH}_4^+\text{-N}$ and $\text{NH}_3\text{-N}$. Nitrite influenced the feeding activity of *Artemia* at least to the same extent as ammonia. This is corroborated by Le Roy (1988) who showed that *Artemia* is more sensitive to nitrite than to ammonia in chronic toxicity tests. Nitrate, which is generally regarded as not toxic for fishes (Poxton & Allouse, 1982), did not affect feeding rate of brine shrimp significantly up to a concentration of 1,000 ppm.

In the earlier literature, reduced filtration efficiency in

Artemia due to agitation of the culture medium has been ascribed to the loss of filtered food particles from the food groove (Bond, 1933; Nimura, 1967). Ryther (1954) found that agitation of the experimental container stopped feeding in *Daphnia magna*. The present data showed that this mechanical feeding rate inhibition in *Artemia* occurred only at extremely high aeration intensities, and was even then not always significant. The lower feeding rates at the extremely low aeration intensity could not be explained by a decrease of food concentration below the incipient limiting level due to sedimentation, since the latter was limited to 10-20% of the initial food concentration. However, the weak agitation of the culture medium resulted in a concentration of the animals in the lower part of the experimental container and a limited water circulation. As a result, the food may have been depleted in the medium surrounding the animals, which may in turn have caused lower feeding rates. A similar increase in observed feeding rates due to stirring was observed for copepods (Anraku, 1964).

Conflicting results are reported in the literature with regard to the effect of light intensity on growth of *Artemia*. Provasoli & Pintner (1980), who reared parthenogenetic *Artemia* from Sète in an artificial biphasic medium, found slower growth and higher mortality in darkness than in continuous light. Conversely, faster growth of brine shrimp in darkness than in light conditions was reported by Sorgeloos (1972) and Royan (1976). The absence of an effect of light intensity on feeding rate does not exclude a possible influence on growth. In this way, the higher browsing activity observed in *Artemia* exposed to light (Sorgeloos, 1972) may not result in a further increase of ingestion at food saturating conditions, but may cause a decreased growth due to the higher metabolic expenditures necessary to maintain the higher activity.

The effect of food digestibility on the feeding rate is poorly documented for filter-feeding organisms. From the data of Sick (1976), it may be concluded that the different digestibility of various algal species did not affect the feeding rate of *Artemia*. In this way, 6 mm brine shrimp ingested a similar

maximal volume of *Chlamydomonas sphagnicolo* and *Chlorella conductrix*, which were assimilated with an efficiency of, respectively, 78 and 9% (total organic matter). However, the present study demonstrated that in the case of poorly digestible food, the cell count method (also used by Sick, 1976) is not measuring the real feeding rate as the food particles may pass the gut unharmed. Although *Artemia* feeding on the laboratory-grown yeasts did not remove a significant amount of untreated yeast from suspension, the gut of these animals was packed with intact yeast cells which were returned to the food suspension after defecation. Although the passage of viable micro-algae through the gut has been observed in various filter-feeders, including *Artemia* (Gibor, 1956), bivalve veliger larvae (Babinchak & Ukeles, 1979; Fretter & Montgomery, 1968), and *Daphnia magna* (Porter, 1976), the resulting artefacts in feeding experiments have never been quantified. The importance of the latter was demonstrated by means of tracer techniques which showed that *Artemia* actually consumes the untreated yeast cells at a 2-5 times higher rate than the treated yeast (see Chapter VI).

The discrepancy between the growth of *Artemia* feeding on yeast concentrations either lower or higher than 800 cells μl^{-1} , indicated that the feeding rate was maximized at a concentration between 800 and 1,200 cells μl^{-1} . Possibly, the animals feeding at a yeast concentration of 800 cells μl^{-1} could maximize their feeding rate only during very short periods, whereupon the cell concentration fell below the incipient limiting level. A critical concentration of at least 800 μl^{-1} for the first stages of *Artemia* feeding in the recirculation system corresponds with the relatively high incipient limiting level found for adults in this system. Furthermore, it confirms that younger stages have a higher critical concentration for maximizing feeding rate. The energy invested in growth is determined by the amount of ingested food as well as the efficiency with which the latter is utilized. A saturation of the food uptake and a constant assimilation efficiency irrespective of yeast concentration (see Chapter VI), would then explain the similar growth of *Artemia* feeding on the

high food concentrations.

It should be emphasized that the present growth experiment aimed at an evaluation of the effect of continuous food loads on growth, whereas other authors have been mainly concerned with the daily food ration (Mason, 1963; Abreu-Grobois *et al.*, 1991). An effect of food concentration on growth rate can only be observed when the food depletion in the course of the experiments is limited, *i.e.* when the absolute amount of available food satisfies the requirement of the animal's feeding rate at each concentration. Mason (1963) fed daily the same amount of *Dunaliella tertiolecta* (0.8×10^6 cells $\text{ind}^{-1} \text{day}^{-1}$) to *Artemia* at various initial cell concentrations, and concluded "... growth of these shrimp was not adversely affected by feeding at the lower concentrations." However, since the latter author reported food clearance of 93-99% in 24 h, growth was food limited irrespective of food concentration. Similarly, Abreu-Grobois *et al.* (1991) concluded on the basis of an analogous experiment that growth rate in *Artemia* fed *D. tertiolecta* is independent of the initial food concentration but mainly determined by the daily amount of food ingested. Although this author applied saturating amounts of food (3×10^6 cells $\text{ind}^{-1} \text{day}^{-1}$), the concentrations tested (100, 400, 1,000 cells μl^{-1}) may have been too high to observe any detrimental effect of lower food concentrations on growth. Since the daily ration largely exceeded the initial requirements (*i.e.* 5 to 20 times higher than the ration used in *Artemia* culture tests; see Chapter VII, Table 44) and the lowest concentration examined was above the incipient limiting level for larger *Artemia*, feeding rate may indeed have been saturated irrespective of the initial concentration.

Chapter VI

STUDY OF FEEDING AND ASSIMILATION IN *ARTEMIA* USING ^{14}C -LABELLED BAKER'S YEAST

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Chapter VI

STUDY OF FEEDING AND ASSIMILATION IN *ARTEMIA* USING ^{14}C -LABELLED BAKER'S YEAST

VI.1. INTRODUCTION

Previous work demonstrated that the ineffectiveness of baker's yeast as a diet for the brine shrimp is mainly due to its poor digestibility rather than to its nutritional composition (see Chapter IV). The chemical treatment applied for improving the digestibility of yeast only affects the cell envelope and leaves the cell contents intact. In this way, treated and untreated yeast are particles of a similar chemical composition and size, and offer a unique test system to study the effect of food digestibility on the feeding kinetics of a filter-feeder. By means of electronic particle counting, differences in the feeding kinetics could be demonstrated between *Artemia* fed either treated or untreated yeast (V.3.3.). However, the cell count method is not necessarily measuring the real feeding rate as food particles of low digestibility may pass the gut unharmed. In the latter case, ingestion rates based on cell counts are a measure of the filter-feeder's capability to clear the food from suspension by ingestion and digestion, rather than the rate of food uptake.

The present study aimed at a quantitative documentation of the differential ingestion and assimilation of treated and untreated yeast by *Artemia* using ^{14}C tracer techniques. Radioactively labelled yeast has been used by several authors to study feeding kinetics in zooplankton. Rigler (1961) used ^{32}P -labelled baker's yeast for his pioneering research on the effect of food concentration on the feeding rate of *Daphnia magna*. Furthermore, ^{32}P -labelled *Rhodotorula* yeast has been applied as a food particle to determine feeding and grazing rates of

zooplankton in laboratory as well as *in situ* experiments (Burns & Rigler, 1967; Haney, 1971; Peters, 1972; Downing & Peters, 1980; Holtby & Knoechel, 1981), and to constitute the phosphorus budget in *D. magna* (Peters, 1972). To date, no radiotracer experiments have been reported using ^{14}C -labelled yeast, probably because of the difficulties associated with the ^{14}C -labelling of heterotrophic organisms. Also, any food organism is assumed to comprise a finite number of food compartments, which are assimilated with different efficiency by the animals. As a result, the measurement of carbon assimilation in zooplankton with ^{14}C tracer techniques requires the uniform labelling of all compartments in the food organism (Conover & Francis, 1973; Lampert, 1977a; Porter *et al.*, 1982; Nielsen & Olsen, 1989). The complexity of the growth media often hampers the uniform labelling of heterotrophic organisms. For these reasons, preliminary tests were run to determine the culture conditions that yield uniformly labelled yeast of sufficiently high ^{14}C -activity in order to detect feeding and assimilation in small numbers of animals.

In the literature, considerable confusion exists about the use of the term "assimilation". Assimilation is usually defined as the passage of material or energy from the lumen of the gut into the body of the animal and is often expressed as assimilation efficiency, *i.e.* the percentage of ingested food which is assimilated (Peters, 1984). There are two general ways of measuring assimilation isotopically. The first is to measure the radioactivity of the animals at two different times after the gut is filled with radioactive food (Peters, 1972; Lampert, 1977ab). The second method, which has been used by most authors and was also adopted for this study, is to feed the animals for a definite period of time with radioactive food, transfer them to non-radioactive food to evacuate the labelled gut content, and measure the remaining radioactivity in the animal (Hayward & Gallup, 1976; Porter, 1976; Pechenik & Fisher, 1979; Porter *et al.*, 1982). However, in radiotracer experiments, assimilation is the sum of the radioactivity retained in the animal after gut evacuation plus the complete metabolic losses of tracer during

the experimental period. Excluding or only partially including the latter leads to the determination of something in between "true assimilation" and "net production" (Lampert, 1977a), which has been referred to as "assimilation efficiency" (Hayward & Gallup, 1976), "net assimilation efficiency" (Porter *et al.*, 1982), or "retention efficiency" (Pechenik & Fisher, 1979).

To avoid ambiguities, we define the terms that are used in this study to express food utilization efficiency in relation to the general energy equation:

$$I = P + R + F + U.$$

where I = ingestion, P = production, R, F, U = losses due to, respectively, respiration, defecation, and excretion

The behaviour of ^{14}C in the animal requires at least a two compartment model, with the assimilated ^{14}C entering a "metabolic pool" from which it goes into a "structural pool" (Conover & Francis, 1973; Lampert, 1975; 1977a). The metabolic pool exhibits a high turnover and is partially respired, whereas the structural pool is of interest in the estimation of production. Respiratory loss of the ingested ^{14}C may occur during feeding on labelled food (R_f) and during the post-feeding when the gut content is replaced with unlabelled food (R_{pf}). Furthermore, the carbon retained after post-feeding may still be partially present in the metabolic carbon pool and thus result in a third respiratory loss term (R_m). In this study, it was assumed that the respiration of ^{14}C before the animals were transferred to the unlabelled food (R_f) was negligible due to the short duration of the feeding on labelled food. The ^{14}C retained in the animals after post-feeding was considered as production plus a potential respiratory loss ($P+R_m$). Assimilation (A) is defined as the amount of carbon retained by an animal ($P+R_m$) plus the amount respired during gut evacuation (R_{pf}):

$$A = P + R = [P+R_m] + R_{pf}$$

The assimilation efficiency (a) is then given by:

$$a (\%) = \frac{A}{I} \times 100 = \frac{[P+R_m] + R_{pf}}{I} \times 100$$

Retention efficiency is the percentage of ingested carbon which

is retained by an animal after gut evacuation during a period of feeding on unlabelled food:

$$\text{retention efficiency (\%)} = \frac{[P+R_m]}{I} \times 100$$

VI.2. MATERIALS AND METHODS

VI.2.1. *Artemia* and general experimental conditions

Artemia franciscana cysts (Great Salt Lake, Utah, USA; Sanders Brine Shrimp Co., lot 31627) were disinfected and hatched in seawater at 25 °C according to Sorgeloos *et al.* (1986). After 24 h of incubation, freshly-hatched nauplii were transferred to 5 l beakers at a density of 1 ml⁻¹ and grown under continuous light at 25 ± 1 °C. The feed consisted of a mixture of fresh baker's yeast, treated according to the standard cysteine treatment (C-yeast, see IV.4.), and the alga *Dunaliella tertiolecta* Butch. The feeding regime was identical to that used for growing *Artemia* on *D. tertiolecta* (see Chapter VII, Table 36), with the replacement of 90% of the algal cells by C-yeast (one algal cell equivalent to three C-yeast cells). For most experiments *Artemia* were harvested after 5 to 6 days of culture and ranged between 3 and 4 mm body length. Variation of body length between individuals used for the same experiment was less than 10% (coefficient of variation, n=15).

The time course (VI.2.5.) and functional response (VI.2.7.) experiments were performed separately for each yeast type on the same day with animals sampled *ad random* from the same population. Mean body length did not differ significantly between groups derived from the same population in the course of the day (ANOVA, P>0.10). Each of the other experiments was performed with the same sample of animals.

All experiments were conducted in beakers, which were agitated regularly to keep the food in suspension, at 25 ± 1 °C and 35 ppt salinity. Seawater used for dilution of the yeast

suspensions and rinsing of the animals was filtered through a Whatman GF/C filter.

VI.2.2. Culture, labelling and chemical treatment^t of baker's yeast

An industrial strain of baker's yeast *Saccharomyces cerevisiae* (Bl, provided by Algist Bruggeman, N.V., Belgium) was grown at 30 °C under continuous shaking in either YPG medium, containing yeast extract (Oxoid L21, 1% weight/volume), peptone (Oxoid L37, 2% w/v) and D-glucose (2% w/v), or YNBG medium, containing yeast nitrogen base without amino acids (Difco 0919, 0.67% w/v) and D-glucose (2% w/v). Shaking cultures were inoculated with 0.3% (v/v) aliquots from static tube cultures, which had been prepared by 12-h incubation of a loop of an agar-slant culture in 6 ml of the same medium.

Initial tests were run to determine the best culture medium for ¹⁴C-labelling of yeast using ¹⁴C-glucose (D-[¹⁴C(U)]-glucose, Nen®). A first test compared yield and specific activity of yeast grown for 24 h and 48 h on either the YPG or the YNBG medium, supplemented with a total concentration of 2 µCi ml⁻¹ ¹⁴C-glucose. In a subsequent test, the concentration of ¹⁴C-glucose in the YNBG medium was varied between 2 and 4 µCi ml⁻¹. Yeast cells were separated from 1 ml samples of the culture medium by centrifugation at 3,000 rpm for 5 min. The yeast pellet was twice rinsed by suspension in 10 ml of distilled water and centrifugation, and diluted to an absorbance of approximately 0.2 measured in a photometer at 750 nm (1 cm cuvette). Yield was estimated using a calibration curve of absorbance (A⁷⁵⁰) versus carbon concentration previously determined for yeast suspensions. Because the rinsing procedure reduced the unincorporated label to negligible levels, activity of the yeast was determined from triplicate 0.1 ml samples of the diluted yeast suspension. Specific activity was then calculated as counts per minute (CPM) per unit of carbon (CPM (µg C)⁻¹). Growth curves of the labelled and unlabelled yeast were determined by measuring absorbance of six-fold dilutions of the yeast cultures with distilled water.

^t: protected by International Patents PCT/BE 89/00009 and EP-89870040.6 (old 09.03.89) "Feed for Aquaculture" filed in Europe, USA, Japan, Canada, Australia (various file numbers; pending; owned by Artemia Systems N.V./S.A., Baasrode, Belgium).

The standard chemical treatment consisted of suspending the rinsed yeast pellet at a concentration of 10 mg wet weight ml^{-1} in a solution of cysteine (L-cysteine hydrochloride 0.05 M, previously adjusted to pH 12 with NaOH) and incubating the suspension at 30 °C for 30 min. The yeast was separated from the treatment medium by centrifugation and rinsed as described above. Since preliminary tests revealed the presence of tracer in the supernatant of the standard treatment medium, a series of experiments were run to quantify the loss of tracer due to treatment under various conditions of pH (cysteine 0.05 M, pH 9-13), concentration (cysteine 0.01 M, 0.05 M; pH 12) and nature of the thiol solution (cysteine 0.05 M, 2-mercaptoethanol 2% v/v; pH 9). The total radioactivity of the supernatant of the treatment medium and the rinsed treated yeast was calculated as a percentage of that of the untreated yeast. A control treatment, in which yeast was incubated for 30 min at 30 °C in distilled water, was run to determine the loss of label due to yeast respiration, sampling, and rinsing.

Following the results of the above experiments yeast used for the feeding experiments was grown on the YNBG medium, to which 4 $\mu\text{Ci ml}^{-1}$ ^{14}C -glucose was added in case labelled yeast was desired. Yeast was harvested after 24 h and 48 h of culture for the unlabelled and labelled yeast, respectively. Besides the untreated yeast (UT), two types of treated yeast were selected: T11 (cysteine 0.05 M, pH 11) and T12 (cysteine 0.05 M, pH 12).

VI.2.3. Preparation of the labelled yeast suspension

The labelled yeast, derived from the culture, or alternatively from the treatment medium, was rinsed twice and diluted to the desired food concentration using the calibration curve of A^{750} versus carbon content. From this stock suspension three aliquots of 5 ml were filtered on a GF/C filter for carbon analysis using a CHN analyzer (Carlo Erba, model 1106) and triplicate 0.1 ml samples were counted for radioactivity. For each stock suspension the specific activity was computed from the determinations of carbon content and radioactivity. The various

yeast concentrations were obtained by diluting the stock suspension with filtered seawater. The exact food concentration was then calculated from radioactivity, measured on triplicate samples of the diluted yeast suspensions, and the specific activity in the stock suspension.

The stability of the incorporated tracer under the experimental conditions was verified by incubating the treated yeast (T12) in seawater at 25 °C. Over a period of 80 min, the total activity of the suspension was found to decrease with less than 7%, whereas the fraction retrieved in the supernatant (after centrifugation for 5 min at 3,000 rpm) increased slightly from 5 to 7% of the total activity.

VI.2.4. Radioassay of the samples

All samples were counted in a Packard Liquid Scintillation Spectrometer, model 1900TR. Radioactivity of yeast suspensions was determined on triplicate samples of 0.1 or 1 ml of yeast suspension, after the addition of 3 ml of scintillation cocktail (Ultimagold and Instagel, Packard; for respectively 0.1 ml and 1 ml samples) at least 2 h prior to counting. Animals were disintegrated through the addition of tissue solubilizer (Soluene-350, Packard) at least 24 h prior to the addition of scintillation cocktail (3 ml Ultimagold).

VI.2.5. Gut passage time and retention efficiency

In a first series of experiments, gut passage time was determined in *Artemia* fed UT, T11, or T12 at a concentration well above the incipient limiting concentration (20 mg C l⁻¹). The method, which has been previously used for *Daphnia* (Rigler, 1961; Peters, 1972) and *Brachionus* (Korstad et al., 1989), consisted of following the amount of tracer accumulated in *Artemia* feeding for varying lengths of time on labelled yeast.

Animals were acclimated to the experimental conditions (temperature, type and concentration of food) for at least 2 h. At 1 min intervals, groups of 35-40 animals were collected on a

sieve, rinsed with filtered seawater, and transferred to 20 to 60 ml of the labelled yeast suspension, depending on the duration of the feeding period. In this way, the yeast concentration did not decrease with more than 20% in the course of the experiment and remained above the incipient limiting level for feeding. The *Artemia* were allowed to feed for 0 to 80 min whereupon they were killed by adding nearly boiling water (50% v/v), rinsed and transferred in groups of five to scintillation vials. For each time interval the uptake of radioactivity per animal was calculated from the mean of six groups and converted to carbon units through division by the specific activity of the yeast stock suspension. In preliminary experiments, no significant difference in tracer content was observed between *Artemia* that were either heat-killed as described above or plated alive immediately following collection.

Simultaneously with the time course experiment, an additional group of animals was fed a 20 min pulse-meal of the labelled yeast, rinsed, and eventually transferred to a suspension of unlabelled yeast of the same type and concentration. After 2 h of post-feeding, the *Artemia* were killed and plated as described above to give the radioactivity retained in the animals after gut evacuation. The retention efficiency was calculated from the amount of carbon retained in the animal after post-feeding ($P+R_m$) and the carbon ingestion after 20 min of feeding (I) using the equation:

$$\text{retention efficiency (\%)} = \frac{[P+R_m]}{I} \times 100$$

VI.2.6. Carbon budget and assimilation efficiency

The carbon budget was determined according to the energy equation $I = P + R + F + U$ using a ^{14}C pulse-labelling technique (Nielsen & Kofoed, 1982; Nielsen & Olsen, 1989) which included a pulse of radioactive food and post-feeding with unlabelled food of the same type and concentration. The rate of ingestion was directly determined immediately after the radioactive pulse meal

(I) and indirectly by summation ($I\Sigma = P+R+U+F$) at the end of the post-feeding period. The retained radioactive carbon in the *Artemia* after the post-feeding period was considered to consist of production (P) and a metabolic carbon pool which may result in further respiratory losses of ^{14}C (R_m). Dissolved inorganic ^{14}C was assumed to express carbon respired during the post-feeding (R_{pf}), and particulate (F_p) and dissolved (F_d+U) organic ^{14}C to express defecated (F_p+F_d) plus excreted (U) carbon. The assimilation efficiency (a) was calculated as

$$a (\%) = \frac{P + R}{I\Sigma} \times 100 = \frac{[P+R_m] + R_{pf}}{[P+R_m] + R_{pf} + F_d + F_p + U} \times 100$$

After acclimation to the experimental conditions for at least 2 h, about 70 animals were transferred at 1 min time intervals to between 75 and 300 ml of radioactive yeast suspension, depending on the food concentration tested. After 20 min, which is shorter than the gut passage time (VI.3.2.), the *Artemia* were sieved from the food suspension, rinsed with filtered seawater and transferred to between 200 and 400 ml of non-radioactive yeast suspension for post-feeding. Animals from an additional beaker were heat-killed and plated in groups of ten to measure radioactivity (*i.e.* direct measurement of I). At the end of the post-feeding, the animals, the particulate carbon, and the dissolved carbon of each beaker were separated and collected on a sieve, a GF/C-filter, and as filtrate in a beaker, respectively. The *Artemia* were plated in groups of ten to give $[P+R_m]$. From the filtrate, two subsamples (2.5 ml) were pipetted into vials. Prior to the addition of scintillation cocktail (2.5 ml Instagel, Packard), one vial was acidified with 250 μl 1 N HCl and aerated for 1 min to purge dissolved $^{14}\text{CO}_2$ (F_d+U), whereas to the other vial 25 μl CO_2 absorber (Carbo Sorb, Packard) was added (F_d+U+R_{pf}). Respired $^{14}\text{CO}_2$ (R_{pf}) was calculated as the difference between radioactivity in the $[F_d+U+R_{pf}]$ and the $[F_d+U]$ samples. The various terms of the carbon budget were calculated as an average rate over the 20 min feeding period and expressed as μg carbon individual $^{-1}$ h $^{-1}$. To correct for variation in the ingestion rate computed by summation ($I\Sigma$), the various components of the

carbon budget were expressed as a fraction of $I\Sigma$ and normalized to the directly measured ingestion rate (I) using the equation:

$$\text{normalized } X = \frac{X}{I\Sigma} \cdot I \quad \text{for } X = [P+R_m], R_{pf}, [F_d+U], F_p$$

In a preparatory series of experiments, the effect of the post-feeding conditions on the distribution of the radioactivity among the various compartments was examined. The effect of the duration of the post-feeding period on the radioactivity retained by *Artemia* fed on untreated yeast at a concentration of 20 mg C l⁻¹ was determined in a first test. Animals, pulse-labelled for 20 min on untreated yeast, were transferred to non-radioactive yeast for 0 to 24 h. To avoid recycling of egested material, the animals were transferred to a new yeast suspension after 4 h and 20 h of post-feeding. The above experiment was repeated for *Artemia* fed treated yeast at 20 mg C l⁻¹ and extended to include measurements of radioactivity in the dissolved (F_d+U+R_{pf}) and the particulate (F_p) carbon compartments. Furthermore, the influence of the food concentration on the change of radioactivity in the various compartments was examined by transferring *Artemia* for 1 to 4-h post-feeding on a low concentration of treated yeast or filtered seawater.

The individual variation of ingestion and retention of carbon was estimated for 8-days old *Artemia* (4.7 ± 0.5 mm) fed untreated yeast at 15 mg C l⁻¹. The ingestion rate of this larger animals allowed plating of individuals after the pulse-meal or the 2-h post-feeding period.

Following the results of the preparatory tests the carbon budget was determined for *Artemia* fed either untreated or treated yeast at various concentrations after 2 h of post-feeding. Furthermore, the influence of acclimation to the experimental food concentration on the estimate of the carbon budget was examined by acclimating the animals either to a saturating food concentration (15 mg C l⁻¹) or to the appropriate experimental food level prior to the start of the pulse-labelling.

VI.2.7. Ingestion rate as a function of yeast concentration

The methodology used for the determination of a functional response curve for the treated and untreated yeasts was identical to that used for the direct measurement of ingestion rate immediately after pulse-labelling for 20 min (VI.2.6.). Concentrations of the non-radioactive and radioactive yeast suspensions during, respectively, acclimation and pulse-feeding, ranged from 0.2 to 30 mg C l⁻¹.

VI.2.8. Treatment and statistical analysis of the data

The methods for statistical analysis of the data were derived from Sokal & Rohlf (1981). The significance of the difference between two regression coefficients was tested by means of the F-test. The specific activities of the various yeast stock suspensions in the different experiments were compared using Friedman's method for randomized blocks without replication, with either the experiments or the yeast types considered as blocks. Statistical analysis of several means included analysis of variance and Tukey HSD multiple range tests. The homogeneity of the variances of the means was checked by Cochran's and Hartley's test. When only two means were to be tested, a t-test was applied.

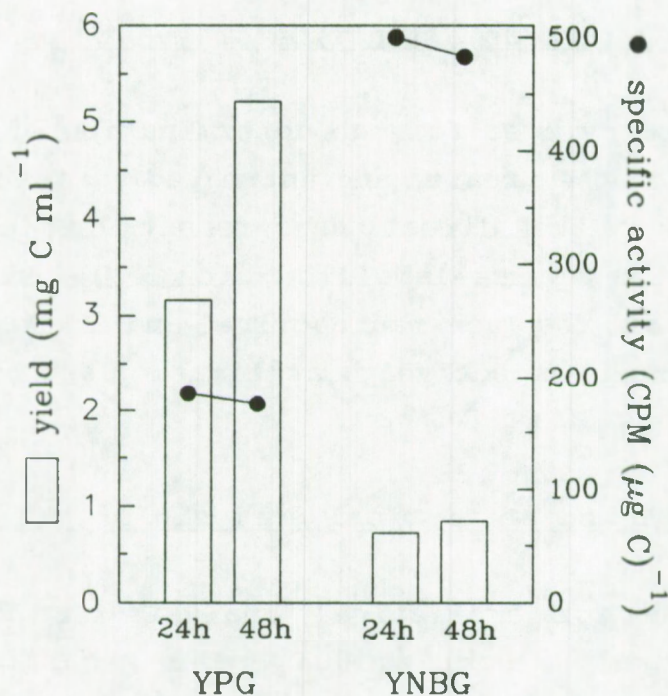


Fig. 28: Yield and specific activity of baker's yeast cultured for 24 h and 48 h in YPG or YNBG medium, supplemented with $2 \mu\text{Ci ml}^{-1}$ of ^{14}C -glucose.

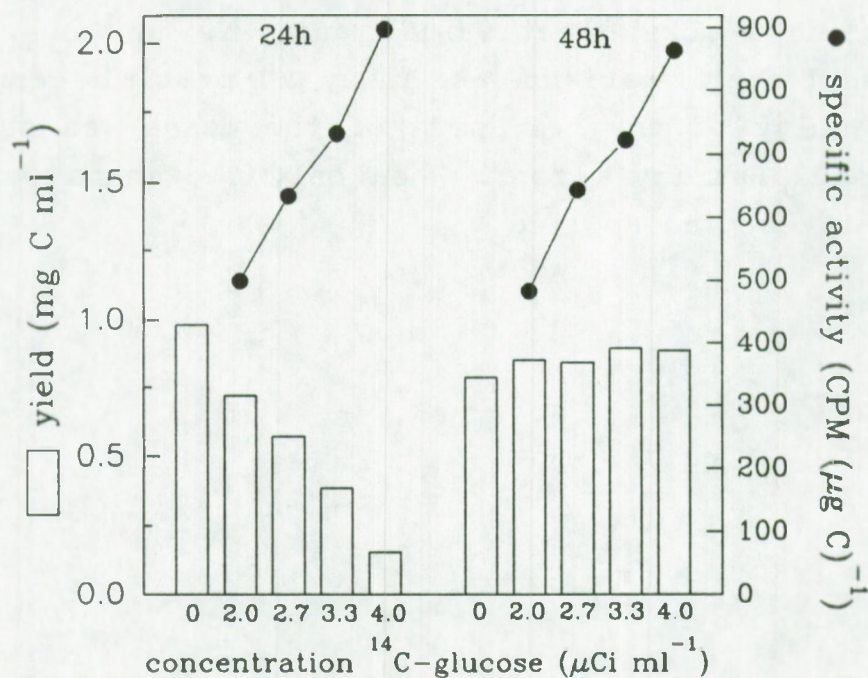


Fig. 29: Yield and specific activity of baker's yeast cultured for 24 h and 48 h in YNBG medium, supplemented with ^{14}C -glucose at final concentrations ranging from 0 to $4 \mu\text{Ci ml}^{-1}$.

VI.3. RESULTS

VI.3.1. Culture, labelling and chemical treatment of baker's yeast

The culture of baker's yeast on the minimal YNBG medium resulted in a 6 times lower yield after 48 h (Fig. 28). However, for yeast grown on the same concentration of ^{14}C -glucose, specific activity was about 2.7 times higher for the YNBG medium compared to YPG. For both media, yeast specific activity was not found to be different after 24 or 48 h of shaking culture.

Increasing the tracer concentration reduced yeast growth rate and yield after 24 h (Fig. 29). However, after 48 h of incubation, the yeast attained stationary phase irrespective of the tracer concentration within the range of 0 to $4.0\ \mu\text{Ci ml}^{-1}$. The specific activity (SA, $[\text{CPM} (\mu\text{g C})^{-1}]$) of yeast harvested either after 24 or 48 h of culture was not different and increased linearly with the tracer concentration ($^{14}\text{C-G}$, $[\mu\text{Ci ml}^{-1}]$) in the medium within the range of 2 to $4.0\ \mu\text{Ci ml}^{-1}$ according to the regression equation

$$\text{SA} = 191\ ^{14}\text{C-G} + 113 \quad (r^2=0.99).$$

The growth curves and the moment of harvesting of the radioactive and non-radioactive yeast cultures used for the feeding experiments is represented in Fig. 30.

The carbon uptake efficiency of the yeast, *i.e.* the amount of incorporated ^{14}C expressed as a percentage of the initial radioactivity present in the medium, amounted to about 9 and 18% for the YNBG and YPG medium, respectively.

The present experiments revealed a significant loss of incorporated tracer from the yeast cells during the standard chemical treatment (cysteine 0.05 M, pH 12). The release of tracer into the supernatant of the treatment medium was strongly dependent on the initial pH and the concentration of the cysteine solution (Fig. 31). The ^{14}C loss showed a sharp increase between pH 11 and pH 12 from $\pm 5\%$ to $\pm 25\%$ of the radioactivity in the untreated yeast. The treatment with 2-mercaptoethanol (2-ME) at pH 9 caused a similar tracer loss as the cysteine treatment at

the same pH (Fig. 31).

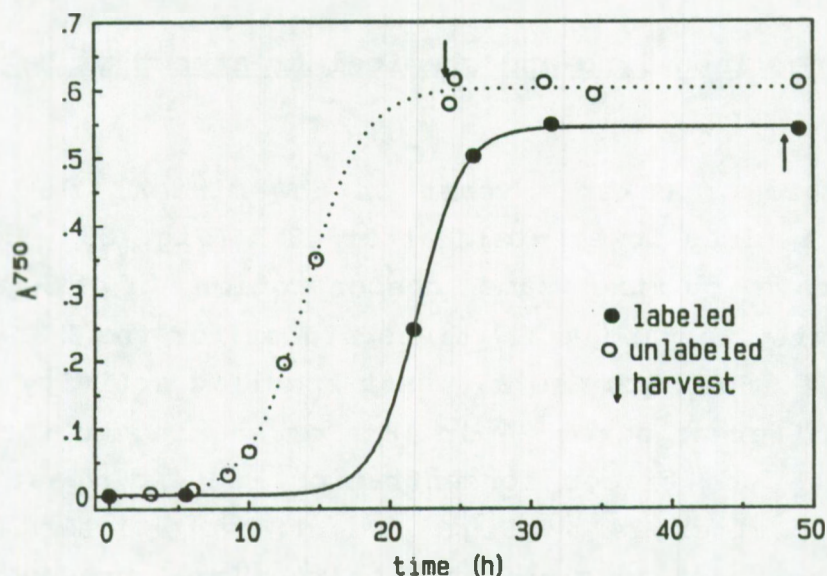


Fig. 30: Growth curves of baker's yeast cultured on YNBG with (●) or without (○) the addition of ^{14}C -glucose ($4 \mu\text{Ci ml}^{-1}$). Vertical arrows indicate the moment of harvesting the culture for use in the feeding experiments.

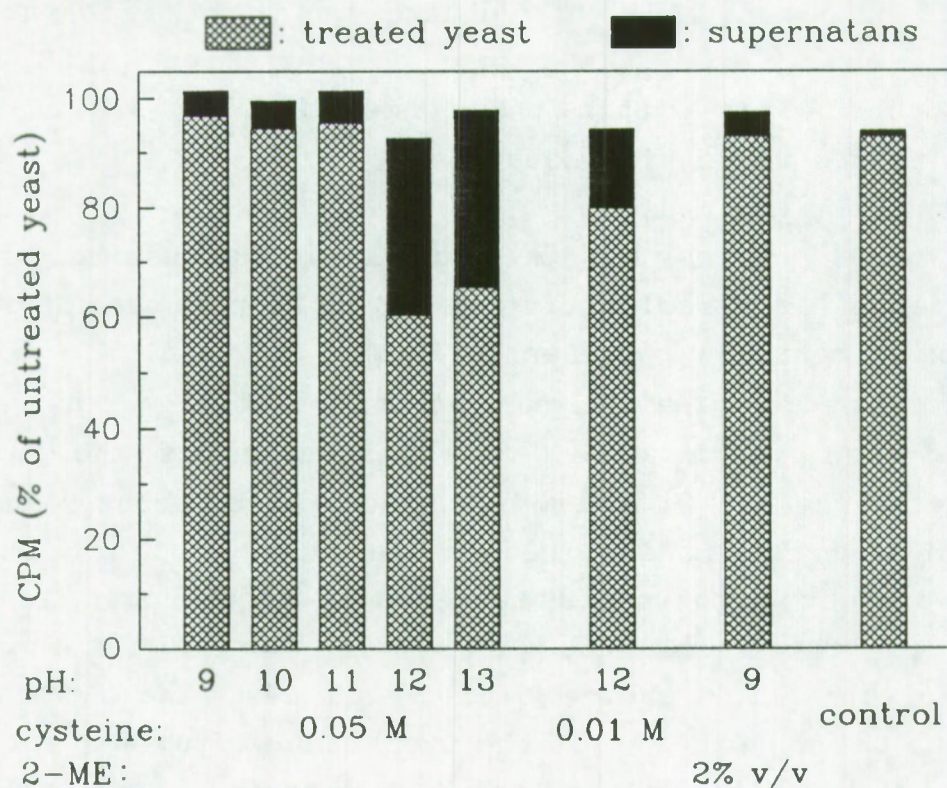


Fig. 31: Loss of incorporated tracer in baker's yeast due to chemical treatment as a function of pH, concentration and nature of the thiol solution (cysteine or 2-mercaptoethanol). The control treatment consisted of incubating the yeast in distilled water. Data represent total ^{14}C -activity retrieved in the supernatant of the treatment medium and the rinsed treated yeast, expressed as a percentage of the total radioactivity in the untreated yeast.

The yeasts treated with cysteine (0.05 M) at pH 11 (T11) and pH 12 (T12) were selected for comparison with the untreated yeast in the study of feeding kinetics. In the course of the experiments the treatments were repeated five times on independent yeast cultures. The ^{14}C fractions retrieved from the supernatant and the treated yeast were consistent for both treatments (Table 26).

Table 26: ^{14}C activity in the supernatant and the yeast after cysteine treatment at pH 11 and 12 (% of total ^{14}C in untreated yeast; mean \pm SD, n=5).

pH treatment	supernatant	treated yeast
pH 11	5.4 \pm 0.4	89.2 \pm 3.8
pH 12	26.5 \pm 4.3	65.6 \pm 6.7

Summation of the ^{14}C fractions retrieved from the treated yeast and the supernatant were in general smaller than 100%. This apparent loss ($\leq 7\%$) also occurred in the control treatment (distilled water, 30 min at 30 °C, Fig. 31) and can be ascribed to yeast respiration during the treatment and the loss of material during rinsing.

The specific activity of the yeast stock suspensions ready for use in the feeding tests did not vary significantly between experiments nor between the three yeast types (Friedman's test for randomized blocks, $P > 0.05$; Table 27). As a result, measurements of radioactivity were converted into carbon units for all experiments using the overall mean specific activity, i.e. 682 CPM ($\mu\text{g C}$) $^{-1}$.

Table 27: Specific activity of the different stock suspensions of the yeast used in the various feeding experiments.

Experiment	Specific activity (CPM ($\mu\text{g C}$) ⁻¹)			mean \pm SD
	untreated yeast	treated yeast (T11)	treated yeast (T12)	
1	668	644	646	653 \pm 13
2	661	611	683	652 \pm 37
3	701	701	773	725 \pm 42
4	682	641	768	697 \pm 65
mean \pm SD	678 \pm 18	649 \pm 38	718 \pm 63	682 \pm 49

VI.3.2. Gut passage time and retention efficiency

Artemia feeding on radioactive yeast accumulated the tracer at a constant rate as the gut filled, until a break in the uptake curve appeared when defecation started (Fig. 32). The positive x-intercept found in all time course experiments indicated an initial lag phase of a few minutes in the feeding of *Artemia* after transfer to the radioactive yeast suspension. The results of the time series experiments revealed similar uptake curves for the untreated and treated T11 yeast, whereas a different pattern was observed for the T12 yeast. The animals ingested the untreated yeast at a 2.3 times higher rate than the T12 yeast, which was coupled with a two-fold reduction of gut passage time (Table 28A). The gut passage time, corrected for the positive x-intercept, amounted to 28 and 29 min for the T11 and untreated yeast, respectively.

The exact gut passage time for the T12 yeast could not be determined conclusively because of the few data beyond the inflection of the uptake curve, but appeared to be about 60 min (Fig. 32C). This was corroborated by the increased variation in the ¹⁴C-activity per animal after 60 min of feeding, which occurred generally when defecation started.

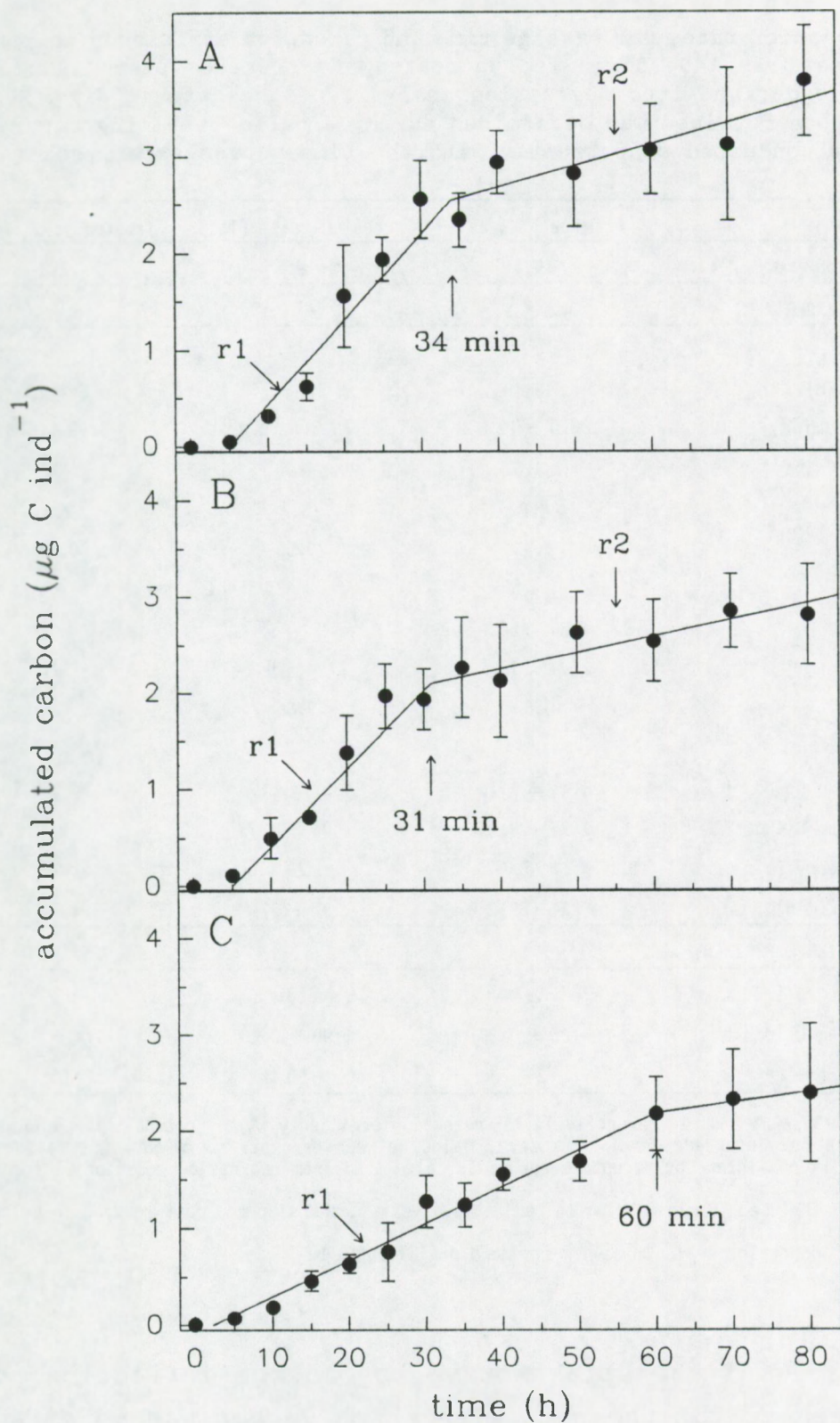


Fig. 32: Accumulation of labelled carbon in *Artemia* (body length = 3.10 ± 0.35 mm) fed untreated yeast (A) and treated yeast (T11: B, T12: C). Data represent mean and standard deviation from six groups of five animals each. Parameters from the linear regression analysis (r1, r2) are presented in Table 28. Gut passage times are indicated (arrow + time in min).

Table 28: Ingestion rate, gut passage time and retention efficiency in *Artemia* (body length = 3.10 ± 0.35 mm) fed untreated and treated yeast (T11, T12). Parameters are derived from regression analysis of the data in Fig. 32. The amount of carbon retained was determined during a pulse-labelling experiment (B) which was conducted concurrently with the time course experiment (A).

	untreated yeast	treated yeast (T11)	treated yeast (T12)
food concentration (mg C l ⁻¹)-	19.1	19.5	21.1
A: TIME COURSE EXPERIMENT			
REGRESSION r1			
time interval (min)	5 - 40	5 - 35	5 - 60
slope (ng C ind ⁻¹ min ⁻¹) [*]	86.9 ± 19.8	75.5 ± 18.0	37.9 ± 4.3
y-intercept (ng C ind ⁻¹)	-416	-240	-87
x-intercept (min)	4.8	3.2	2.3
r ²	0.95	0.96	0.98
REGRESSION r2			
time interval	30 - 80	25 - 80	
slope (ng C ind ⁻¹ min ⁻¹) [*]	22.4 ± 12.2	17.4 ± 6.4	NA
r ²	0.82	0.88	
RESULTS			
ingestion rate (μg C ind ⁻¹ h ⁻¹) ^{*.1}	5.21 ± 1.19	4.53 ± 1.08	2.27 ± 0.26
retention rate (μg C ind ⁻¹ h ⁻¹) ^{*.2}	1.34 ± 0.73	1.04 ± 0.38	NA
retention efficiency (%) ³	26	23	NA
gut passage time (min) ⁴	34 (29)	31 (28)	± 60
B: PULSE-LABELLING EXPERIMENT [§]			
ingestion rate (μg C ind ⁻¹ h ⁻¹) ⁵	3.97	3.81	1.99
retention rate (μg C ind ⁻¹ h ⁻¹) ⁵	1.25 ± 0.20	1.90 ± 0.26	1.74 ± 0.23
retention efficiency (%) ³	31	50	87

1,2: derived from slope r1 and r2, respectively; 3: retention efficiency (%) = (retention/ingestion)×100; 4: time coordinate for intersection of r1 and r2, value between brackets represents gut passage time corrected for positive x-intercept of uptake curve; 5: calculated from regression equation r1 for time = 20 min

§: pulse-labelling included 20 min feeding on radioactive yeast suspension and post-feeding on unlabelled yeast for 2 h.

*: mean ± 95% confidence interval; ±: mean ± standard deviation (n=6)

The slope of the uptake curve beyond the inflection point may be a measure for the rate with which carbon was retained in *Artemia* feeding on radioactive yeast. The carbon retention rate derived from the time course experiment for the untreated yeast (Table 28A) was similar to that measured in animals which received a radioactive pulse-meal and were fed for two hours on

unlabelled yeast (Table 28B). By contrast, the retention rate calculated from the slope of the regression equation r_2 obtained for T11 yeast (Table 28A) amounted to only 50% of the retention rate computed from the pulse-labelling experiment (Table 28B). The strong inflection of the uptake curve and the high retention rate observed in the pulse-labelled animals suggested an even stronger discrepancy for the T12 yeast. As a result, the retention efficiency, estimated from the ratio $(\text{slope } r_2)/(\text{slope } r_1)$ corresponded to that derived from the pulse-labelling test only in the case of untreated yeast.

It is interesting to note that, although T11 yeast is ingested at a significantly higher rate than T12 (F-test on r_1 slopes, $P < 0.001$), no significant differences were found between the amount of carbon retained from a pulse-meal of either of the yeasts (t-test, $P = 0.31$). The significantly higher feeding rate (F-test, $P < 0.001$) and lower carbon retention (t-test, $P < 0.01$) in *Artemia* fed untreated yeast compared to those fed T12, resulted in a strongly reduced retention efficiency (Table 28).

VI.3.3. Ingestion rate as a function of yeast concentration

The feeding response of *Artemia* as a function of the concentration of untreated yeast was a typical saturation response curve (Fig. 33A) and could be described by either the rectilinear or the Ivlev model, with the former yielding the highest correlation coefficients (Table 29). The rectilinear model predicted an incipient limiting concentration of 2.6 mg C l⁻¹. The response curves obtained for the treated yeast were characterized by a five-fold lower maximal feeding rate and lower incipient limiting concentrations, ranging from 1.0 to 1.4 mg C l⁻¹ (Figs. 33BC).

Table 29: Parameters of the rectilinear and Ivlev models fitted to the ingestion rate data presented in Fig. 33. Y represents ingestion rate (μg C ind⁻¹ h⁻¹), X represents yeast concentration (mg C l⁻¹).

YEAST TYPE	MODEL rectilinear: $X < 2: Y = aX$ $X > 3: Y = b$ Ivlev: $Y = b(1 - e^{-ax})$	parameters		ilc [†] (mg C l ⁻¹)	df	r ² ‡
		a	b			
UT	rectilinear	1.537	4.057	2.64	17	0.97
	Ivlev	0.670	4.075	-	55	0.79
T11	rectilinear	0.782	0.810	1.04	10	0.84
	Ivlev	1.365	0.814	-	41	0.75
T12	rectilinear	0.624	0.863	1.38	17	0.74
	Ivlev	1.533	0.854	-	52	0.59

†: incipient limiting concentration calculated as the intercept of the two regression equations from the rectilinear model

‡: df and r² for the rectilinear model are specified for the equation $Y = aX$

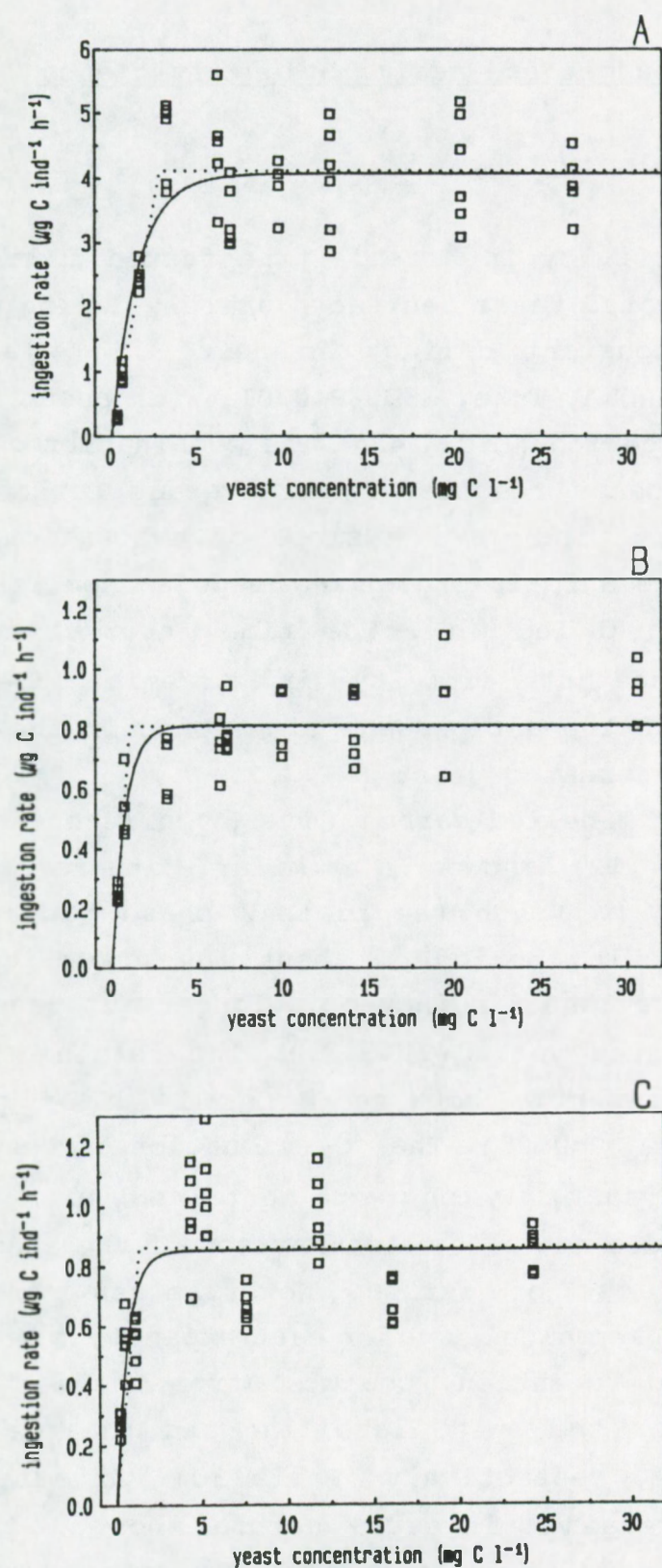


Fig. 33: Ingestion rate in *Artemia* (body length = 3.04 ± 0.36) as a function of concentration of untreated yeast (A) or treated yeast (B: T11, C: T12). Curves are fitted by least squares non-linear regression according to the rectilinear (...) or the Ivlev (—) model (see Table 29).

VI.3.4. Carbon budget and assimilation efficiency

VI.3.4.1. Preliminary tests

The radioactivity in *Artemia* transferred from labelled to unlabelled untreated yeast decreased sharply during the first 30 min, remained constant during the next 3.5 h, and declined significantly (ANOVA, Tukey HSD, $P < 0.01$) with about 30% over the subsequent 20-h period (Fig. 34A). The combination of defecation and metabolic losses resulted in an average carbon evacuation rate of $2.61 \mu\text{g C ind}^{-1} \text{h}^{-1}$ (= slope of evacuation curve, Fig 34A), whereas the animals ingested the pulse meal at an average rate of $5.34 \mu\text{g C ind}^{-1} \text{h}^{-1}$. The time interval required for attaining a constant activity in *Artemia* (i.e. 29 min) corresponded with the gut passage time found in the time course experiment for untreated yeast (VI.3.2.).

The loss of labelled carbon from juvenile brine shrimp fed treated yeast (T12) showed a similar pattern as that for untreated yeast, although the plateau phase was reached only after a post-feeding period of about 100 min (Fig 34B). This confirmed the previous findings of a longer gut passage time in *Artemia* fed treated yeast (IV.3.2.). The retained carbon also decreased significantly between 4 and 24 h of post-feeding (ANOVA, Tukey HSD, $P < 0.05$). The ^{14}C -evacuation curves for treated and untreated yeast, which were obtained with *Artemia* of different size, did not allow a comparison of the absolute values of uptake and loss of carbon. Nevertheless, the retention efficiency could be calculated for each experiment from the rates of ingestion and retention, computed from the carbon retained immediately after the pulse labelling and the plateau of the evacuation curve, respectively (Table 30). *Artemia* feeding on treated yeast retained ingested carbon about 1.7 times more efficiently compared to those ingesting untreated yeast.

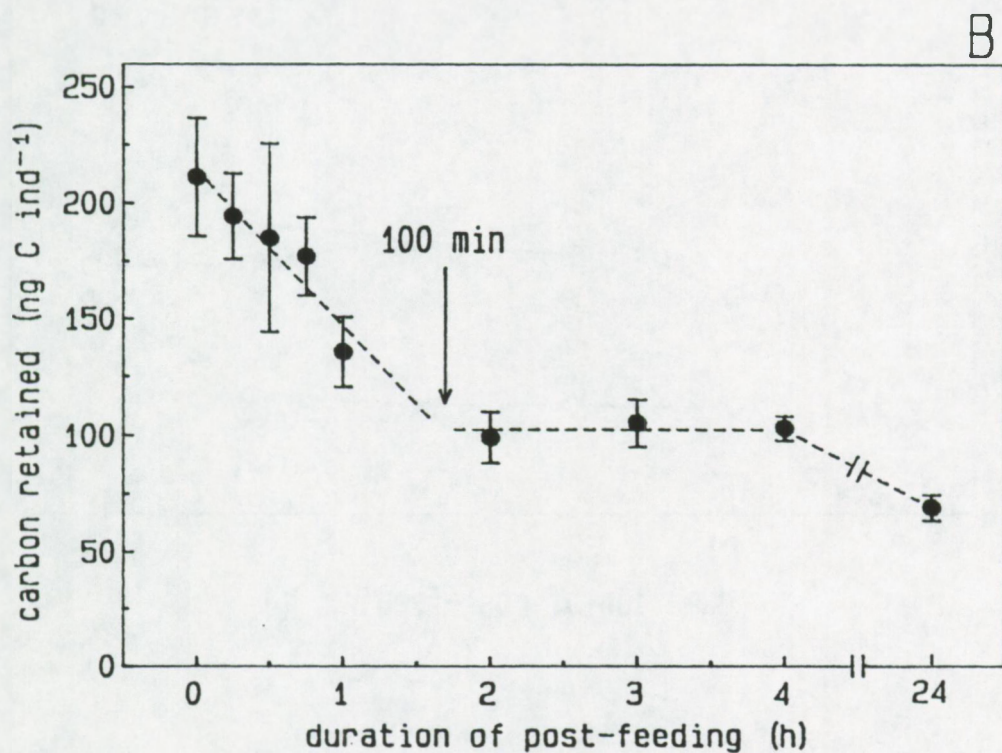
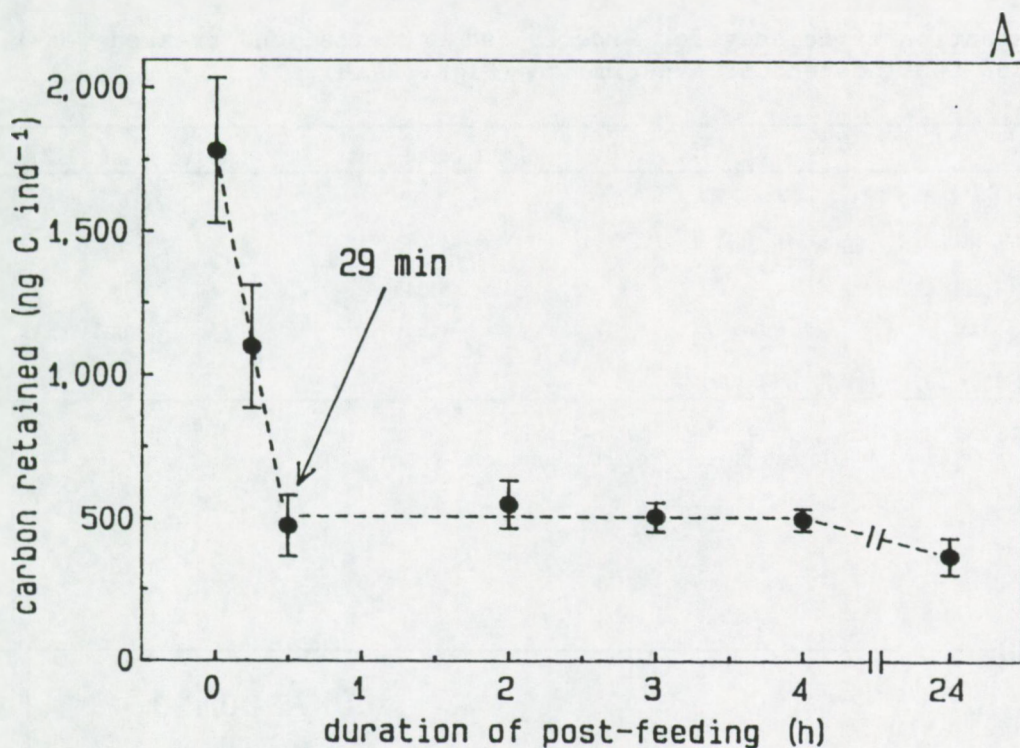


Fig. 34: Retention of labelled carbon by *Artemia* as a function of time after transfer from a radioactive to a non-radioactive yeast suspension. Gut passage time (GPT) is indicated.

A: *Artemia* (body length = 3.04 ± 0.37 mm) fed untreated yeast (17.2 mg C l^{-1}).
 B: *Artemia* (body length = 2.02 ± 0.20 mm) fed treated yeast (T12, 15.0 mg C l^{-1}).

Table 30: Retention efficiency for *Artemia* fed untreated and treated yeast, calculated for two independent experiments (Figs. 34AB).

	untreated yeast	treated yeast (T12)
yeast concentration (mg C l ⁻¹)	17.2	20.0
<i>Artemia</i> body length (mean \pm SD, n=15, mm)	3.04 \pm 0.37	2.02 \pm 0.20
ingestion rate (I, μ g C ind ⁻¹ h ⁻¹) [†]	5.343	0.633
retention rate ([P+R _m], μ g C ind ⁻¹ h ⁻¹) [‡]	1.527	0.306
retention efficiency (%) = ([P+R _m]/I) \times 100	29	48

[†]: I = carbon retained immediately after 20 min pulse-labelling

[‡]: [P+R_m] = carbon retained in plateau phase of evacuation curve

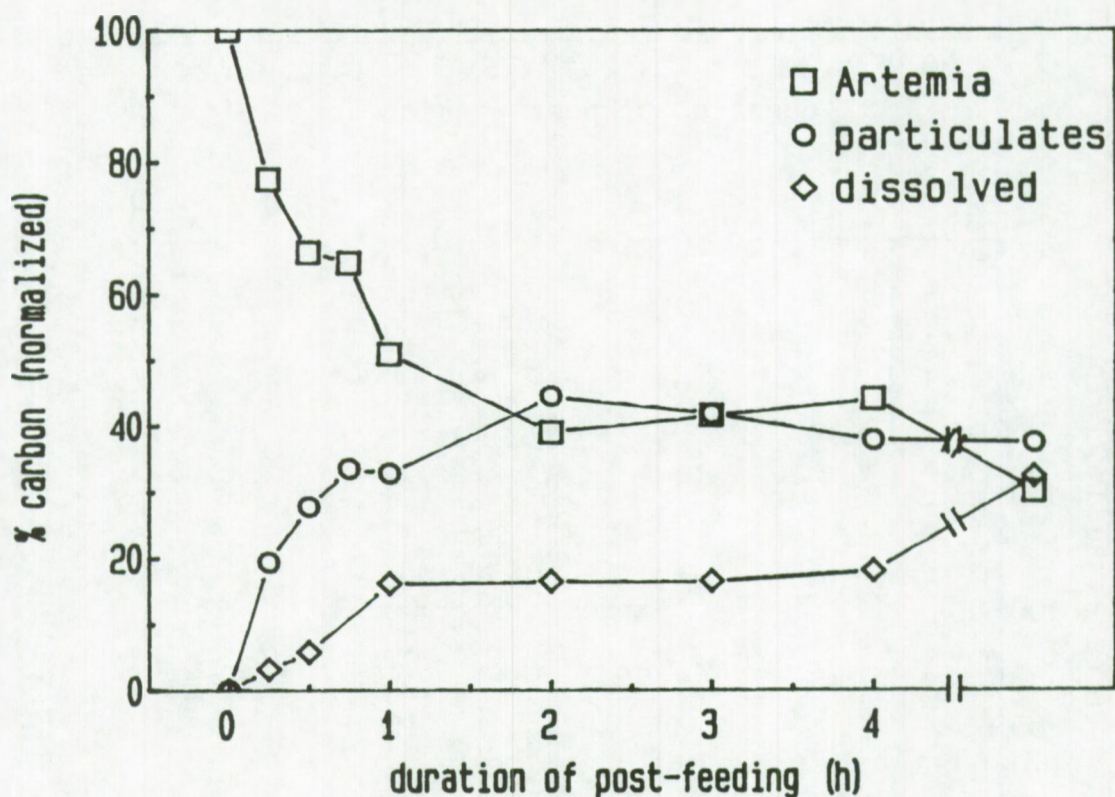


Fig. 35: Relative distribution of the ¹⁴C-activity among the *Artemia* (body length = 2.02 \pm 0.20 mm; \square), particulate (\circ), and dissolved (\diamond) carbon compartments as a function of time after transfer from a radioactive to a non-radioactive yeast suspension of the same concentration (treated yeast T12, 15.0 mg C l⁻¹). The ¹⁴C-activity in each compartment is expressed as a percentage of the total activity retrieved after post-feeding.

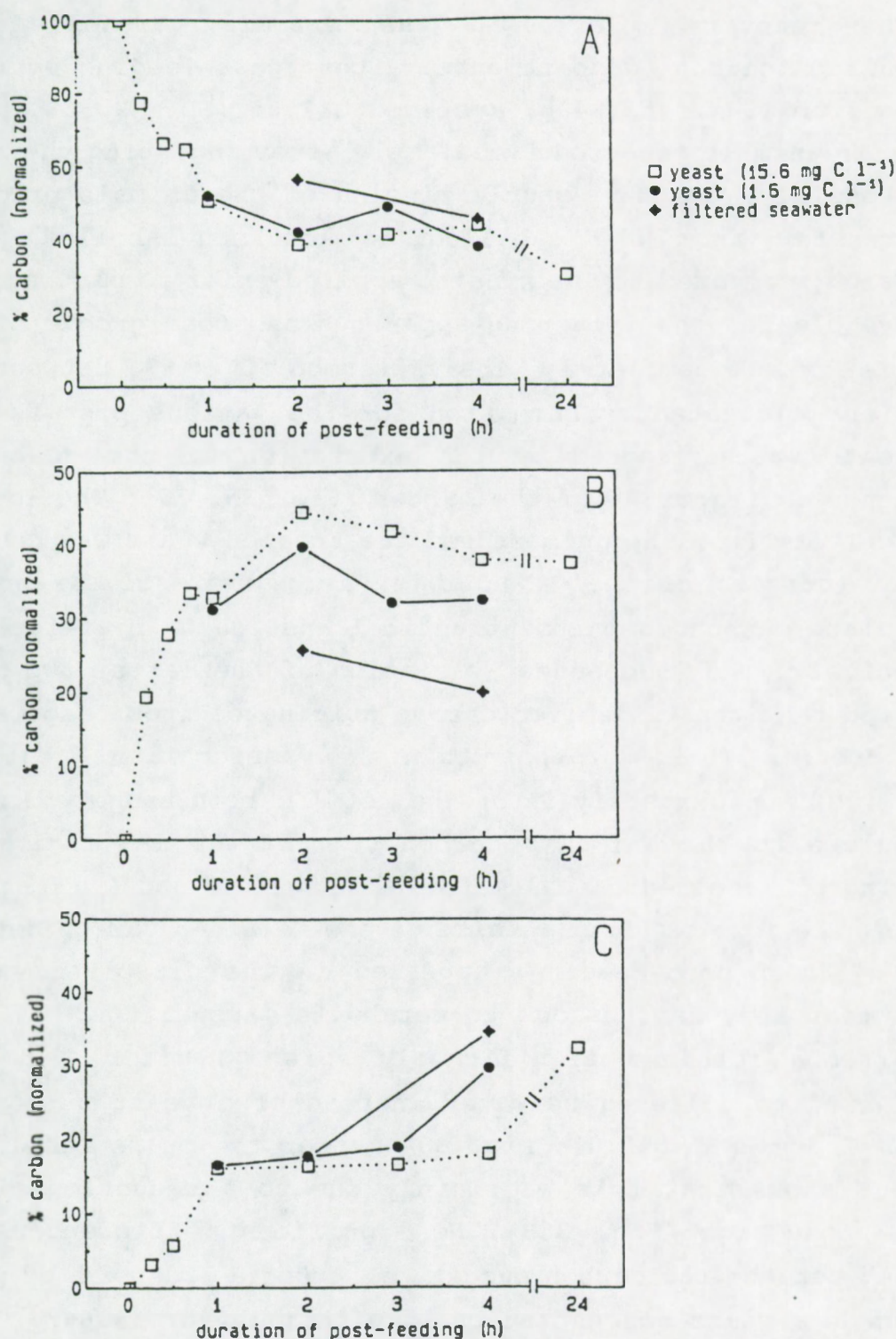


Fig. 36: ¹⁴C-activity in the *Artemia* (body length = 2.02 ± 0.20 mm; A), particulate (B), and dissolved (C) carbon compartments as a function of time after transfer from a radioactive (treated yeast T12, 15.0 mg C l^{-1}) to a non-radioactive yeast suspension of either high (□) or low (●) concentration (T12, respectively, 15.6 and 1.6 mg C l^{-1}), or to filtered seawater (◆). The ¹⁴C-activity in each compartment is expressed as a percentage of the total activity retrieved after post-feeding.

The recovery of ^{14}C in the *Artemia*, dissolved and particulate carbon compartments after post-feeding periods ranging from 15 min to 24 h, averaged 117.5% (SD = 9.36%, n=14) of that in animals measured immediately after ingesting the pulse meal. Possibly, the more gentle rinsing of the animals prior to the transfer from the labelled to the non-labelled yeast suspension, compared to the rinsing applied prior to plating, may not have cleared the filtering apparatus and food groove of the juvenile *Artemia* completely. The variation (CV < 8%) between the ingestion calculated by summation for the various post-feeding treatments was corrected by the expression of the ^{14}C -budget components as fractions of the latter (Figs 35, 36). The loss of radioactivity from the animals fed the treated yeast was coupled with an increase of the ^{14}C -activity in the dissolved and the particulate carbon compartment until 1 and 2 h of post-feeding, respectively. The independent variation of the latter two pools indicated that the dissolved carbon originated from respiration and excretion ($U+R_{pf}$) rather than from defecation (F_d). The transfer of approximately 5% of the total carbon budget from the particulate to the animal's carbon compartment may indicate a recycling of labelled faecal material between 2 and 4 h of post-feeding. The loss of about a third of the retained carbon between 4 and 24 h of post-feeding, appeared in the dissolved carbon compartment and was thus due to metabolic expenditure.

Artemia retained significantly more ^{14}C after 2 h when transferred to filtered seawater instead of a yeast suspension (ANOVA, Tukey HSD, $P < 0.01$; Fig. 36A) after the pulse labelling. Fig. 36B shows that this was mainly due to a reduction of the faeces production (Fig. 36B). No significant differences were observed between the carbon budgets of *Artemia* after 2 h of post-feeding on a yeast concentration of either 1.6 or 15.6 mg C l⁻¹. However, the transfer of carbon from the particulate to the animal's carbon pool between 2 and 3 h of post-feeding was more pronounced at the low yeast concentration. This may indicate a more important recycling of faecal material, due to the higher clearance rate maintained by *Artemia* at concentrations below the incipient limiting level. The metabolic loss of retained carbon

was initiated earlier in *Artemia* switched to starvation or a limiting food concentration (Figs. 36AC). As a result, carbon retention did not differ significantly between animals that were switched to either of the feeding regimes for 4 h (ANOVA, $F_{4,14}=0.849$, $P=0.45$).

Ingestion as well as retention rate in 8-days old brine shrimp of similar size (± 5 mm), showed a variation between individuals of nearly 30% (CV, $n=11$; Fig. 37).

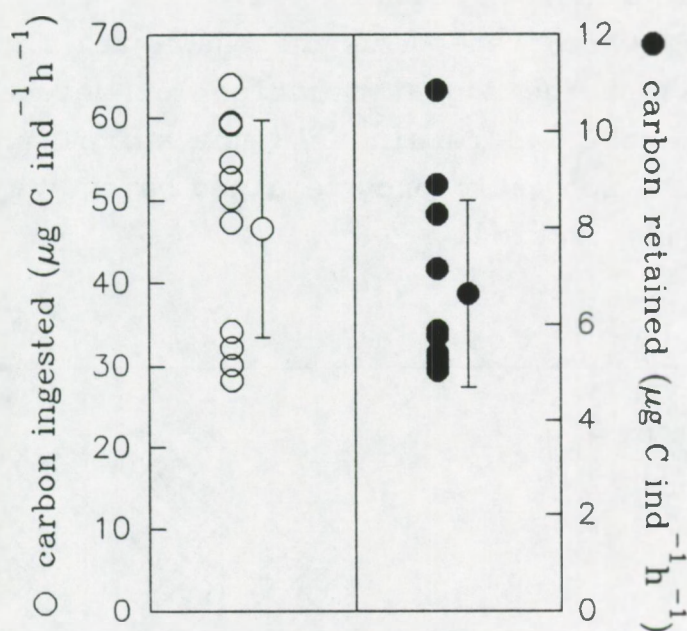


Fig. 37: Individual variation of ingestion (I) and retention ($P+R_m$) rate in *Artemia* (body length = 4.7 ± 0.5 mm) fed untreated yeast. Means and standard deviations ($n=11$) are indicated.

VI.3.4.2. Effect of yeast treatment and concentration on carbon budget

The carbon budgets of *Artemia* fed at various concentrations of untreated yeast following a 2-h acclimation period to a saturating concentration (15 mg C l^{-1}) of the same yeast, are presented in Table 31 and Fig. 38. Ingestion was saturated at a yeast concentration between 3.4 and 8.2 mg C l^{-1} . The yeast concentration did not affect the proportion of the various components of the carbon budget, with the exception of the respiration term which tended to increase with increasing food level. 52 to 64% of the ingested ration was defecated, whereas only about a quarter was retained in the animal (Table 31). As a result, assimilation efficiency remained below 37% irrespective of the yeast concentration.

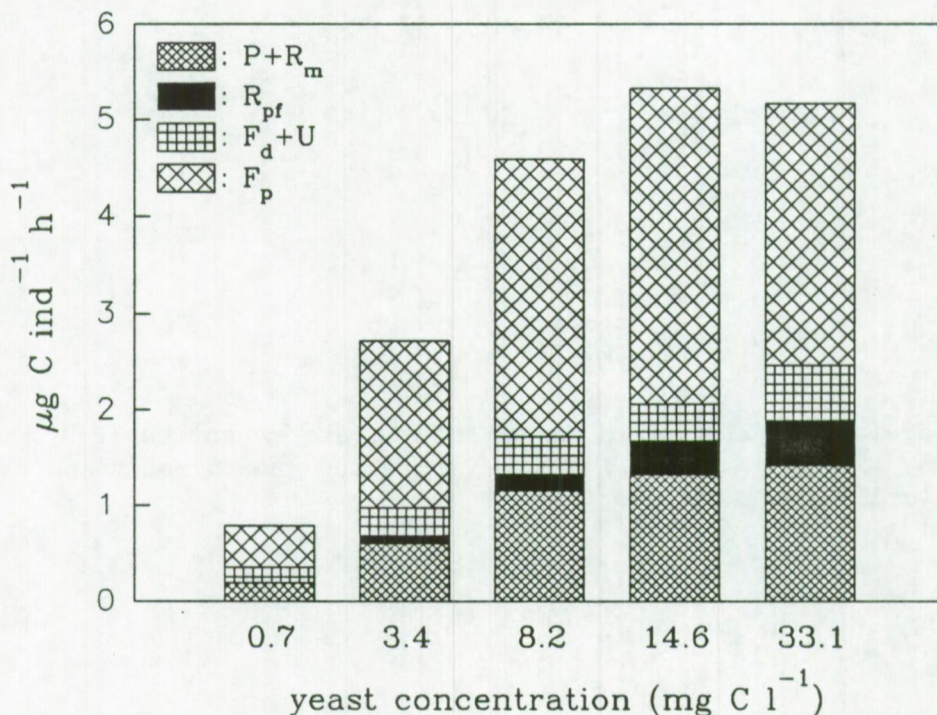


Fig. 38: Carbon budgets for *Artemia* (body length = $3.04 \pm 0.37 \text{ mm}$) fed untreated yeast at various concentrations following acclimation to a standard saturating yeast concentration (15 mg C l^{-1}). Data for the various carbon compartments are normalized to the directly measured ingestion rate, which is represented by the total bar (see also Table 31).

Table 31: Carbon budgets for *Artemia* (body length = 3.04 ± 0.37 mm, $n=15$) fed untreated yeast at various concentrations following acclimation to a standard saturating yeast concentration (15 mg C l^{-1}). All data, unless otherwise stated, represent rates expressed as $\mu\text{g C ind}^{-1} \text{ h}^{-1}$. Standard deviation is given for the estimates from radioactivity in animals (mean \pm SD from 6 samples of 10 animals).

	yeast concentration (mg C/l)				
	0.7	3.4	8.2	14.6	33.1
I	0.787 ± 0.119	2.720 ± 0.624	4.590 ± 0.615	5.324 ± 0.761	5.165 ± 1.167
P+R _m	0.248 ± 0.031	0.517 ± 0.097	1.172 ± 0.106	1.278 ± 0.252	1.181 ± 0.354
F _p	0.548	1.503	2.905	3.144	2.246
F _d +U	0.199	0.248	0.389	0.367	0.473
R _{pt}	N.D.	0.080	0.181	0.340	0.394
IΣ	0.995	2.348	4.648	5.130	4.294
A	0.248	0.597	1.353	1.618	1.574
a (%)	24.9	25.4	29.1	31.6	36.7
<u>expressed as percentage of IΣ:</u>					
I	79	116	99	104	120
P+R _m	25	22	25	25	27
F _p	55	64	63	61	52
F _d +U	20	11	8	7	11
R _{pt}	N.D.	3	4	7	9

abbreviations: I = ingestion (estimated directly), P+R_m = retained carbon after 2-h post-feeding, F_p = particulate carbon (faeces), F_d+U = dissolved organic carbon (dissolved faeces + excretion), R_{pt} = dissolved inorganic carbon (respiration during post-feeding), IΣ = ingestion, calculated by summation ($I + [P+R_m] + F_p + [F_d+U] + R_{pt}$), A = assimilated carbon ($[P+R_m] + R_{pt}$), a (%) = assimilation efficiency = $(A/I\Sigma) \times 100$
 N.D. = not detectable

The comparison of the ^{14}C -budget in *Artemia* fed either treated or untreated yeast confirmed the previous findings with regard to the differential ingestion of these yeasts. At a food concentration above 3 mg l^{-1} , carbon ingestion rates of *Artemia* feeding on untreated yeast were 1.6 to 1.8 times higher than those in animals fed treated yeast at comparable concentrations (Table 32; Fig. 40A). As opposed to the previous experiment, the carbon budget of *Artemia*, which had been acclimated to the experimental food concentration, was greatly affected by the concentration of the untreated yeast. The ratio of retained carbon to faecal carbon was reversed in the concentration range

of 1.4 to 16.1 mg C l⁻¹, while the dissolved carbon pool maintained a constant proportion in the same concentration range (Table 32, Fig 39). As a result, assimilation efficiency increased with decreasing concentration of untreated yeast (Fig. 40C). By contrast, *Artemia* assimilated the T12 yeast with a constant efficiency of 72-79% in the concentration range from 0.6 to 13.5 mg C l⁻¹ (Fig. 40C). As a result, the assimilation rate as a function of yeast concentration followed the same pattern as the ingestion rate for the treated yeast, whereas the untreated yeast was assimilated at a maximal rate at a concentration of 3.1 mg C l⁻¹ (Fig. 40B). The ¹⁴C-fraction spent for respiration was significantly higher in brine shrimp fed the treated yeast compared to those ingesting untreated yeast (respectively, 12.3 ± 0.8% and 4.4 ± 0.6%; Table 32; t-test P<0.001).

Table 32: Carbon budgets for *Artemia* (body length = 4.72 ± 0.50 mm) fed either untreated or treated (T12) yeast at various concentrations following acclimation to the experimental yeast type and concentration. All data, unless otherwise stated, represent rates expressed as a percentage of IΣ.

UNTREATED YEAST	yeast concentration (mg C/l)			
	1.4	3.1	7.8	16.1
I (μg C ind ⁻¹ h ⁻¹)	2.482 ± 0.503	10.525 ± 2.401	14.908 ± 1.594	15.596 ± 1.702
IΣ (μg C ind ⁻¹ h ⁻¹)	2.302	13.580	24.175	18.409
P+R _m	72.9	54.1	27.2	19.6
F _p	27.1	37.1	66.3	73.5
F _d +U	N.D.	3.8	2.6	2.7
R _{pt}	N.D.	5.0	3.9	4.2
a (%)	(>) 72.9	59.1	31.2	23.8

TREATED YEAST	yeast concentration (mg C/l)			
	0.6	2.6	6.8	13.5
I (μg C ind ⁻¹ h ⁻¹)	1.565 ± 0.132	6.519 ± 0.715	9.244 ± 1.213	8.424 ± 0.641
IΣ (μg C ind ⁻¹ h ⁻¹)	2.025	7.787	8.999	10.003
P+R _m	67.7	63.0	60.1	63.0
F _p	16.6	21.2	24.1	19.1
F _d +U	4.1	3.9	3.5	4.6
R _{pt}	11.6	11.9	12.3	13.4
a (%)	79.3	74.9	72.4	76.4

abbreviations: same as in Table 31.

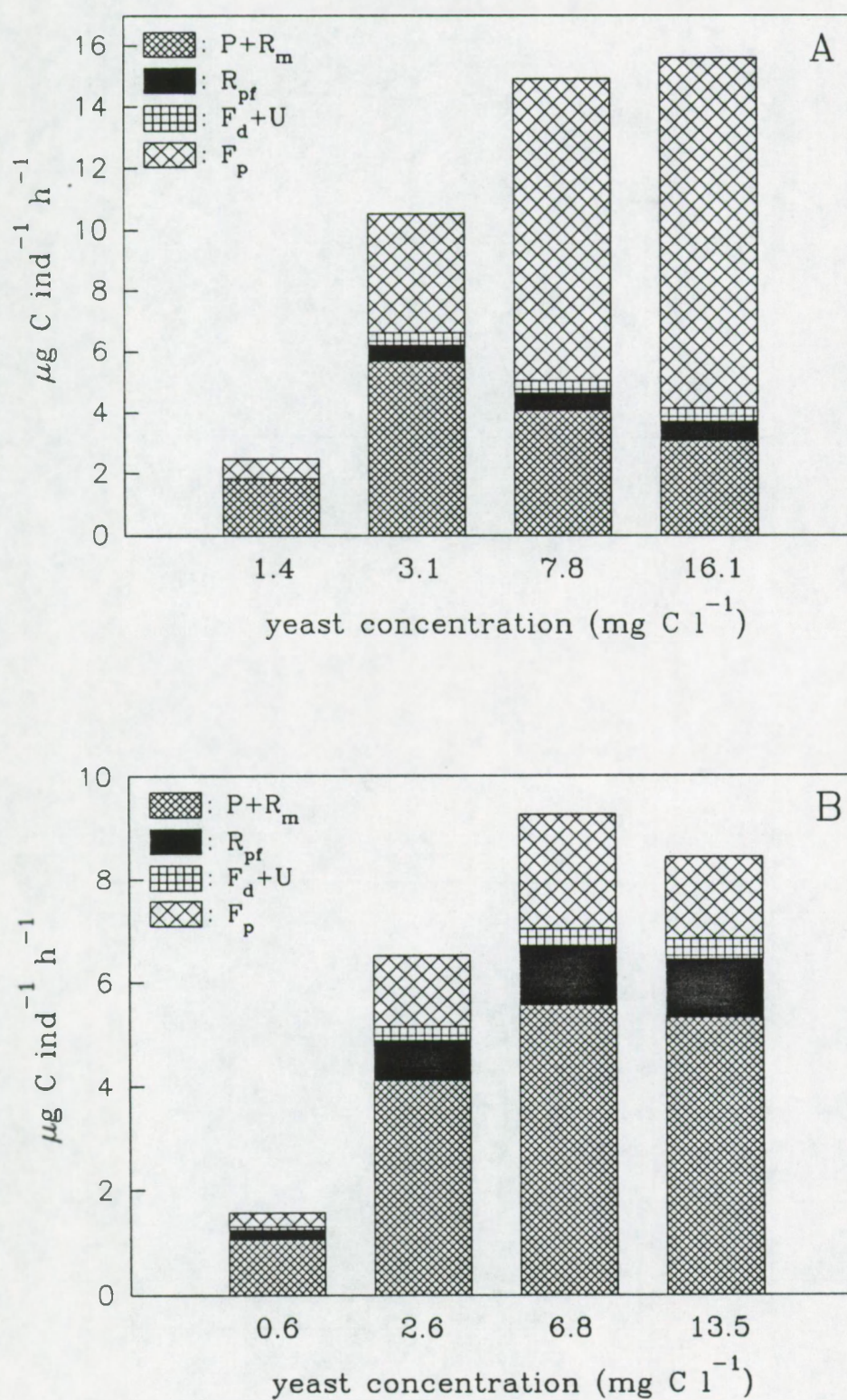


Fig. 39: Carbon budgets for *Artemia* (body length = 4.72 ± 0.50 mm) fed untreated (A) and treated (T12: B) yeast at various concentrations following acclimation to the experimental food concentration. Data for the various carbon compartments are normalized to the directly measured ingestion rate, which is represented by the total bar (see also Table 32).

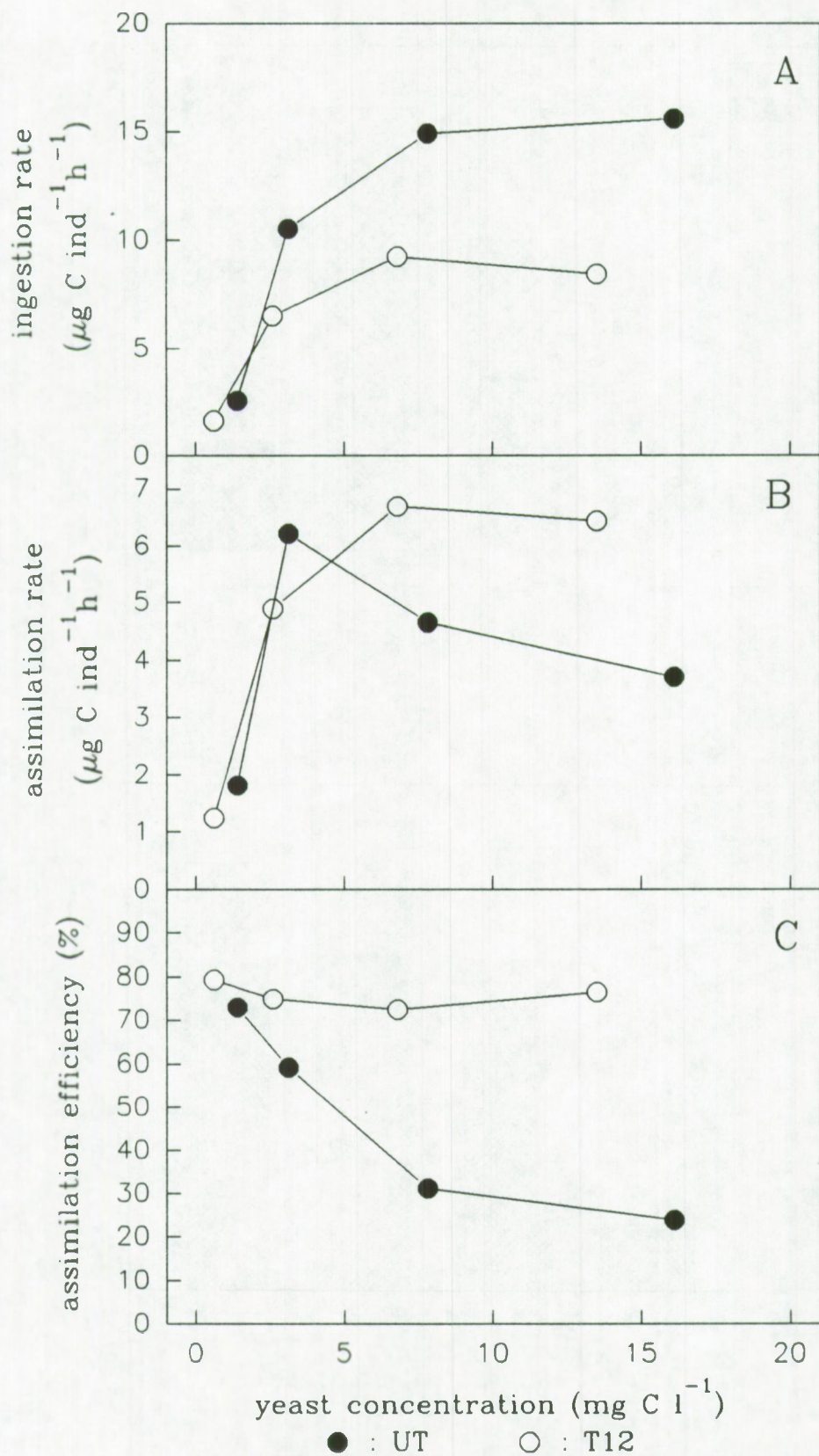


Fig. 40: Ingestion (A) and assimilation rate (B), and assimilation efficiency (C) in *Artemia* (body length = 4.72 ± 0.50 mm) as a function of concentration of untreated (UT: ●) and treated (T12: ○) yeast.

VI.4. DISCUSSION

VI.4.1. Culture, labelling and chemical treatment of baker's yeast

The complex YPG medium was not suitable for labelling baker's yeast with ^{14}C -glucose. The incorporation of non-radioactive carbon compounds present in the yeast extract and the peptone (e.g. amino acids) yielded yeast of a low specific activity and may have resulted in non-uniform labelling of the various cell compartments. In particular, the compartments with high carbohydrate content (e.g. the cell wall, containing over 70% of carbohydrate in *S. cerevisiae*; Killick, 1971) may be more labelled than for example the more protein rich compartments. Uniformly labelled food is a prerequisite for measuring carbon assimilation in zooplankton with ^{14}C tracer techniques (Conover & Francis, 1973; Lampert, 1977a; Porter et al., 1982). Whereas non-uniform labelling in algae usually will cause an overestimation of assimilation rate due to the incomplete labelling of the less digestible, complex structures (Nielsen & Olsen, 1989), the opposite could be expected when using yeast labelled on YPG medium with ^{14}C -glucose. Uniform labelling was obtained by growing baker's yeast on a minimal medium containing glucose as the only carbon source. The only effective way to check for uniform labelling of food cells is to divide the cells into two or more arbitrary fractions and determine the specific activity of each fraction (Peters, 1984). In the present study this was evidenced by the identical specific activities of the untreated and treated yeast, since the latter was a fraction containing about 70% of the carbon of the former.

The ^{14}C uptake efficiency by baker's yeast was lower than 20%, though comparable with that of $\text{H}_3^{32}\text{PO}_4$ reported by Downing & Peters (1980) for *Rhodotorula* cultured in the presence of low concentrations of yeast extract. The latter authors could stimulate ^{32}P uptake to more than 50% by eliminating non-radioactive P-sources from the medium for one day and promoting cell growth by the addition of small amounts of P-containing

yeast extract. Although the latter method produced highly radioactive yeast, the uniformity of the tracer distribution is doubtful. Furthermore, the ^{14}C uptake in yeast corresponds with the carbon assimilation and thus depends primarily on the efficiency of the energy metabolism, which is in turn determined by the culture conditions (e.g. aeration intensity, medium composition). Higher specific activities than those reported in this study may be obtained by further increasing the ratio of tracer to carrier glucose in the YNBG medium. However, the latter may be limited by the decrease of yeast growth rate with increasing tracer concentrations and/or the cost of the tracer.

Numerous studies on various species of yeast have shown that the susceptibility of the cell wall of whole yeast to *in vitro* digestion is increased by the treatment with thiol compounds, such as 2-mercaptoethanol, dithiothreitol, and cysteine (reviewed by Arnold, 1981; Davis, 1985). It is generally hypothesized that thiol reagents disrupt the outer layer of the cell wall which is made of mannoprotein molecules that are cross-linked by disulfide bridges (see also Chapter IV). The cross-linking forms a barrier against the escape of periplasmic enzymes as well as against the penetration of extracellular glucanases to the internal glucan layer, which is the main skeletal component of the wall (Farkas, 1985). Although a thiol treatment is routinely applied in genetic studies that require enzymatic removal of the yeast cell wall, the release of material from intact yeast cells due to this pretreatment is poorly documented in the literature. Weinberg & Orton (1966) showed that periplasmic enzymes could be eluted from intact cells of *Saccharomyces fragilis* through a treatment with 2-mercaptoethanol, although this was not observed for *S. cerevisiae*. Treatment with hot, dilute alkali has been applied to extract mannoproteins as well as glucans from yeast (reviewed by Bacon, 1981). However, these drastic treatments (e.g. 3% NaOH at 75 °C dissolved 83% of the yeast's dry matter; Bacon *et al.*, 1969) are not comparable with the treatments applied in this study (e.g. NaOH 0.6%, partially neutralized by cysteine 0.05 M, at 30 °C). Furthermore, Bacon *et al.* (1969) could not extract the cell wall glucan at room temperature in 3% sodium hydroxide

unless a pretreatment with acid was applied. The enhanced ^{14}C release by treating yeast at elevated pH with cysteine may indicate that cysteine facilitated the alkali extraction of carbohydrates from the cell wall.

The carbon loss during the cysteine treatment appears to be in conflict with the earlier observations that the microscopic appearance and dry weight of yeast cells are not affected by the thiol treatment (see Chapter IV). However, the specific requirements of the radiotracer experiments (use of yeasts grown on a minimal medium, small quantities of yeast incubated at low densities in the treatment medium; see IV.4.) may have caused a more drastic treatment of the yeast compared to the standard cysteine treatment (C-yeast: commercially available yeast, incubated at 50 times higher densities in the treatment medium). *In vitro* studies have indeed demonstrated that the effectiveness of sulfhydryl treatments depends on the growth conditions of the yeast, such as medium composition and shaking intensity (Killick, 1971; Kratky *et al.*, 1975; Kaneko *et al.*, 1973). The more extreme treatment conditions may thus have resulted in a leaching of cell contents for the most sensitive fraction of the yeast cell population.

VI.4.2. Gut passage time, retention efficiency and ingestion rate

The discontinuity in tracer uptake after 5 min exposures to either of the yeast types, revealed a disturbance of the feeding of *Artemia* due to the transfer from the pre-feeding to the labelled food suspensions. The data from Peters (1972) suggest a similar disturbance in *Daphnia rosea*. Rigler (1961) avoided removing *D. magna* from the water by adding the non-radioactive food to the suspension of unlabelled food, and did not observe any interruption of feeding. The initial lag in the uptake of the labelled yeast means that the rates of ingestion and assimilation measured in the pulse-labelling experiments are slightly underestimated.

The gut passage time (GPT) in rotifers and cladocerans increases with body size, decreasing temperature, and with decreasing food concentration (reviewed by Peters, 1984). This feeding parameter is less well documented for the brine shrimp. The GPTs reported in this study were determined under food saturating concentrations and thus correspond with a minimal value. The minimal GPT of about 30 min in *Artemia* (3 mm body length) feeding on untreated yeast was comparable with that observed for brine shrimp (1.2 mm) ingesting ^{14}C -labelled *Isochrysis galbana* (28 min: Evjemo, in prep.). Similarly, Nimura (1989) reported a shortest gut passage time of about 30 min irrespective of the body size for *Artemia* fed either a marine chlamydomonad or starch particles. Korstad (1990) observed a GPT of less than 20 min for *Artemia* in the size range of 0.7 to 4 mm feeding on various radioactively labelled algal species. Apparently, the GPT of *Artemia* consuming the untreated yeast is similar to that when feeding on micro-algae, whereas the treated yeast passed at a much lower rate through the gut. The higher retention efficiencies indicated that the treated yeast was more efficiently digested than the untreated yeast. In this way, the digestive physiology of the brine shrimp differs from that of gastropod veligers, where low digestibility is associated with a longer processing time in the gut (Fretter & Montgomery, 1968; Pechenik & Fisher, 1979). Alternatively, the untreated yeast may be processed by the digestive system as an "inert particle" and forced through the gut as was observed in gastropod larvae (Fretter & Montgomery, 1968) and bivalve juveniles fed undigestible algae (Bricelj et al., 1984a), and *Artemia* fed sand particles (Reeve, 1963b).

A general inverse relationship between gut passage time and ingestion rate is apparent from Fig. 41, which relates the various gut passage times determined from the uptake or evacuation of radioactive yeast by *Artemia* with the corresponding feeding rates.

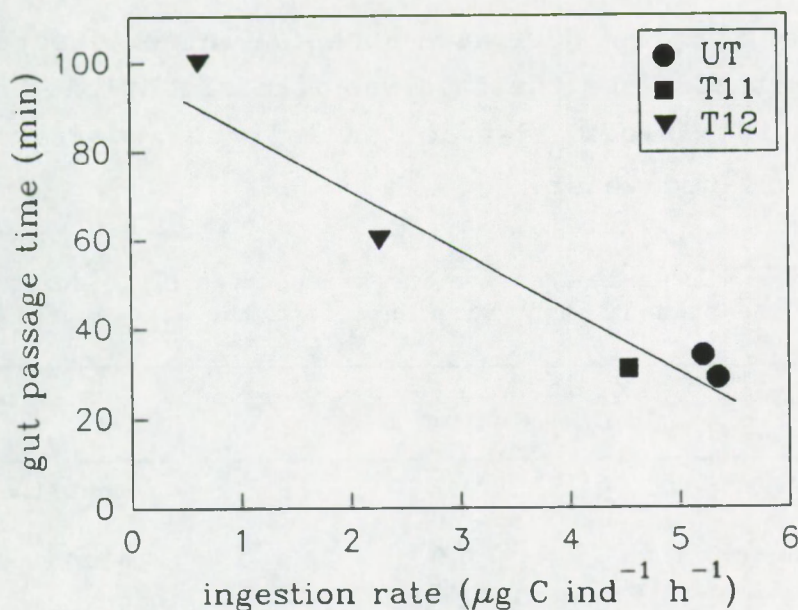


Fig. 41: Gut passage time as a function of ingestion rate in *Artemia* feeding on the various yeast types at saturating concentration.

The apparent discrepancy between the retention rate calculated from the slope of the ^{14}C -uptake curve beyond the inflection point and the value obtained from the pulse-labelling experiment may have several reasons. The higher variation of the retained tracer per animal, due to an asynchronous start of defecation in the animals, resulted in less dependable regression analysis beyond the break in the uptake curve. In addition, the metabolic loss of tracer may have been relatively more important in the time course experiment where the animals are fed labelled food for a longer period of time. Lampert (1977a) found that the percentage of assimilated ^{14}C lost through respiration during feeding on labelled food was largely dependent on the duration of the latter, with losses of up to 34% and 20% for respectively 3- and 1-h feeding experiments. Holm *et al.* (1983) reported similar values for the retention efficiency (*i.e.* "assimilation efficiency uncorrected for respiration") calculated either from the ^{14}C -uptake by *Daphnia pulex* between 1 and 3 h after the start of feeding on radioactive algae, or from animals fed for 1 h on labelled algae and allowed to clear their gut for 1 h on unlabelled food. However, these authors found retention

efficiencies lower than 36%. Our data showed a larger discrepancy between the two methods when the food was more digestible, as can be expected from the larger ^{14}C -fraction lost to respiration in *Artemia* fed the treated yeast.

Table 33: Comparison of the maximal carbon ingestion rates (I_{\max}) for juvenile *Artemia* obtained in the present study with data from the literature.

<i>Artemia</i> body length (mm)	food	I_{\max} ($\mu\text{g C ind}^{-1} \text{ h}^{-1}$)	method	source
1.89 3.94	<i>Isochrysis galbana</i>	1.60 3.10	^{14}C	Korstad (1990)
1.78 3.56 4.63	<i>Isochrysis galbana</i>	0.50 1.67 4.36	^{14}C	Evjemo (in prep.)
2.43 5.66	treated baker's yeast (dried C-yeast)	2.42 8.09	cell counting	Table 22 (see V.3.1.) [†]

	untreated baker's yeast (UT)	treated baker's yeast (T11)	treated baker's yeast (T12)	^{14}C	present study (VI.3.)
3.10	5.21	4.53	2.27		Table 28
3.04	4.06	0.81	0.86		Table 29
3.04	5.34				Table 30
2.02			0.63		Table 30
3.04	5.03				Table 31 [‡]
4.72	15.25		8.83		Table 32 [‡]

†: conversion factor: $18.3 \text{ pg C (yeast cell)}^{-1}$, based on $36.5 \text{ pg DW cell}^{-1}$ (see IV.4.3.) and carbon content of 50% (Evjemo, unpubl. data)

‡: mean of the ingestion rates measured at concentrations above the incipient limiting level

The comparison of the ingestion rates obtained by different investigators is hampered by differences in experimental conditions (e.g. animal size, food type and concentration) and applied methodology. Furthermore, the present study demonstrated that the variation of feeding rate between experiments performed under similar conditions differed according to the food type. The ingestion rate of similarly sized *Artemia* fed untreated yeast was consistent between experiments, whereas that of animals fed the treated yeast ranged from about 20 to 50% of the former (Table 33). Also, the T11 yeast was consumed at a rate corresponding

with that of either the untreated or the T12 yeast, depending on the experiment. Nevertheless, the highest ingestion rates measured in this study for *Artemia* fed T12 yeast are comparable with those reported by Korstad (1990) and Evjemo (in prep.), using the same ^{14}C -techniques for brine shrimp fed algae, and with those found by means of the cell count method (V.3.1.) for animals of approximately the same size (Table 33). *Artemia* thus appears to ingest the untreated yeast at abnormally high rates, whereas the T12 yeast is consumed at rates which are comparable with those reported in the literature for brine shrimp fed algae.

It is generally believed that the maximal ingestion rate of *Artemia* is determined by physical limits, i.e. the total volume of the ingested particles, regardless of the species of food (Reeve, 1963b; Sick, 1976; Yanase & Shiraishi, 1972). However, microscopic observation and analysis of the particle size distribution by means of a Coulter counter could not detect any differences between the size of treated and untreated yeast cells. Also, the loss of about 30% of the yeast's carbon content during the chemical treatment cannot explain the 2 to 5 fold lower ingestion rates observed for the treated yeast. The present data thus demonstrate that ingestion capacity of particles of the same volume can differ considerably and may be inversely related with the digestibility of the food. This is further supported by the observation of Reeve (1963b) that *Artemia* ingested sand particles at 10 times higher maximal rates in terms of volume than when feeding on algae of the same average particle volume. The higher ingestion rate of particles of a low digestibility is coupled with a shorter gut passage time.

The digestibility of the treated yeast depends on the efficiency of the chemical treatment as well as on the digestive capability of the animals. Variation of the efficiency of the yeast treatment between experiments is unlikely because of the high degree of standardization of the treatment conditions, as demonstrated by the reproducibility of the ^{14}C -loss. Although the *Artemia* used for this study were grown in controlled lab cultures, the physiological condition of the animals at the moment of the harvest may have differed between experiments and

may not have been completely standardized after the short-term acclimation. Differences in the digestive activity may thus explain the variation of the uptake of the treated yeasts between (T12) and within (T11 versus T12) experiments. The extremely low digestibility of untreated yeast under saturating food conditions would exclude a dependence of feeding on the level of the digestive enzymes and thus explain the consistently elevated feeding rates.

The incipient limiting concentration of 1.4 mg C l^{-1} for *Artemia* feeding on the treated yeast was in agreement with that observed by the cell count method for brine shrimp in the size range of 2.4 to 7.2 mm (Table 34). The somewhat higher incipient limiting concentration reported by Evjemo (in prep.) for *Artemia* fed *Isochrysis* may be due to the use of smaller animals than in this study.

Table 34: Comparison of the incipient limiting concentration for feeding in juvenile *Artemia* obtained by ^{14}C -techniques and the cell count method.

Artemia body length (mm)	food	incipient limiting level		method	source
		cells μl^{-1}	mg C l^{-1}		
2.43	treated baker's yeast (dried C-yeast)	141	2.58	cell counting	Table 22 (see V.3.1.) [†]
5.66		77	1.41		
7.23		82	1.50		
3.04	treated baker's yeast (T12)	-	1.38	^{14}C	Table 29 (VI.3.3.)
1.05	<i>Isochrysis galbana</i>	-	3-4	^{14}C	Evjemo (in prep.)

†: conversion factor: $18.3 \text{ pg C (yeast cell)}^{-1}$, see Table 33 for explanation

Large variation among individuals is a generally occurring phenomenon in feeding studies of zooplankton. For example, individual variability in *Daphnia magna* feeding on fluorescent beads amounted to over 60% (CV, $n=10$, Gerritsen & Sanders, 1987). In this regard, the variation in carbon ingestion and retention by similar sized *Artemia* of about 30% (CV, $n=11$) was acceptable.

VI.4.3. Carbon budget and assimilation efficiency

The measurement of the ^{14}C distribution in the animal's, particulate and dissolved carbon compartments allowed to attribute changes in the tracer retained in the animals during the feeding on unlabelled food. The short-term loss of tracer from *Artemia* feeding on treated yeast was mainly due to gut evacuation, but also for about 25% the result of respiratory losses within one hour after transfer to non-radioactive food. The latter loss corroborates the existence of a metabolic carbon pool in *Artemia* as has been described for cladocerans and copepods (Conover & Francis, 1973; Lampert, 1975, 1977a). This intermediate carbon pool, which is the source for the instantaneous respiration of assimilated carbon, was largely replaced in *Artemia* by unlabelled carbon within one hour after the transfer to non-radioactive food.

Various authors have reported a considerable percentage loss of assimilated isotope due to respiration during the feeding on labelled food (R_f , see introduction), e.g. more than 40% in 5-h experiments (Hargrave, 1970) and even up to 20% in 1-h feeding periods (Lampert, 1977a). For the experiment represented in Fig. 35, this loss may be estimated to be less than 5% of the total ^{14}C -budget, assuming that the production of $^{14}\text{CO}_2$ starts 10 min after transferring the animals to the labelled food at a rate similar to that measured in the first hour of post-feeding (i.e. about $30\% \text{ h}^{-1}$). As a result, the assimilation efficiencies reported in this study approximate the "true" efficiencies very closely.

A more important metabolic loss, amounting to about 25% of the assimilated carbon, occurred between 4 and 24 h of post-feeding. This loss is comparable with the average carbon turnover rate of $1.6\% \text{ h}^{-1}$ found by Bourne (1959, in Conover & Francis, 1973) for *D. magna* during feeding on unlabelled food for up to 71 h, and may thus be ascribed to the turnover of the structural carbon pool. Alternatively, the animals were fed discontinuously during the long-term post-feeding and may thus have mobilized carbon from the structural pool to maintain metabolism during

periods of food depletion. In either case, the considerable loss of retained carbon between 2 h and 24 h of post-feeding indicated that the latter consists for a large part of carbon that is destined for respiration. As a result, $[P+R_m]$ determined after 2 h of post-feeding is seriously overestimating the actual production (P). This was supported by the calculation of the specific growth rate from the growth curve of *Artemia* reared for one week after hatching on a saturating concentration of treated baker's yeast (same batch of *Artemia*, 25 °C; see Fig. 42). The latter empirical value amounted to 0.47 day^{-1} , whereas the estimate projected by the present short-term radiotracer experiments ranged between 0.84 and 1.41 day^{-1} (see Table 35).

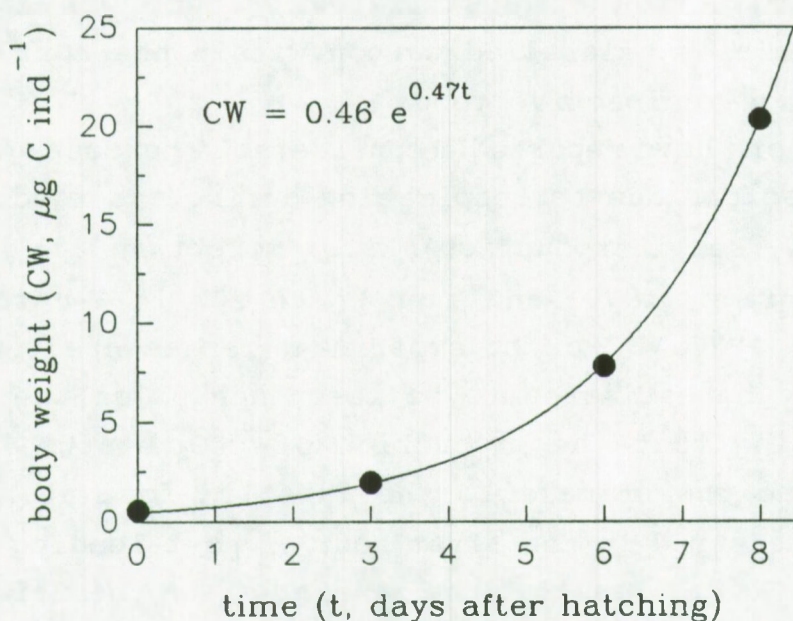


Fig. 42: Estimation of specific growth rate in *Artemia* reared at a saturating concentration of treated baker's yeast ($2,000 \text{ C-yeast cells } \mu\text{l}^{-1}$) during 8 days after hatching. Data derived from Table 25 (see Chapter V). Conversion of *Artemia* body length (L, mm) to carbon content (CW, $\mu\text{g C ind}^{-1}$) was based on the equation $CW = 0.289 e^{1.24L}$ (Evjemo, in prep.).

Table 35: Calculation of maximal weight-specific growth rate (μ) from the amount of carbon retained after 2 h of post-feeding ($[P+R_m]$) in *Artemia* fed the T12 yeast in three independent experiments.

<i>Artemia</i>		$[P+R_m]$		μ^\ddagger	source
body length (mm)	carbon content ($\mu\text{g C ind}^{-1}$) [†]	$\mu\text{g C ind}^{-1} \text{ h}^{-1}$	$\mu\text{g C ind}^{-1} \text{ day}^{-1}$	(day^{-1})	
3.10	13.5	1.74	41.8	1.41	Table 28
2.02	3.54	0.306	7.34	1.12	Table 30
4.72	101	5.55	133	0.84	Table 32

†: Conversion of *Artemia* body length (L, mm) to carbon content (CW, $\mu\text{g C ind}^{-1}$) was based on the equation $\text{CW} = 0.289 e^{1.24L}$ (Evjemo, in prep.).

‡: $\mu = \ln(\text{CW} + [P+R_m]) / \text{CW}$

Animals switched from a saturating food concentration of labelled food to a limiting concentration of unlabelled food or starvation, did not evacuate the gut to the same extent as those fed under saturating food conditions. This indicates that *Artemia* responds nearly instantaneously to limiting food conditions by decreasing the gut evacuation rate. In this regard, the few studies that have compared the gut evacuation rates of feeding and non-feeding copepods reach conflicting conclusions (discussed by Ellis & Small, 1989). Reeve (1963c) observed a gut content in *Artemia* after two weeks of starvation and associated the rate of gut passage to the pressure resulting from the ingestion of food. The higher metabolic ^{14}C loss which occurred 4 h after transferring the animals to a low yeast concentration or filtered seawater may have several causes. The food-limited animals may have partially depended on the labelled gut content, which was retained for a longer period, and the recycling of egested labelled material. Furthermore, starvation may have initiated an earlier turnover of the carbon previously incorporated in the structural carbon pool. Finally, high respiration rates may also have been the result of the higher grazing activity of the animals feeding at concentrations below the incipient limiting level. The determination of the assimilation efficiency on the basis of the ^{14}C distribution after 2 h of post-feeding appeared to be most accurate, as it resulted in a maximal gut clearance and minimal recycling of egested material, irrespective of the

concentration of unlabelled food.

The consistently low retention and assimilation efficiencies in *Artemia* fed the untreated yeast, evidenced its low digestibility. However, the assimilation efficiency of untreated yeast increased with decreasing concentrations when the animals were acclimated to the experimental food concentration. In the same way, Sushchenya (1962) found a decrease of the assimilation efficiency in *Artemia* grown on yeast from 78 to 33% at concentrations ranging from 0.2 to 1×10^6 cells ml^{-1} . By contrast, animals that were acclimated to saturating concentrations of the untreated yeast showed a constant assimilation efficiency irrespective of the yeast concentration. Apparently, the capability of *Artemia* to digest low concentrations of untreated yeast was inversely related to the food level during acclimation. This is in accordance with the lower digestive enzyme activities detected in brine shrimp (Samain et al., 1975; Samain & Moal, 1982) as well as copepods (Hassett & Landry, 1983) after acclimation to high food conditions. The 20 min feeding period on the low concentrations of labelled food was probably too short for inducing an increase of the digestive enzyme activity as expected from the model of Samain & Moal (1982).

Artemia feeding on low concentrations of untreated yeast exhibited carbon assimilation rates which were comparable with the maximal rates observed in animals fed the treated yeast (Fig. 40). This suggests that *Artemia* would be able to grow on untreated yeast if the latter would be offered at extremely low concentrations. However, it should be emphasized that the short-term radiotracer experiments are not necessarily reflecting the feeding kinetics of animals that are grown on untreated yeast from start-feeding onwards. Furthermore, rearing brine shrimp at food concentrations as low as 3 mg C l^{-1} would be practically possible only at extremely low animal densities.

The present study yielded interesting data with regard to the controversy in the literature about the concept of "superfluous feeding" in zooplankton, conceived by Beklemishev (1962) as "... there is a superfluous feeding when actively feeding animals stop responding to an increase in standing crop

of their food by an increase of assimilation". Few laboratory experiments were able to demonstrate a decrease of assimilation rate with increasing food concentrations (Hayward & Gallup, 1976) or a constant assimilation rate with further increasing feeding rates (Richman, 1958). Most investigators found that the assimilation rate depended on food concentration in a similar way as feeding rate, with a linear increase at low food densities and a plateau at higher concentrations (Conover, 1966; Sushchenya, 1970; Lampert, 1977b; Gulati et al., 1987). The latter saturation response was also found for *Artemia* feeding on the treated yeast. On the contrary, the animals fed the untreated yeast showed a typical superfluous feeding response at yeast concentrations above 3 mg C l^{-1} . From this, it seems that Beklemishev's concept is strongly related to the digestibility of the food. When the food is assimilated at a constant efficiency, irrespective of the rate of ingestion and gut passage, the relation between assimilation rate and food concentration is expected to resemble the model of ingestion rate. However, high concentrations of a refractory food particle may lead to a gut retention time which is too short for an efficient digestion. If the latter occurs at a concentration below the incipient limiting level for feeding, a further increase of the food density will result in reduced assimilation efficiencies. Depending on the digestibility of the food particle, i.e. the relative drop of assimilation efficiency with increasing ingestion rates, assimilation rate will further increase, level off, or decrease with increasing food concentrations. The latter is demonstrated by *Artemia* feeding on the untreated yeast after acclimation to the experimental food conditions. It is self-evident that the food digestibility is also determined by the state of the digestive system, which may in turn vary with the acclimation conditions and the physiological status of the animals. This may explain the observations of increasing as well as decreasing assimilation rates (*Artemia* feeding on untreated yeast, respectively, without or with appropriate acclimation) and constant as well as decreasing assimilation rates (*D. pulex* feeding on *Asterionella*; Lampert, 1977b) with increasing food concentration under similar

experimental conditions.

Since the efficiency of the digestion processes is influenced by the amount of food passing through the gut per unit of time rather than by the food concentration surrounding the animal, assimilation efficiency should be analyzed as a function of ingestion rate. In the same way, gross growth efficiency of *Artemia franciscana* fed *Dunaliella tertiolecta* was independent of initial food concentration but determined by the daily amount of food ingested (Abreu-Grobois *et al.*, 1991) and a mathematical model of the assimilation in the copepod *Calanus finmarchicus* predicted a decrease of the assimilation efficiency with increasing ration (Slagstad & Tande, 1981). Figure 43 indicates that assimilation rate increases linearly and assimilation efficiency remains approximately constant up to an ingestion rate of $10 \text{ mg C ind}^{-1} \text{ h}^{-1}$. Higher rates of consumption, measured only for *Artemia* fed the untreated yeast, depressed the rate and efficiency of assimilation. This suggests that the low assimilation efficiency for untreated yeast is at least partially due to the enhanced feeding response of *Artemia*. At low food concentrations, the increased feeding activity balanced the lower digestibility of the untreated yeast, resulting in an assimilation rate which was comparable with that of animals feeding on similar concentrations of the treated yeast. A balancing of assimilation through modulation of ingestion was also demonstrated in *Artemia* fed *Tetraselmis* of different composition (Samain *et al.*, 1981). The latter authors showed that *Artemia* fed the same algal species containing either low or high protein levels, ingested similar amounts of protein by consuming the former algae at a higher rate. Obviously, the same response to compensate for low protein assimilation when feeding on high concentrations of undigestible yeast fails due to the decrease of assimilation efficiency.

The present results demonstrate that the differential ingestion and digestion of untreated and treated baker's yeast by *Artemia* offer a unique test system to study the effect of food digestibility on feeding kinetics in a filter-feeder. Further research is needed to unravel the role of the structure and

configuration of the yeast cell envelope in the response of the feeding and digestive system of *Artemia*. Knowledge of the effect of the chemical treatment on the surface of the yeast cell may contribute to the understanding of the mechanisms that regulate ingestion and digestion in filter-feeding zooplankton.

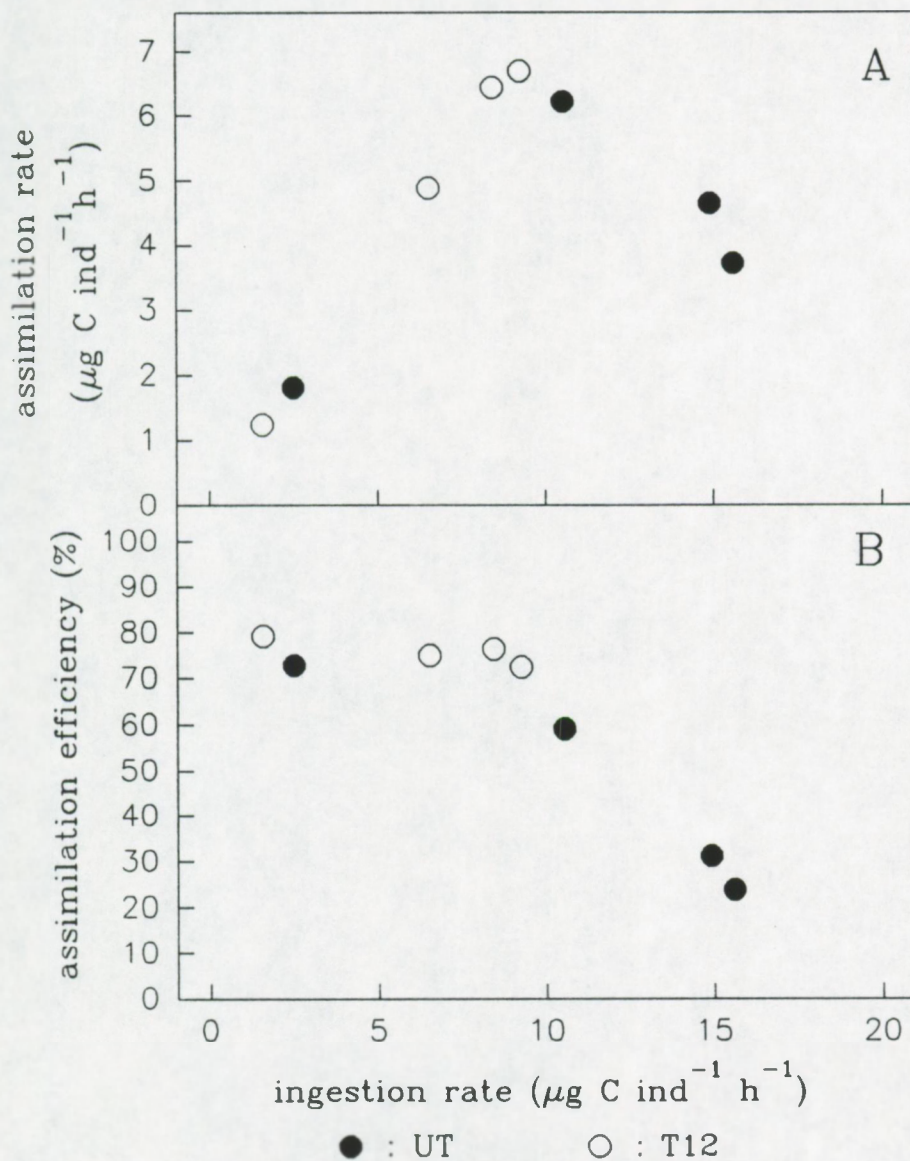


Fig. 43: Assimilation rate (A) and assimilation efficiency (B) in *Artemia* (body length = 4.72 ± 0.50 mm) as a function of ingestion rate of untreated (UT: ●) and treated (T12: ○) yeast.

Chapter VII

MANIPULATED BAKER’S YEAST AS AN ALGAL SUBSTITUTE
FOR THE LABORATORY CULTURE OF *ARTEMIA*

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Chapter VII

MANIPULATED BAKER'S YEAST AS AN ALGAL SUBSTITUTE FOR THE LABORATORY CULTURE OF *ARTEMIA*

VII.1. INTRODUCTION

So far, unicellular algae remain an indispensable food for rearing aquatic filter-feeders at laboratory scale for research purposes. Furthermore, in spite of all efforts to replace algae by artificial feeds, the culture from the egg-stage onwards of many commercially important fish, mollusc, and crustacean species still depends on the production of large volumes of micro-algae. The culture of these unicellular algae, however, is expensive and labour-intensive. The development of a cost-effective algal substitute is therefore of primary importance for large-scale culture systems.

In this regard yeasts offer several interesting characteristics. Because of their suitable particle size and good buoyancy in the water column, yeasts can easily be ingested by filter-feeding organisms. Furthermore, the rigid cell wall prevents the nutrients from leaching into the culture medium and subsequent deterioration of the water quality. In addition, yeasts have a high protein content and can be produced on the basis of various raw materials, independently of the climate, and at relatively low production costs (Kihlberg, 1972).

The use of yeasts for sustaining laboratory cultures of *Artemia* for genetic and morphological studies has already been described by several researchers (e.g. Bond, 1937; Weisz, 1946; Bowen, 1962; and Bowen *et al.*, 1985). Yeast products have also been included as a protein source in mixed diets for the production of brine shrimp biomass, e.g. baker's yeast (Talloen, 1978; James & Makkeya, 1981), *Kluyveromyces* (Lavens *et al.*, 1987), brewer's yeast and methanol yeasts (Robin *et al.*, 1987).

Most trials with various species of marine yeasts lead to poor culture results (Johnson, 1980; Nimmanit & Assawamunkong, 1985), unless the experiments were run in small scale systems with low animal densities (Shimaya *et al.*, 1967; Kawano *et al.*, 1976; Johnson, 1980). Only James *et al.* (1987a) reported high production yields with marine *Candida* yeast when culturing *Artemia* in 10 m³ batch cultures without water renewal. However, the latter culture conditions were suitable for the development of microbial blooms which may have served as a food supplement for the brine shrimp and in this way may have masked the diet's deficiency.

In fact, little work has been done on the evaluation of pure yeast diets under standardized culture conditions. Preliminary trials in 5 l aquaria by Blanco Rubio (1987) indicated that *Torula* yeast (*Candida utilis*) might be a promising food for cultivating *Artemia*. On the other hand, feeding a diet consisting solely of fresh baker's yeast (*Saccharomyces cerevisiae*) under controlled conditions lead to poor culture results (see Chapter IV). Since culture performances could be significantly improved by removing or permeabilizing the yeast cell wall by an enzymatic, respectively, a chemical treatment, the ineffectiveness of untreated baker's yeast appears to be mainly due to its low digestibility.

The present study documents the use of chemically treated baker's yeast as a potential algal substitute for the culture at laboratory scale of the brine shrimp *Artemia franciscana*.

VII.2. MATERIALS AND METHODS

Artemia franciscana cysts (Great Salt Lake, Utah, USA; Sanders Brine Shrimp Co., lot 31627) were disinfected for 20 min in seawater containing 200 ppm hypochlorite and incubated under optimal hatching conditions (Sorgeloos *et al.*, 1986) in artificial seawater (Dietrich & Kalle, 1963; in Kinne 1971). After a cyst incubation period of 24 h, 200 (tests 1-3) or 300 (test 4) freshly-hatched nauplii were transferred to test tubes containing 0.2-µm filtered artificial seawater. Initial animal

density was 2 ml larva⁻¹, which was reduced to 4 ml individual⁻¹ after 7 days of culture. The tests were carried out at 25 ± 1 °C in continuous darkness in cylindro-conical tubes with aeration from the bottom and covered with perforated Petri dishes to minimize evaporation. The experiments were run in duplicate (tests 1-3) or triplicate (test 4) and the algal control was fed *Dunaliella tertiolecta* Butch following the feeding schedule presented in Table 36. After 7 days of culture, experiments 1 and 2 were continued for another week with 100 of the surviving larvae per culture unit. In the fourth experiment, the number of surviving animals per culture was reduced to 150 and 100 after 7 and 11 days of culture, respectively. After two weeks of culture, the remaining animals from the triplicate cultures per treatment were pooled and the test was continued for another three days.

Table 36: Feeding schedule for *Artemia* cultured with *Dunaliella tertiolecta* (modified from Vanhaecke *et al.*, 1984).

day	<i>Dunaliella</i> ration (10 ³ cells ind ⁻¹ day ⁻¹)
1	150
2, 3, 4	300
5, 6	450
7	600
8	750
9	1,220
10, 11	1,440
12, 13	1,800
14, 15	2,160
16, 17	2,250

Survival and average body length were determined at each water renewal, *i.e.* after 7, 11, and 14 days of culture, depending on the duration of the experiment, and at the termination of the test. Body length was measured on 30 animals

per culture from the tip of the head till the end of the telson by means of a dissecting microscope, equipped with a camera lucida, and a digitizing tablet. In the prolonged culture tests, sexual differentiation was followed by determining the percentage of couples (%C) and gravid females (%GF), calculated respectively as

$$\%C = \frac{n_c \times 2}{n} \times 100$$

$$\%GF = \frac{n_{GF}}{n} \times 100$$

where n_c : number of couples, n_{GF} : number of gravid females, n : total number of animals per culture.

The parameter %GF, which was determined during experiment four, could maximally amount to the total percentage of females, which was found to be similar in all cultures as a result of the relatively large number of animals (> 90 culture⁻¹ throughout the experiment) and the *ad random* culling of the cultures.

Two yeast diets were evaluated at various substitution levels for the micro-algae. One yeast product consisted of baker's yeast *Saccharomyces cerevisiae* (provided by Algist Bruggeman N.V., Belgium), which was treated according to the standard cysteine treatment (C-yeast, see IV.4.) to improve its digestibility and stored at -22 °C until use. The second diet (made available by Artemia Systems N.V.-S.A., Belgium) was composed of chemically treated baker's yeast which was processed into a dry product and eventually enriched with highly unsaturated fatty acids. The proximate composition of both yeast diets is given in Table 37.

Different algae/yeast ratios were tested for both the dried (test 1) and the fresh yeast (test 2). Ratios are expressed as a percentage of the algal cells being substituted. In experiments 1 and 2, one algal cell was replaced by four yeast cells. In a third set of experiments different substitution levels were applied for the 100% fresh yeast diet, i.e. one algal cell was replaced by either two (Y2), three (Y3) or four (Y4) yeast cells. In addition, the effect of higher water quality was examined by

renewing the seawater on the fifth day of culture. During the fourth experiment, in which one algal cell was replaced by three yeast cells, various mixed diets consisting of dried yeast for at least 50% were evaluated during a longer culture period.

Differences between treatment means were tested for experiment 4 by means of the t-test (Sokal & Rohlf, 1981).

Table 37: Proximate composition of the yeast diets. Figures are percent of the dry weight of the diet, unless stated otherwise.

	fresh yeast ¹	dried yeast ²
protein	48-54	40
carbohydrate	± 30	35
lipid	< 2	18
Σ(n-3) HUFA (% of total lipid)	-	28
moisture (% of total diet)	± 68	< 3

¹: data provided by Algist Bruggeman N.V.

²: data provided by Artemia Systems N.V.-S.A.

VII.3. RESULTS

Body length and survival in *Artemia* after 7 days of culture is presented in Figs. 44A, 44B, and 45 for experiments 1, 2, and 3, respectively. For the latter, body length was expressed as a percentage of the length measured in the algal control treatments (100% algae), except for experiment three where the 5/95 algae/yeast treatment was used as a control. The results of the cultures that were prolonged after the first week are presented in Table 38.

The 25/75 algae/yeast diet yielded higher growth than the 100% algal diet, while survival rates were not found to be different. This was true for both the fresh and the dried yeast, although a higher level of substitution (95%) was only successful with the fresh yeast (Fig. 44). Feeding both yeasts as a sole diet resulted in a poor survival, except for the case where the amount of fresh yeast was adapted by replacing one algal cell by

three yeast cells (Fig. 46). Nevertheless, in the latter case survival hardly exceeded 50% and could not be improved by renewing the culture medium on the fifth day of culture. After 14 days, larger *Artemia* and a higher percentage of couples were obtained with the 25/75 algae/dry yeast diet compared to the algal control (Table 38: test 1), which in turn performed better than the algae/fresh yeast diets (Table 38: test 2).

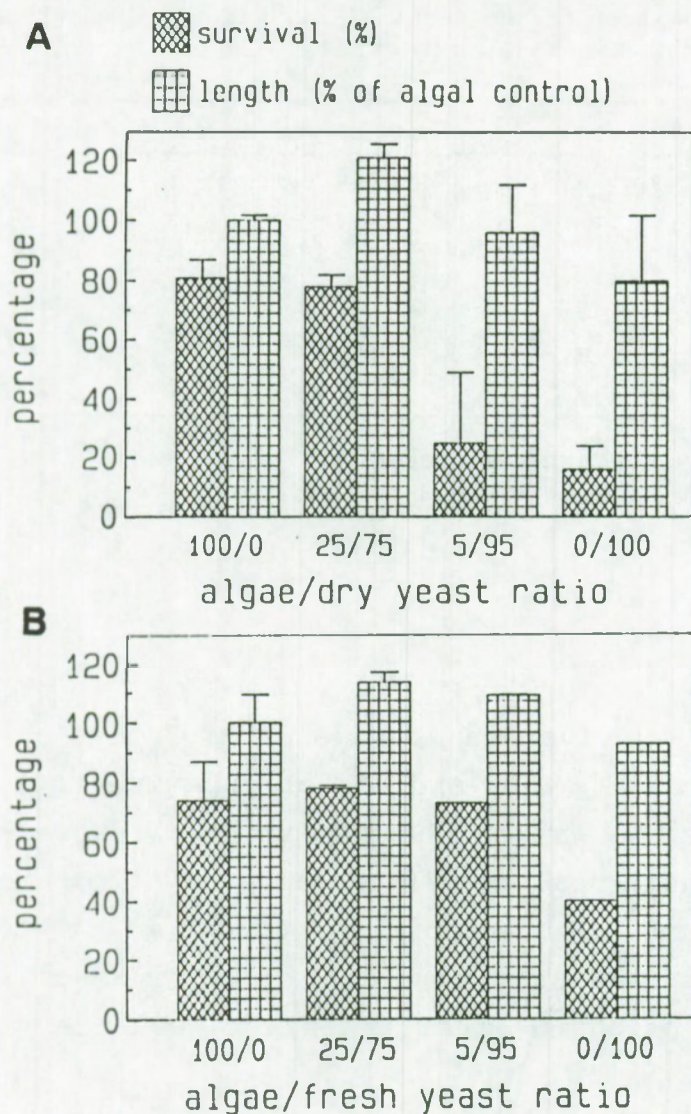


Fig. 44: Survival and growth of *Artemia* fed various mixtures of algae and either dry yeast (A: Test 1, 100% growth = 4.15 mm) or fresh yeast (B: Test 2, 100% growth = 4.82 mm) after 7 days of culture. Body length is expressed as a percentage of that obtained for the algae-fed controls. Data represent means and standard deviations from two replicates.

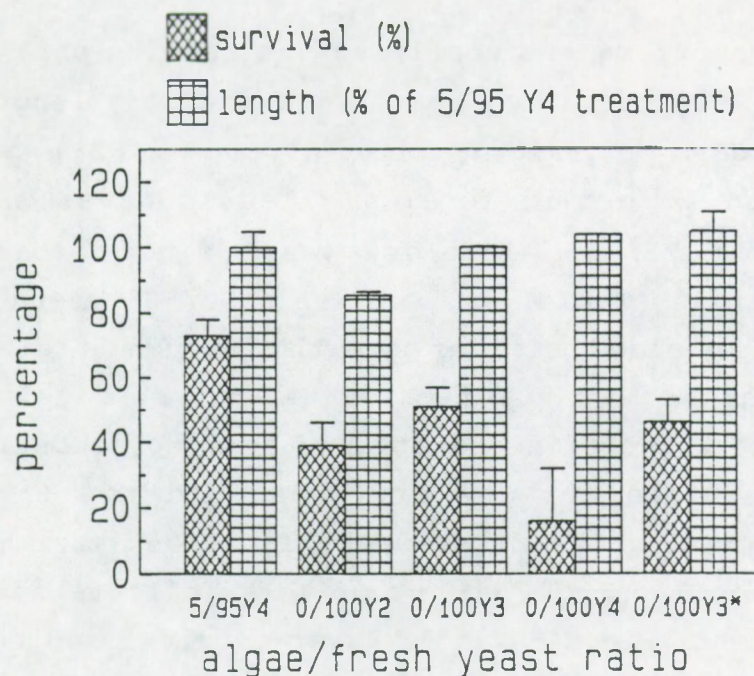


Fig. 45 (Test 3): Survival and growth of *Artemia* fed various rations of fresh yeast after 7 days of culture (100% growth = 5.58 mm). Replacement of one algal cell by two, three, or four fresh yeast cells is indicated by Y2, Y3, or Y4. The culture medium is renewed on the fifth day for the treatment denoted with *. Body length is expressed as a percentage of that obtained for the control treatment (5/95 algae/yeast). Data represent means and standard deviations from two replicates.

Table 38 (Test 1 & 2): Survival, body length and percentage of couples in *Artemia* fed various mixtures of *Dunaliella* and yeast after 14 days of culture. Data represent means and standard deviations from two replicate cultures.

		survival (%) [†]	body length (mm)	% of couples
test 1	algae/dry yeast ratio			
	100/0	89 ± 6	7.13 ± 0.23	30 ± 2
	25/75	88 ± 7	7.81 ± 0.14	43 ± 6
test 2	algae/fresh yeast ratio			
	100/0	96 ± 1	8.19 ± 0.05	31 ± 9
	25/75	95 ± 2	7.71 ± 0.09	5 ± 1
	5/95	96 ± 1	7.50 ± 0.52	7 ± 7

†: survival after 7 days of culture is 100%

The fourth experiment confirmed the results obtained during test one, *i.e.* significantly faster growth (body length after 7, 11, and 14 days: $t_s=3.88^*$, 4.06^* , and 2.82^*) and sexual differentiation (% gravid females after 14 days: $t_s=2.86^*$) in *Artemia* fed the 25/75% algae/dry yeast diet compared to the algae-fed controls (Table 39). Feeding a 50/50% mixture of algae and dry yeast yielded similar growth, though a significantly lower percentage of gravid females (after 14 days: $t_s: 2.86^*$) compared to the 25/75% mixed diet. Furthermore, substituting 90% of the algal ration for the dry yeast did not significantly affect growth during the first week (Table 39: $t_s=1.87$ ns), but resulted in a collapse of two out of three culture in the course of the experiment (Fig. 46) and a lower survival during the final period (Table 39). Feeding *Artemia* solely on dry yeast resulted in a limited survival in only one replicate after one week of culture.

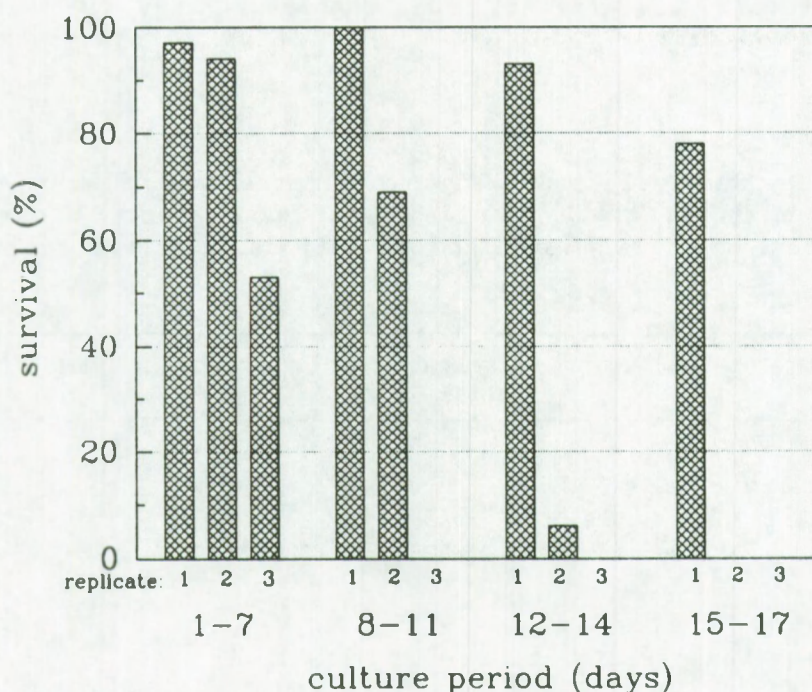


Fig. 46 (Test 4): Survival as a function of time in three replicate cultures fed the 10/90% *Dunaliella*/dry yeast diet. Survival is expressed as a percentage of the number of animals at the start of each culture period.

Table 39 (Test 4): Survival, body length, percentage of couples and percentage of gravid females in *Artemia* fed various mixtures of *Dunaliella* and dry yeast after 7, 11, 14 and 17 days of culture. Data represent means and standard deviations from three replicate cultures, unless otherwise indicated.

treatment A= <i>Dunaliella</i> Y= dried yeast	culture period			
	7 DAYS	11 DAYS	14 DAYS	17 DAYS†
survival (%)§				
100 A	95 ± 2	95 ± 3	99 ± 1	95
50/50 A/Y	96 ± 1	97 ± 4	98 ± 3	99
25/75 A/Y	94 ± 5	99 ± 1	97 ± 3	99
10/90 A/Y	81 ± 24	85 ± 22 (n=2)	93 (n=1)	78
100 Y	57 (n=1)	0	-	-
body length (mm)				
100 A	3.91 ± 0.12	6.02 ± 0.10	7.16 ± 0.38	ND
50/50 A/Y	4.16 ± 0.07	6.69 ± 0.21	7.89 ± 0.36	ND
25/75 A/Y	4.38 ± 0.16	6.88 ± 0.35	8.10 ± 0.43	ND
10/90 A/Y	3.59 ± 0.38	5.05 ± 0.82	6.68 (n=1)	ND
100 Y	2.50 (n=1)	-	-	-
% couples				
100 A	0	1.0 ± 0.8	12.1 ± 0.1	12.3
50/50 A/Y	0	0	12.9 ± 4.4	36.8
25/75 A/Y	0	0.9 ± 1.6	14.5 ± 4.2	25.4
10/90 A/Y	0	0	0	16.3
100 Y	0	0	-	-
% gravid females				
100 A	0	0	4.1 ± 3.1	8.7
50/50 A/Y	0	0	4.1 ± 1.8	10.0
25/75 A/Y	0	0	10.7 ± 2.7	21.8
10/90 A/Y	0	0	5.4 (n=1)	8.2
100 Y	0	0	-	-

†: data obtained from one culture per treatment which was derived from pooling the remaining animals (± 200) from the triplicate cultures after 14 days of culture.

§: survival is expressed as a percentage of the number of animals transferred during the previous water renewal, i.e. 100% survival = 300, 150, and 100 animals after 7, 11, and 14 days of culture, respectively.
ND: not determined

VII.4. DISCUSSION

Most of the studies performed so far on the use of yeast as a food source for *Artemia* do not allow the evaluation of its nutritional value: *i.e.* the yeast often forms only a component of a mixed diet, or may be subjected to microbial decomposition and lysis which in turn may lead to uncontrolled development of other food sources, *e.g.* algae and bacteria, in the culture medium. Besides, culture tests under axenic conditions have shown that colonization of adventitious micro-organisms is essential to the nutrition of *Artemia* when feeding baker's yeast or other particulate diets (Douillet, 1987). Because the present study was not performed under axenic conditions, the effect of the developing microflora was not eliminated. Nevertheless, the high standardization of the experiment (disinfection of the cysts; use of 0.2- μ m filtered artificial seawater; frequent water renewal) prevented uncontrolled microbial colonization, such as algal and bacterial blooms.

Feeding the chemically treated yeast as a sole diet resulted in low survival rates. Possibly, the feeding regime adapted from Vanhaecke *et al.* (1984) for the culturing of *Artemia* with *Dunaliella*, was not optimal. Over-feeding appears to be at least partially responsible for the low survival rates, since survival could be improved by replacing one algal cell by only three yeast cells instead of four. However, feeding the yeast on an equivalent dry weight base, *i.e.* approximately two yeast cells for one algal cell, resulted in reduced growth. Supplementing the fresh yeast diet with only 5% of the algal ration, which is equivalent to less than 3% of the total dry weight of the 5/95 algae/yeast diet, increased the survival of *A. franciscana* by 30 to 50%. The presence of algae in the culture medium might play a role in maintaining a good water quality (Hanaoka, 1977), although renewing the culture medium did not result in a higher survival. More likely, the small quantities of algae may function as a supplement to the nutritionally deficient yeast. In the same way, several authors have supplemented yeast with *Spirulina* (James & Makkeya, 1981; Robin *et al.*, 1981), agricultural by-

products (Talloen, 1978; Lavens *et al.*, 1987) or pure nutrients (Shimaya *et al.*, 1967; Robin *et al.*, 1981) in order to improve growth and/or survival of the brine shrimp. A larger algal supplement, *i.e.* 25% of the full algal ration, was required to support good survival when feeding the dried yeast. This might be explained by the lower buoyancy and stability in the water column of the dried product, which in turn resulted in a lower availability of the yeast cells for *Artemia* and a faster deterioration of the water quality.

Although the fresh yeast supplemented with *Dunaliella* sustained good growth and survival during the first week, a lower growth rate as well as percentage of couples were obtained after 14 days compared to the algal control. Contrary to the first week, survival was not affected by the diet during the prolonged cultures. In this way, our findings confirm those of Provasoli *et al.* (1970) who concluded that deficiencies of nutrients in artificial media, rather than resulting in mortality, arrest growth in *Artemia* at a certain stage. However, in our tests this was only the case once the larvae passed through the critical period of days 3-6, during which the transition from the larval to the adult filtration apparatus takes place and the larvae strongly depend on dietary inputs (Provasoli & Shiraishi, 1959; Mason, 1963).

The dry yeast supplemented with 25% of the algal ration was more nutritious than the algal control diet. This may indicate that lipids and fat soluble vitamins, which are enriched in the processed yeast diet, may promote growth to the adult stage as well as sexual differentiation. Very little is known about the requirement for lipid factors in *Artemia*. The addition of lipids and fat soluble vitamins to the artificial medium of Provasoli & D'Agostino (1969) was indispensable for supporting good growth to adults and enhancing fertility of the parthenogenetic strain of Sète (Provasoli & Pintner, 1980). However, one generation growth studies with the bisexual strain from Utah could not demonstrate any requirement for fat soluble vitamins and fatty acids (D'Agostino, 1980). Also, Robin *et al.* (1981) could not detect any effect of supplementing cod liver oil on the growth

of *Artemia* from Brazil or San Francisco Bay origin when fed brewer's yeast. D'Agostino (1980) suggested that the requirements for lipid factors in bisexual *Artemia* may depend on maternal supplies to the eggs during ovogenesis. Since this transition of nutritional components varies according to the feeding conditions of the females (Vos *et al.*, 1984; Lavens *et al.*, 1989) the lipid requirements of first-generation nauplii might differ as a function of the origin and batch of *Artemia*.

The present study demonstrated that the requirement for unicellular algae to culture *Artemia* from freshly-hatched nauplii to the adult stage can be reduced for 75% by the use of the dry yeast product as a partial substitute. Although not the object of this investigation, preliminary experiments have shown that the yeast diet may also be used as a partial algal replacement for growing multiple generations of bisexual as well as parthenogenetic strains of *Artemia* (Abatzopoulos, pers. comm. 1991). Further research is needed to eliminate completely the requirement for algal cultures to maintain laboratory cultures of *Artemia* under standardized conditions. In this regard, the supplementation of the nutritional deficiencies in the dry yeast diet through the addition of dried algal extracts or cells, offers interesting possibilities in the development of a complete off-the-shelf diet for laboratory rearing of the brine shrimp.

Chapter VIII

STUDY OF FEEDING AND GROWTH IN *ARTEMIA* USING BAKER'S YEAST AS A FOOD SOURCE: GENERAL CONCLUSIONS

I. BAKER'S YEAST AS A FOOD SOURCE FOR THE BRINE SHRIMP *ARTEMIA*

1. DEMONSTRATION OF THE LIMITED DIGESTIBILITY OF BAKER'S YEAST

In the present study, it was evidenced by various experimental approaches that the nutritional value of baker's yeast *Saccharomyces cerevisiae* for the brine shrimp is primarily limited by the low digestibility of the yeast cell wall.

-Attempts to culture brine shrimp on a sole diet of baker's yeast failed due to extremely poor growth.

-Standardized culture tests demonstrated that growth and survival of *Artemia* fed baker's yeast was significantly improved when the yeast cell wall was completely removed by enzymatic treatment. Furthermore, a treatment with 2-mercaptoethanol, which is known to improve the susceptibility of the yeast cell wall to *in vitro* digestion by microbial and snail gut enzymes, was also effective in rendering baker's yeast digestible for *Artemia in vivo*.

-Faecal material produced by *Artemia* fed untreated baker's yeast consisted largely of cells which retained their cell contents after gut passage and often contained a viable cytoplasm. By contrast, brine shrimp fed enzymatically or chemically treated yeast excreted few intact cells and faecal pellets consisted mainly of fine-granular cell debris.

-A study of the carbon budget of *Artemia* fed ^{14}C -labelled baker's

yeast under food saturating conditions demonstrated an assimilation efficiency as low as 24-31% for the untreated yeast, whereas the chemically treated yeast was assimilated with an efficiency ranging between 72-76%.

2. IDENTIFICATION OF THE OUTER MANNOPROTEIN LAYER OF THE CELL WALL AS THE POSSIBLE BARRIER FOR THE DIGESTION OF BAKER'S YEAST

The yeast cell wall is generally believed to consist of mainly two layers, an outer mannoprotein layer and an inner glucan layer. The latter is the main skeletal constituent of the cell wall and is protected against extracellular enzymes by the low permeability of the outer layer, consisting of mannoproteins that are cross-linked by disulfide bridges. The present study provided various arguments for the hypothesis that the external mannoprotein layer forms a permeability barrier for the digestive enzymes of *Artemia* and thus prevents the penetration of the cell wall.

-An extensive literature study on the *in vitro* enzymatic digestion of the yeast cell wall indicated that efficient mycolytic enzyme extracts contain either a mannanase or a specific proteolytic enzyme that attacks the mannoprotein layer, and a glucanase that dissolves the glucan microfibrils. Furthermore, various authors have shown that the former component can be substituted by a sulfhydryl treatment, which is claimed to increase the permeability of the mannoprotein layer by reducing the disulfide bridges. The dependance of the yeast digestibility in *Artemia* on the thiol treatment suggested that *Artemia* only lacks the enzymes for attacking the mannoprotein component of the cell wall. This was confirmed by the knowledge on the carbohydrase spectrum in *Artemia*, which was shown to include glucanase, but excludes mannanase activity.

-The hypothesis that the improvement of the digestibility is due to a reduction of wall compounds was further substantiated by the

nature of the effective compounds. Chemical treatments which improved the nutritional value of yeast for *Artemia* comprised a sulfhydryl compound (2-mercaptoethanol, cysteine), whereas treatments with other sulfur containing reagents (methionine), alkali and acid were either not or far less effective.

-Light and electron microscopy revealed that thiol-treated yeast maintained its cell integrity and viability, which proved that the skeletal glucan component of the cell wall was still functional. Furthermore, the increased stainability of the cell wall after thiol treatment, which was also reported for phosphomannanase-treated *Saccharomyces*, supports the hypothesis that the sulfhydryl reagents act on the mannoprotein layer.

-Cell wall mutant strains of *S. cerevisiae*, which were found to be more susceptible to digestion by *Artemia* than their parental strains, exhibit an increased permeability due to a deviating structure of the mannan component (osmotic-dependent fragile mutants) or synthesize a cell wall with an increased glucan/mannan ratio (helicase-sensitive mutants).

3. IMPROVING THE DIGESTIBILITY OF BAKER'S YEAST

The traditional methods used to improve the digestibility of single-cell protein for animal and human consumption destroy the cell integrity, and are thus unsuitable for preparing yeast as food for filter-feeders. The present study identified several alternatives to improve the yeast digestibility without causing the release of the cytoplasmic contents of the yeast cells.

-Chemical treatment with sulfhydryl compounds (2-mercaptoethanol and cysteine)

The various conditions of the sulfhydryl treatments were optimized by means of standard culture tests evaluating growth and survival of *Artemia* fed different yeast preparations. The action of the thiol reagents was enhanced in treatment media with

a more alkaline pH, and a complementary effect of ethylenediaminetetraacetate was observed for the 2-mercaptoethanol treatment. A simple cysteine treatment and subsequent storage by deepfreezing was proposed to prepare baker's yeast for use as a food particle in feeding studies of *Artemia*.

-Heat treatment

Autoclaving yeast improved its nutritional value for *Artemia*, although the integrity of the yeast cell was strongly affected. The reduced water quality, due to lysis of the yeast cells shortly after the heat treatment, restricts the applicability of this treatment.

-Cell wall defective mutants of *S. cerevisiae*

Two types of cell wall defective mutants of baker's yeast (helicase-sensitive and osmotic-dependent fragile mutants) were found to be of a higher nutritional value for *Artemia* than their parental wild type strains. This demonstrated that digestibility of baker's yeast may be genetically improved, although the effect of yeast mutations was less important than that of the sulfhydryl treatment. Further research is needed to identify the mutations that are responsible for the increased digestibility and to construct mutant yeast strains which can be propagated under industrial conditions while maintaining their sensitivity to digestion.

4. USE OF TREATED BAKER'S YEAST FOR REARING ARTEMIA AT A LABORATORY SCALE

To date, the controlled rearing of *Artemia* for research purposes depends on the laboratory culture of unicellular algae of a suitable and consistent quality. The application of the cysteine-treated baker's yeast, preserved either in a fresh form by deepfreezing or as a dried, lipid-enriched product, seriously reduced the requirement for micro-algae to cultivate brine shrimp at a laboratory scale.

-A total substitution of the alga *Dunaliella tertiolecta* by the fresh yeast was achieved in small scale growth tests (40 *Artemia* per 90 ml) resulting after one week of culture in an average survival and body length of 70% and 4 mm, respectively. Feeding the yeast for longer periods resulted in reduced survival, which revealed that a sole diet of yeast is nutritionally deficient.

-A partial substitution of up to 95% of the algal diet by the fresh yeast diet resulted in similar growth and survival compared to the algae-fed controls after 14 days of culture in a system which is routinely applied for laboratory culture of *Artemia* (0.4 - 1 liter scale). However, the 5% algal supplement did not satisfy the nutritional requirements for a normal sexual differentiation. By contrast, a substitution of 75% of the *D. tertiolecta* diet by the dried, lipid-enriched yeast yielded similar survival and significantly faster growth and sexual differentiation compared to *Artemia* fed solely on algae.

II. STUDY OF FEEDING, ASSIMILATION, AND GROWTH IN *ARTEMIA* USING BAKER'S YEAST

Despite the extensive literature on feeding biology of zooplankton, the knowledge of feeding in *Artemia* under intensive culture conditions and using non-algal foods is very limited. The thiol-treated baker's yeast offered several advantages compared to unicellular algae as a food particle to study feeding kinetics of *Artemia*.

-The possibility of preserving one batch of yeast guarantees a consistent food quality between experiments.

-The high stability of the yeast cells under the experimental conditions excludes the requirement for control vessels without animals, which are used to correct feeding rates determined by the cell count method for spontaneous changes of the food concentration.

-The use of treated and untreated yeast, which are food particles

with a similar size and composition, but different digestibility, offers a unique test system to study the effect of food digestibility on the feeding kinetics of a filter-feeder.

Using the electronic cell count method and radiotracer techniques, the present study contributed to the knowledge of the following three aspects of the feeding biology of *Artemia*:

1. THE EFFECT OF FOOD CONCENTRATION ON FEEDING AND GROWTH

-Feeding rate, determined by means of the cell count method for various stages of *Artemia* feeding on treated yeast in rotating tubes, varied as a function of yeast concentration according to the rectilinear type of functional response curve. The incipient limiting concentration for feeding decreased during the first week of development from over 500 cells μl^{-1} to 80 cells μl^{-1} for brine shrimp of, respectively, 0.9 and 5.7 mm in size. The maximal clearance and ingestion rate increased with increasing *Artemia* size. Based on the present observations and data reported in literature, an allometric relationship of the form $\text{CR}_{\text{max}} = a\text{DW}^b$ ($a=0.047$, $b=0.918$) was established for *Artemia* between maximal clearance rate (CR_{max}) and dry body weight (DW). Weight-specific maximal feeding rates attained a maximum of 630% of body dry weight per day in 2.4-mm sized *Artemia* (5 days old) and decreased with increasing age to 170% day^{-1} for 14-days old animals.

-The incipient limiting concentration and maximal feeding capacity, determined by the cell count method, was confirmed by means of short-term radiotracer experiments for juvenile *Artemia* (3-4 mm) fed treated yeast. The ^{14}C -tests revealed a large variation of feeding rate between experiments performed with *Artemia* of similar size.

-The functional response curve of adult *Artemia* was influenced by the experimental environment. A higher incipient limiting concentration and lower CR_{max} were found for brine shrimp grazing

in a flow-through recirculating system compared to those feeding in closed rotating tubes. It was hypothesized that differences in physical conditions caused a higher filtration efficiency in the latter system.

-*Artemia*, grown at various continuous concentrations of the treated yeast in the recirculating system, maximized its growth rate during the first week after hatching at a concentration between 800 and 1200 cells μl^{-1} .

2. THE EFFECT OF CULTURE CONDITIONS ON FEEDING

The ingestion rate of adult *Artemia* at food saturating concentrations of treated yeast was measured by means of the cell count method at various conditions of animal density, water quality, mechanical disturbance, and light intensity in short-term grazing tests.

-Crowding did not affect feeding of *Artemia* up to densities of 6.7 animals ml^{-1} in the recirculating system, which allowed to exclude side-effects from changes in water quality. By contrast, feeding rate was depressed at animal densities of 3 adults ml^{-1} in closed, rotating tubes due to oxygen depletion and/or accumulation of excretory products.

-*Artemia* was found to be resistant to short-term exposures to high concentrations of inorganic nitrogen compounds. Feeding rate was significantly reduced by concentrations of ionized ammonia and nitrite as high as, respectively, 1,000 and 100 ppm, whereas nitrate did not influence feeding in the range of 0-1,000 ppm.

-In aerated culture systems, maximal feeding rates were observed at an intermediate aeration intensity, which prevented sedimentation of the food and maintained an optimal distribution of the animals over the culture volume without causing mechanical disturbance.

-Light intensity did not alter feeding rate in a measurable manner.

3. THE EFFECT OF FOOD DIGESTIBILITY ON FEEDING AND ASSIMILATION

-Strongly reduced ingestion rates were observed by means of the cell count method in *Artemia* fed untreated yeast. This was more pronounced in experiments using the laboratory-grown yeast (no detectable feeding rates for untreated yeast) than those where commercially available yeast was applied (40-60% lower feeding rates compared to treated yeast). Conversely, radiotracer experiments revealed 2 to 5 times lower ingestion rates in *Artemia* fed the treated yeast compared to animals fed untreated yeast. The discrepancy between the indirect (cell counting) and the direct (^{14}C) estimates of ingestion evidenced an important recycling of defecated yeast cells in brine shrimp fed untreated yeast. In the latter case, feeding rates were underestimated by means of the cell count method.

-Gut passage time in *Artemia* fed the various yeast types was inversely related to ingestion rate. Elevated feeding rates in *Artemia* fed untreated yeast were associated with a minimal gut passage time of about 30 min, which was in accordance with data reported in literature for animals of a similar size feeding on algae. The lower and more variable feeding rates in animals fed the treated yeast was coupled to a minimal gut passage time of 60-100 min.

-The effect of food concentration on the assimilation efficiency depended on the yeast type and the acclimation conditions prior to the experiment. After acclimation to the experimental food concentration, assimilation efficiency decreased with increasing concentrations of untreated yeast, whereas a high assimilation efficiency (> 72%), irrespective of food concentration, was observed in animals fed the treated yeast. When *Artemia* was acclimated to a high concentration of untreated yeast, the latter

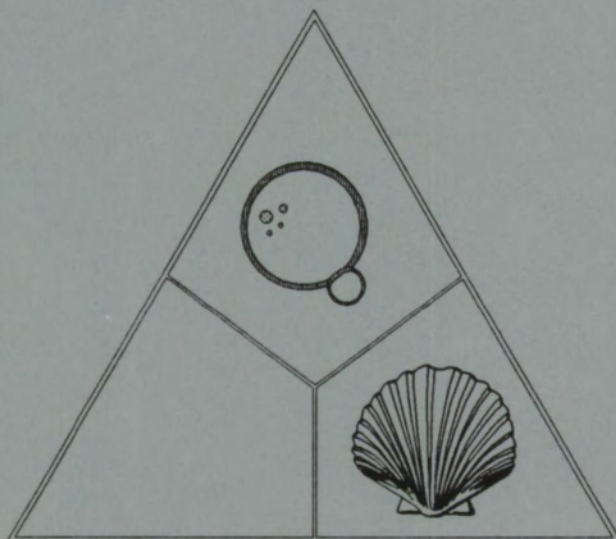
was assimilated at a low efficiency ($< 37\%$) independent of the food concentration. This was discussed in terms of the response of the digestive enzyme activity to short-term food acclimation.

-Assimilation rate in *Artemia* showed a saturation response towards increasing concentrations of treated yeast, whereas this parameter decreased with increasing levels of the untreated yeast.

-The above observations allowed to relate the concept of superfluous feeding in zooplankton to the digestibility of the food. Furthermore, the variation of assimilation rate and assimilation efficiency as a function of ingestion rate, irrespective of the yeast type or concentration, revealed a critical ingestion rate of about $10 \mu\text{g C ind}^{-1} \text{ h}^{-1}$. The latter was exceeded by *Artemia* feeding on untreated yeast, which resulted in decreased assimilation rate and efficiency. A compensatory mechanism adjusting ingestion rate as a function of assimilation was postulated to explain the enhanced feeding activity of *Artemia* fed yeast of a low digestibility.

EXPERIMENTAL PART 2:**SUBSTITUTION DIETS FOR LIVE MICRO-ALGAE IN THE
HATCHERY AND NURSERY REARING OF BIVALVE MOLLUSCS**

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Chapter IX

THE USE OF MANIPULATED YEAST DIETS AS AN ALGAL SUBSTITUTE IN THE CULTURE OF BIVALVE SEED

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Chapter IX

THE USE OF MANIPULATED YEAST DIETS AS AN ALGAL SUBSTITUTE IN THE CULTURE OF BIVALVE SEED

IX.1. INTRODUCTION

Rearing bivalve molluscs in commercial hatchery and nursery systems has so far relied on the production of live algae, which is a costly and often unpredictable venture (see III.4.1.). In addition, the capacity and cost of algal production restricts the controlled nursery culture of seed to a size of 1 to 2 mm. The suitable planting size for grow out (7-10 mm) is achieved by growing the seed under less-controlled conditions, which often yield lower survival and growth rates. Therefore, the development of a cost-effective artificial diet could greatly reduce the operating costs and improve the efficiency of bivalve seed production. The latter is supported by the strong efforts that have been put into the evaluation of various algal substitution diets for bivalve molluscs by commercial operators as well as scientists all over the world (see Chapter X).

The development of techniques to improve the digestibility (see Chapter IV) and the nutritional composition (Léger *et al.*, 1985) of yeast-based diets resulted in a product with great potential as a substitute for unicellular algae. A similar product has proven to be a valuable algal substitute in the larval culture of marine shrimp (Naessens-Foucquaert *et al.*, 1990). The present study focussed on the evaluation of manipulated yeasts as an algal substitute for small bivalve seed, which demand the largest volumes of algal culture in commercial hatchery operations (see III.4.1.).

A preliminary test, performed at the hatchery Tinamenor S.A. (Spain) with juvenile Manila clams, *Tapes philippinarum*, aimed at the evaluation of manipulated yeasts as a partial or complete

substitute for the routinely applied food, which consisted of a mixture of five algal species (IX.3.).

The promising results of this experiment were verified under more standardized conditions in two different sets of laboratory tests. One series of experiments was performed at the Laboratory of Aquaculture & Artemia Reference Center with juvenile *T. philippinarum* supplied by commercial hatcheries in Spain and UK (IX.4.). Another sequence of tests was carried out at the South Carolina Wildlife and Marine Resources Department (SC, USA) with seed of the hard clam *Mercenaria mercenaria* (IX.5.). Since literature data are lacking with regard to the quantitative food requirements of small bivalve seed, several experiments were run to obtain a better understanding of the effect of algal ration on growth of spat. Furthermore, an attempt was made to improve the nutritional value of the yeast diets by the addition of various compounds.

Finally, a second hatchery trial was run at Guernsey Sea Farms Ltd. (Guernsey, UK) with seed of *T. philippinarum* and the pacific oyster *Crassostrea gigas* (IX.6.).

IX.2. MATERIALS AND METHODS

An overview of the culture conditions in the various experiments is presented in Table 40.

IX.2.1. Origin and acclimatization of the animals

The juvenile bivalves used in these studies were obtained from several sources. The seed for the laboratory experiments with the Manila clam *Tapes philippinarum* and the hard clam *Mercenaria mercenaria* was obtained from commercial hatcheries (*T. philippinarum*: Guernsey Sea Farms Ltd. (GSF), UK; Seasalter Shellfish Whitstable Ltd., UK; Tinamenor S.A., Spain; *M. mercenaria*: Aquaculture Research Corporation, MA, USA). The hatchery trials were performed with juveniles produced in the facilities of the counterpart (Tinamenor S.A.: *T. philippinarum*; Guernsey Sea Farms Ltd.: *T. philippinarum*, *Crassostrea gigas*).

The initial live weight of the seed (mostly 1-5 mg) is detailed for the various experiments in Table 40.

Table 40: Culture conditions during the recirculation tests with bivalve juveniles at the various locations.

LOCATION -	Tinamenor S.A. (Spain)	Lab. of Aquaculture & Artemia Reference Center (Belgium)	SCWMRD (SC, USA)	Guernsey Sea Farms (UK)
CODE -	TM	ARC	SC	GSF
bivalve species (initial live weight)	<i>Tapes philippinarum</i> (1.3 mg ind ⁻¹)	<i>Tapes philippinarum</i> (1.3 - 5.7 mg ind ⁻¹)	<i>Mercenaria mercenaria</i> (0.4 - 1.7 mg ind ⁻¹)	<i>Tapes philippinarum</i> (2.8 mg ind ⁻¹) <i>Crassostrea gigas</i> (± 2 mg ind ⁻¹)
scale culture system (l)	50	5	18	28
seawater treatment	sand-filtered	1) sand-filtered + storage in settling tanks 2) 0.22 µm capsule, or 1µm bag + UV	1 µm bag + UV	sand-filtered
temperature (°C)	21 ± 1	21 ± 1	room temperature (25 - 30)	room temperature (± 18)
salinity (ppt)	30 - 33	30 - 33	25 - 30	34 - 35
initial stocking density (mg WW l ⁻¹)	80	100	100	80
duration of the experiment (weeks)	4	3	2-3	3
frequency water renewal	2 week ⁻¹	3 week ⁻¹	1 day ⁻¹	3 week ⁻¹
algal reference diet:				
- composition	<i>Isochrysis galbana</i> , <i>Skeletonema</i> <i>costatum</i> , <i>Thalassiosira</i> <i>pseudonana</i> , <i>Chaetoceros</i> <i>gracilis</i> , <i>Tetraselmis suecica</i> (25:25:22.5:22.5:5 on DW)	<i>Chaetoceros gracilis</i> (concentrated + stored at 4 °C, or directly from culture) or <i>Chaetoceros gracilis</i> + <i>Isochrysis galbana</i> (T-Iso) (50:50 on DW)	<i>Chaetoceros gracilis</i> + <i>Isochrysis galbana</i> (T-Iso) (50:50 on DW)	<i>Tetraselmis suecica</i> + <i>Skeletonema</i> <i>costatum</i> (50:50 on ODW)
- daily ration (% DW WW ⁻¹ day ⁻¹)	4	1	2	2 (ODW)

The spat were mostly transported in a refrigerated styrofoam box from the hatchery to the lab. Upon arrival they were gradually acclimated to the experimental temperature (temperature increase rate < 0.25 °C h⁻¹) and fed the algal control diet *ad libitum* for three to seven days prior to the start of the experiment.

IX.2.2. Culture systems and conditions

Because the experiments were performed at various locations during relatively short stays, the culture system often consisted of a modified version of the *in situ* available system. Except for the preliminary flow-through test in GSF, the juvenile bivalves were batch-fed in recirculating systems consisting basically of a seed tray mounted in a reservoir of varying size and equipped with an air-water lift to maintain water circulation (see Fig. 47).

The methodology used for the preliminary trials in Tinamenor was based on a recirculating system designed by Walne & Spencer (1974). The spat were held in trays with a base of 500 μm mesh. Four trays, each containing initially one gram of seed, were hung at the surface of each 50 l tank (Fig. 47A). An air-water lift (AWL) sucked water from the bottom and delivered it through a perforated PVC distribution pipe to the trays at a rate of about 300 ml min⁻¹ tray⁻¹. Excess water was diverted through overflows at the ends of the distribution pipe. The water in the tank was aerated through two aeration points to keep the food in suspension.

The experiments with *T. philippinarum*, conducted at the Laboratory of Aquaculture, were accomplished in a small scale recirculating system consisting of a 350 μm mesh tray which was partially submerged in a five liter aquarium (Fig. 47B). An AWL maintained a flow of about 300 ml min⁻¹ through the tray, which was stocked at the start of the experiment with 0.5 g of spat. During a first set of experiments the air-water lifts sprayed the water on to the surface of the cultures. More consistent flow rates could be obtained when the outflow of the AWL penetrated the trays at the water level (Fig. 47B). An additional aeration point minimized settling of the food.

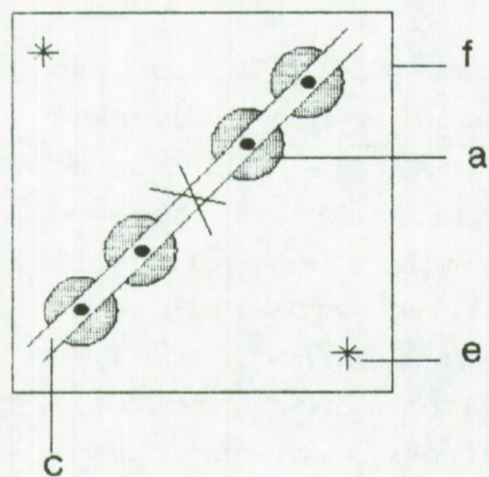
Juveniles of the hard clam *M. mercenaria* were grown at the SCWMR Department in a 18 l recirculating system (Fig. 47C). Each culture unit consisted of a 20 l bucket in which a seed cylinder was mounted. The system was filled with 18 l of seawater and was stocked initially with 1.7 g of seed. An AWL, penetrating the

side wall of the seed cylinder, maintained a water flow through the seed. Water circulation in the bucket was created by an additional AWL.

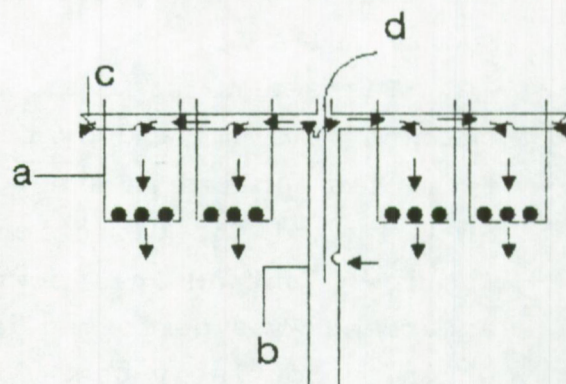
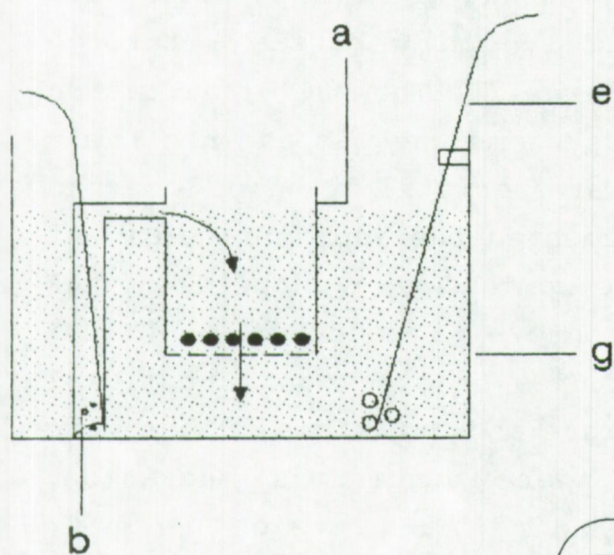
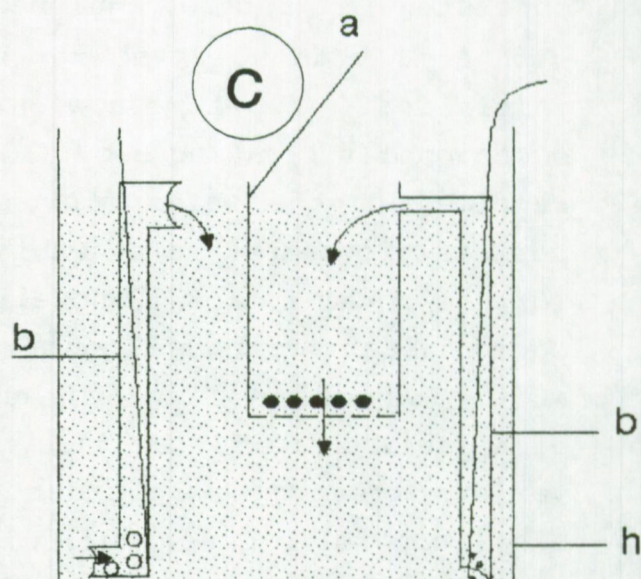
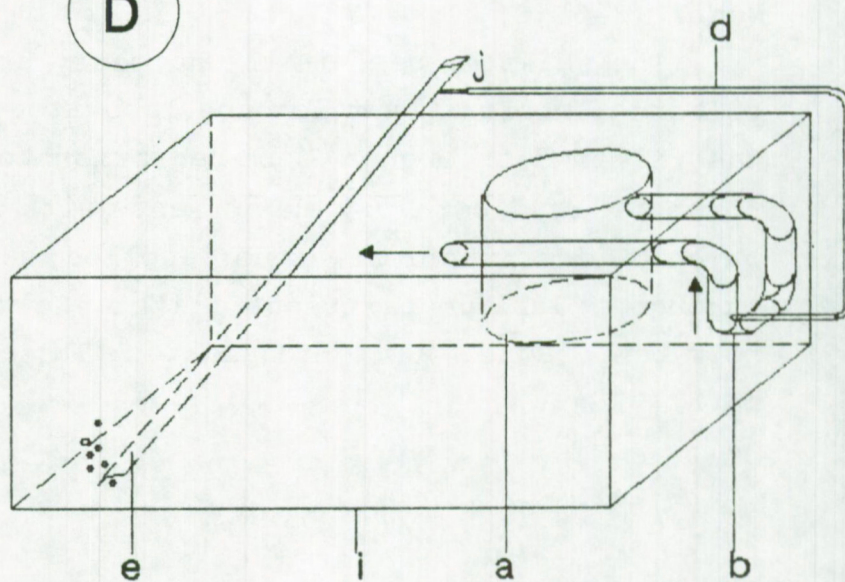
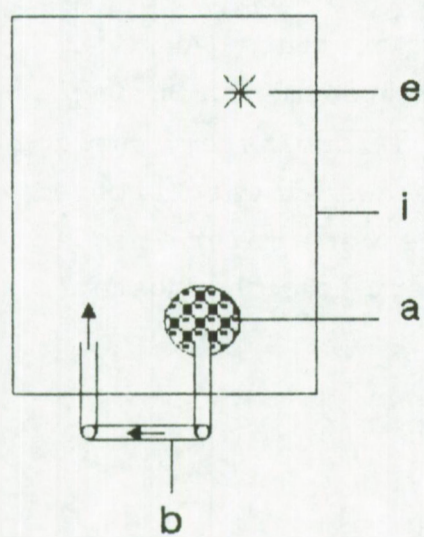
The set-up used in Guernsey Sea Farms (Fig. 47D) for the culture of clams and oysters in a recirculation system differed from that applied in Ghent with regard to the scale (28 l instead of 4 l) and the direction of the water flow through the seed (upflow instead of downflow). Furthermore, a preliminary experiment was performed in a forced upwelling system (Fig. 47E) which is routinely used for rearing seed in the size range from 800 μm to 2 mm (Dravers, pers. comm.). Each flow-through unit consisted of a bottle, equipped for the purpose of the experiment with a tray to hold the relatively small amount of seed (*i.e.* initially 70 g). A mesh at the outflow prevented the loss of occasionally floating seed. Algal food and artificial diets were supplied to the water flow (about 1 l min^{-1} bottle $^{-1}$) through peristaltic pumps, as shown in Figure 47E. The 20% algal ration for the total set of 10 upwelling systems was pumped into their common water supply through a separate peristaltic pump. A second multi-channel metering pump supplemented the appropriate amount of algae and artificial food to the common water supplies of the various treatments in order to achieve, respectively, a 100% algal control diet and 20/80% mixtures of algae and artificial diets. Stock suspensions of the algae and artificial diets were prepared daily and stored under continuous aeration, respectively, at room temperature ($\pm 18^\circ\text{C}$) and outside ($3\text{--}11^\circ\text{C}$, mostly $3\text{--}6^\circ\text{C}$).

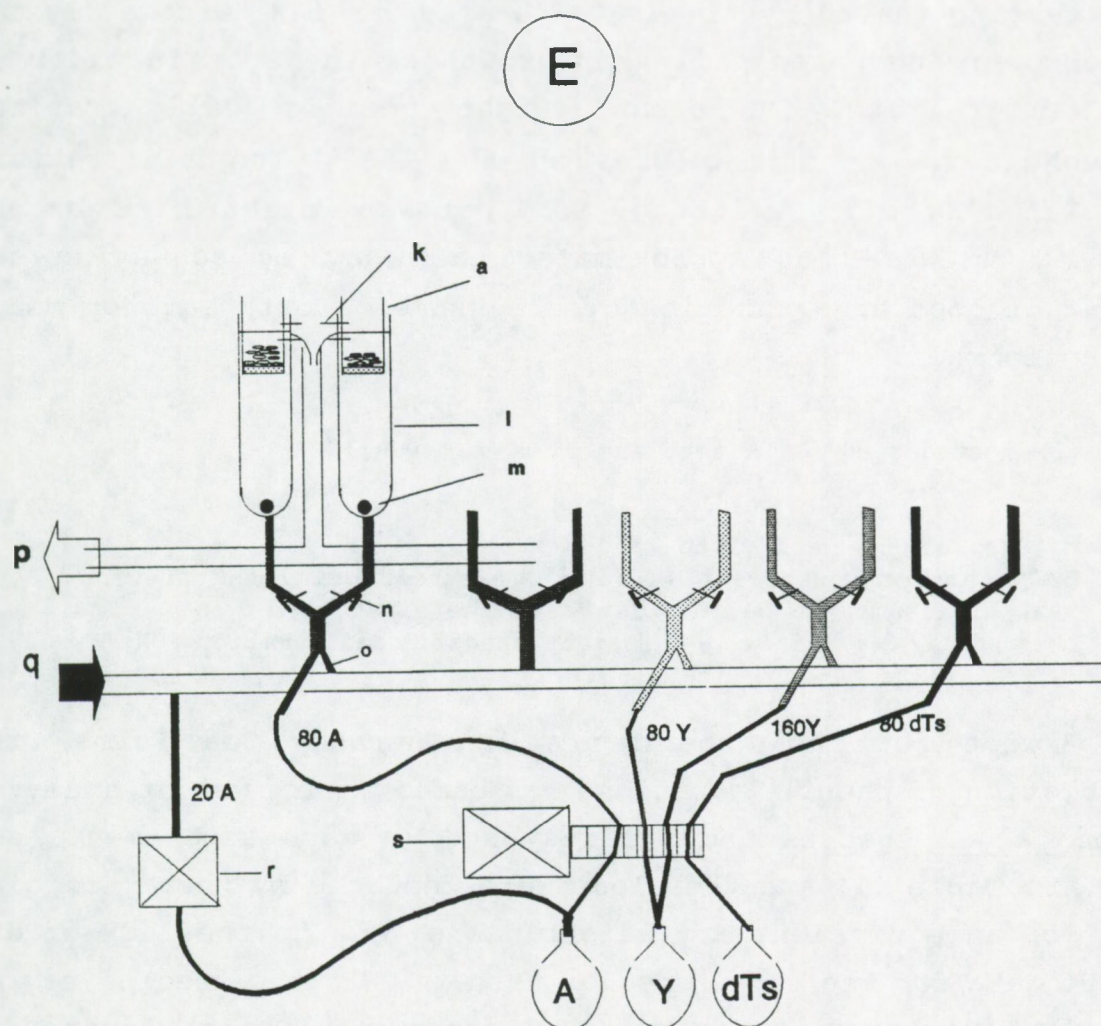
The seawater used in the hatchery trials (TM & GSF) was sand filtered, whereas that for the laboratory experiments (ARC & SC) was filtered through a 1 μm bag prior to UV treatment. During a first set of laboratory experiments with *T. philippinarum*, during which UV disinfection was not applied, the seawater was filtered through a 0.22 μm cartridge filter. Cultures were maintained at room temperature (GSF, SC), heated (TM) or kept in a thermostatic bath (ARC).

Top view

**A**

Side view

**B****C****D**



~! Fig. 47: Schematic representation of the experimental culture systems used in the different locations for rearing bivalve seed.

A: 50 l recirculating system (Tinamenor S.A., Spain)

B: 5 l recirculating system (Laboratory of Aquaculture, Belgium)

C: 18 l recirculating system (SCWMR Department, SC, USA)

D: 28 l recirculating system (Guernsey Sea Farms Ltd., UK)

E: forced upwelling system (Guernsey Sea Farms Ltd., UK)

a: seed cylinder with mesh bottom, b: air-lift pump, c: perforated water distributing tube, d: air supply, e: aeration point, f: 50 l tank, g: 5 l aquarium, h: 18 l bucket, i: 28 l tank, j: needle supplying air to airlift pump, k: mesh preventing loss of floating seed, l: upwelling bottle, m: marble, n: tap to regulate the flow rate per bottle, o: water supply (common per treatment), p: drain to heat exchanger, q: heated water supply, r: peristaltic pump for feeding 20% algal ration, s: multi-channel metering pump for feeding the various supplements (80-160%) from the stock suspensions (A= algae, Y= yeast, dTs= dried *Tetraselmis*).

IX.2.3. Feeding regime

In the recirculation experiments, the seed was fed a weight-specific daily ration of 1 to 4%, based on experiments in which the effect of ration size on growth was determined (laboratory trials) or on the routinely applied feeding regime in commercial seed rearing (hatchery trials). The seed was fed twice a day and rations were adjusted daily for growth of the spat in order to feed approximately constant weight-specific daily rations throughout the experiment (Urban *et al.*, 1983). The daily ration, calculated as % dry weight of food per wet weight of clams ($DW\ WW^{-1}\ day^{-1}$), was thus approximated each day by adjusting the amount of food as a function of an assumed growth rate by means of the formula:

$$[DW\ food\ day\ n] = [DW\ food\ day\ 1] \times (1 + DGR/100)^{n-1}$$

with n = day of the week (1 to 7)

DGR = daily growth rate ($\% day^{-1}$) measured during the previous week or assumed to be 10 $\% day^{-1}$ for the first week

DW food day 1 = initial WW x weight-specific daily ration ($DW\ WW^{-1}\ day^{-1}$)

Except for the experiments in Guernsey Sea Farms, the calculation of the daily rations was based on dry weight analysis of the algal species and strains used in each experiment (see IX.2.4., Table 41) and the algae were replaced by the artificial diets on an equivalent dry weight basis. Due to the lack of dry weight data for the algal species used in GSF, the feeding regime and algal replacement was based on organic dry weight data from Helm (1990b).

The feeding strategy used in the flow-through experiment at GSF was derived from the regime that is normally applied in the hatchery and aimed at maintaining a constant food concentration in the inflowing current. The algal control diet received an algal mixture which was equivalent to 50 *I. galbana* (clone T-Iso) cells μl^{-1} (see IX.6., Table 58). Assuming a constant flow rate of 1 $l\ min^{-1}$, the latter food concentration resulted in an initial weight-specific ration of 1.5% algal ODW $WW^{-1}\ day^{-1}$.

IX.2.4. Algal diets

Each experiment contained a control treatment which was fed the full standard algal ration (100% SAR), consisting of one alga or a mixture of various algal species (see IX.2.1., Table 40).

Dry weights of algal cells were determined by filtering algae from aliquots of suspension of known concentration. Algae were retained on tared, glass-fiber filters (1 μ m pore size) which were subsequently washed with a solution of ammonium formate (0.5 M) to remove sea salts. Filters were then dried at 100 °C for 4 h to volatilize the ammonium formate, and weighed on an analytical balance (modified from Epifanio & Ewart, 1977). The same procedure was followed with control filters on which an equal amount of seawater was filtered. In some cases, depending on the strength of the applied vacuum, salts were retained on the control filters. In order to improve the correction for salt residues and the variation among samples, cellular dry weight was determined from regression analysis of DW retained on the filter *versus* number of algal cells filtered (Fig. 48). A comparison of the present data with values reported in the literature is hampered by the great variability between the latter (Table 41).

For the experiments in Tinamenor S.A. and Guernsey Sea Farms Ltd. algae were provided by the algal production unit of the hatchery. In both cases, the latter consisted of a battery of 400 l plastic bags supported by wire cages, which were maintained as continuous cultures for 3-6 weeks (described for Seasalter Shellfish Whitstable by Holliday, 1986). Algae in the SCWMRD experiments were derived from semi-continuous cultures in 150 l fibreglass containers. Sterilization was done by chlorination/dechlorination (5 ppm sodium hypochlorite, 30 min) and a commercial fertilizer (Fritz f/2 medium) was used as a nutrient supplement. At the laboratory in Ghent algal culture was based on the method described by Liao *et al.* (1983). Algae were grown in 4 or 25 l carboys using Walne medium and 0.45- μ m filtered seawater. Only algal cells in the logarithmic phase of growth were used in the feeding experiments.

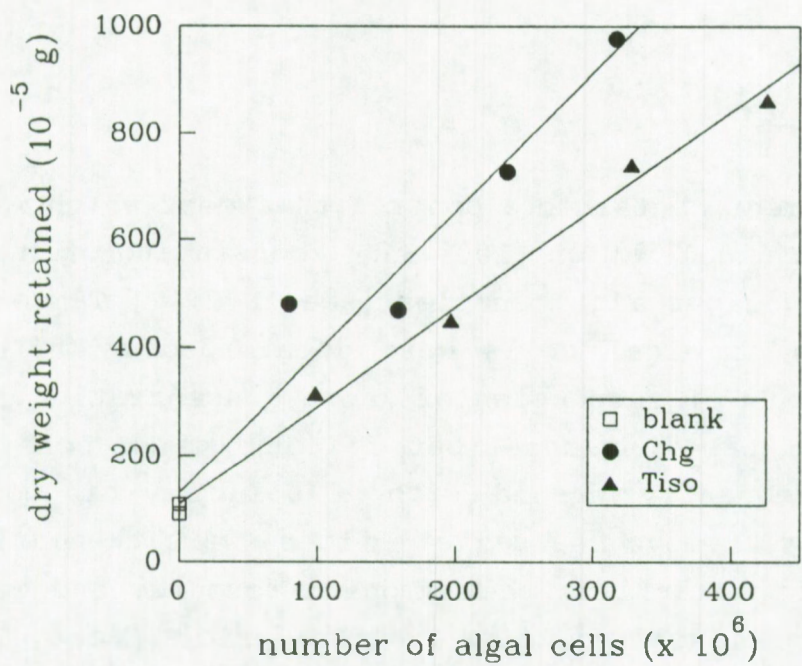


Fig. 48: Dry weight analysis of algae by means of linear regression of dry weight retained on the filter *versus* number of algal cells filtered. Each data set represents the dry weight determination for algae obtained from one culture of, respectively, *Isochrysis galbana*, clone T-iso (Tiso) and *Chaetoceros gracilis* (Chg). Linear regression equations are:

Tiso: $y = 1.83 x + 107.9$ $r^2=0.99$
Chg: $y = 2.61 x + 130.1$ $r^2=0.95$
with y = cell numbers [10^6], x = dry weight [10^{-5} g]

Table 41: Dry weight (pg cell⁻¹) of the algal species used in the experiments. Mean and standard deviation from n replicate cultures.

	Tinamenor S.A. (Spain) [§]	Lab. of Aquaculture (Belgium)	SCWMD (SC, USA)	Guernsey Sea Farms (UK) [†]	values reported in literature [‡]
<i>Isochrysis galbana</i>	12.2				8.0 ³ , 16.1 ⁵ , 20.1 ⁶ , 23.5 ⁴ , 30.5 ⁷
<i>Isochrysis galbana</i> (clone, T-ISO)		14.1 ± 0.8 (n=5)	17.3 ± 0.9 (n=4)		29.7 ⁷
<i>Skeletonema costatum</i>	22.0			29 (ODW)	52.2 ⁷
<i>Thalassiosira pseudonana</i>	12.6				13.2 ⁴ , 17.8 ⁵ , 28.4 ⁷
<i>Chaetoceros gracilis</i>	24.0	23.8 ± 3.8 (n=6)	30.6 ± 1.7 (n=4)		74.8 ⁷
<i>Tetraselmis suecica</i>	165.1			200 (ODW)	66 ² , 168 ⁷ , 194-244 ¹ , 247 ⁸ , 292 ⁵

§: Gutierrez (pers. comm., 1989)
†: organic dry weight data from Helm (1990b)
‡: 1: Walne & Spencer (1974), 2: Thompson & Bayne (1974), 3: Winter & Langton (1976), 4: Epifanio & Ewart (1977), 5: Romberger & Epifanio (1981), 6: Ali (in Urban *et al.*, 1983), 7: Brown (1991), 8: Laing & Verdugo (1991).

In the first series of experiments with *T. philippinarum*, conducted at the Laboratory of Aquaculture, algae were separated from the culture medium by centrifugation and stored prior to use (Winter & Langton, 1976). The algal pellet was resuspended and diluted in filtered seawater to obtain a standard density of $20 \cdot 10^6$ cells ml^{-1} using a haemocytometer or Coulter counter, model Zf. The stock suspension was stored in total darkness at 4 °C for maximal three days. In all other experiments, algal suspensions were drained from the cultures and counted immediately prior to each feeding.

IX.2.5. Artificial diets

The basis of the experimental yeast diets used in this study consisted of baker's yeast *Saccharomyces cerevisiae* which was chemically treated in order to improve its digestibility (see IV.4.). The basic formulation of the diet as well as the technology to produce dry yeast-based diets with high lipid and HUFA content was provided by Artemia Systems N.V.-S.A. (Belgium). Modifications of the basic formulation, including the addition of rice starch, kaolinite, fat-soluble vitamins, and an extract from macro-algae, resulted in experimental diets of various composition (Table 42).

Table 42: Proximate composition of the experimental yeast diets. Figures are percent of diet dry weight (moisture < 3%).

	Y1	Y2	Y3	Y4 [§]	Y5	Y6	Y7
protein	40	40	40	30	30	30	38
carbohydrate	35	35	35	50	27	27	33
lipid	18	18	18	14	14	14	17
Σ (n-3) HUFA (% of total lipid)	28	28	28	28	28	28	28
supplement fat-soluble vitamins [†]	-	+	-	-	-	-	-
kaolin	-	-	-	-	23	23	-
concentrated extract from macro-algae	-	-	1	-	-	1	5

†: supplement fat-soluble vitamins (per g of diet): vitamin A: 27 IU, vitamin D: 16 IU, vitamin E: 9 IU, vitamin K: 22 μg

§: higher carbohydrate content was achieved by the addition of rice starch (23% of total DW)

The yeast diets were formulated and added to the clam cultures in such a way that the silt and starch components were fed as supplements to the basic diet Y1. For example, feeding Y5 (containing 23% kaolinite and 77% Y1) occurred at a 30% higher rate than Y1, and was thus equivalent to feeding 100% Y1 (77% x 1.3) supplemented with 30% (23% x 1.3) of kaolinite.

In some of the experiments, the nutritional value of the yeast-based diets was compared with other commercial (dried *Tetraselmis suecica*: Algal 161, Celsys, Cambridge, UK) or experimental (dried *Cyclotella cryptica*, Sea Ag Inc., Florida, USA) dried algal preparations.

The artificial diets were prepared by mixing in a kitchen blender until the cell conglomerates were separated into individual cells, i.e. after 3-5 min and 0.5-1 min for dried yeast and algae, respectively. New suspensions were prepared daily and stored till use in the refrigerator.

IX.2.6. Determination of clearance and intake rate for *T. philippinarum* (Experiments ARC 1 & 5)

IX.2.6.1. Average intake rate during a growth test

During the *Tapes* experiments evaluating the effect of *C. gracilis* ration on growth, food concentration was measured either at short time intervals of 2 to 6 h (experiment ARC 1) or before and after each feeding (experiment ARC 5). The observed decreases in cell concentration could be related to the total live weight present in the culture system by calculating an average weight-specific intake rate (ir) over the elapsed time interval (t) by means of the formula:

$$ir = \frac{V (C_0 - C_t)}{WW_n t} \quad [\text{cells g}^{-1} \text{ h}^{-1}]$$

where V= volume of the suspension, C_0 and C_t = initial and final (i.e. after time t (h)) concentration, WW_n = total clam live weight (g) present at the moment of the measurement. The clam weight on day n (WW_n) is estimated from the daily growth rate

(DGR) and the initial live weight (WW, day 1) from:

$$WW_n = WW (1 + DGR/100)^{n-1}$$

The intake rates were not corrected for algal growth during the experiment, since the latter was found to be fluctuating in time, but negligible compared to the amount of cells removed by the clams.

IX.2.6.2. Clearance and intake rate as a function of food concentration

During a short term grazing experiment, filtration and clearance rate were recorded for *T. philippinarum* (live weight 20 mg) at various concentrations of *C. gracilis*. The animals were derived from a population which was fed *C. gracilis* and transferred to 5 l culture systems (see IX.2.2., Fig. 47B) which were placed in a thermostatic water bath at 21 ± 1 °C. In order to achieve 10 to 20% decreases of cell concentration over a 2 h period, stocking density was varied between 0.3 and 0.7 g live weight per 5 l system, depending on the food concentration tested. The seed was acclimated to the food concentration for 1 h prior to the experiment (Sprung & Rose, 1988). Algal concentration was monitored with a Coulter counter (model Zf) during two consecutive periods of 2 h. A control experiment with no animals present was run in duplicate for all concentrations tested.

Weight-specific clearance rate (CR) was calculated using the equation (Coughlan, 1969):

$$CR = \frac{V}{WW \ t} \left[\ln \frac{C_0}{C_t} - \ln \frac{C'_0}{C'_t} \right] \quad [\text{ml g}^{-1} \text{ h}^{-1}]$$

where WW= total clam live weight (g), V= volume of the suspension (ml), C_0 , C'_0 = initial, and C_t , C'_t = final concentration of, respectively, the experimental and the control aquarium, and t= elapsed time (h).

The rate at which cells are removed from suspension, to be referred to as intake rate (ir) since pseudofaeces production was not quantified (III.2.3.), was computed as:

$$ir = C_m \times CR \quad [\text{cells g}^{-1} \text{ h}^{-1}]$$

where $C_m = (C_t + C_0)/2$ the mean concentration (cells ml⁻¹) encountered by the animals during the measurement of the clearance rate. Clearance and intake rates were graphically presented as a function of C_m .

IX.2.7. Parameters followed

Animals were initially selected from a single population of juveniles and divided *ad random* in groups of equal weight, which were distributed among the culture systems. Initial parameters (shell length and/or individual live weight) were measured on three subsamples.

At 7-day intervals, the seed was removed from the respective trays and the total live weight determined. Enough clams were removed to return the weight to the initial value and individual live and dry weight were determined on the culled animals.

The total live weight per tray was determined by collecting the clams on a mesh, which was blotted dry on paper towel. To avoid differences in water content between samples due to air-drying, the total biomass was then immediately weighed and reduced to the initial weight prior to returning to the culture systems. The culled animals from each cylinder were weighed and counted for the determination of the individual live weight. The seed samples were subsequently transferred to Teflon pots, previously dried in an oven at 60 °C for 4h and weighed. The pots were returned to the oven for 24h at 60 °C and then weighed to give the dry weight. Live weight of *T. philippinarum* showed a high correlation with dry weight (DW):

$$DW = 0.571 WW + 0.431 \quad (r^2 > 0.99)$$

A constant relationship between live and dry weight was also reported by Urban & Langdon (1984) for *Crassostrea virginica* and demonstrated that the drying procedure used for determining the live weight resulted in a constant water content.

Daily growth rate was calculated from the weekly increase of total wet weight per cylinder (DGR) or from the increase of individual live weight over the total culture period (DGRE) using the equation of Gutierrez (1990):

$$DGR = \left(\sqrt[n]{\frac{WW_n}{WW_0}} - 1 \right) \times 100 \quad [\% \text{ day}^{-1}]$$

DGRE represented an average growth rate for the total experimental period, whereas DGR was used to reveal variation of growth in the course of the experiment.

In addition to wet weight and, from that derived, daily growth rate, which were used as parameters in all experiments, shell length was measured in the *Mercenaria* and in some of the *Tapes* experiments. Shell length was measured on 30 clams per tray using a dissecting microscope equipped with a calibrated ocular. Live weight (WW) increased with increasing shell length (L) according to the general equation $WW = aL^b$.

For *M. mercenaria* (Fig. 49A):

$$WW = 0.372 L^{2.575} \quad (r^2 = 0.98)$$

For *T. philippinarum* (Fig. 49B):

$$WW = 0.463 L^{2.477} \quad (r^2 = 0.98)$$

A similar relationship between live weight and shell length was reported by Pellizzato (1990) for *T. philippinarum* in the size range of 3-35 g.

During the experiments testing the effect of algal ration on growth of *T. philippinarum* (ARC 1 & 5) and *M. mercenaria* (SC 1), cell concentration was monitored in one replicate of the various treatments using a Coulter counter (model Zb) and a Turner fluorometer, respectively.

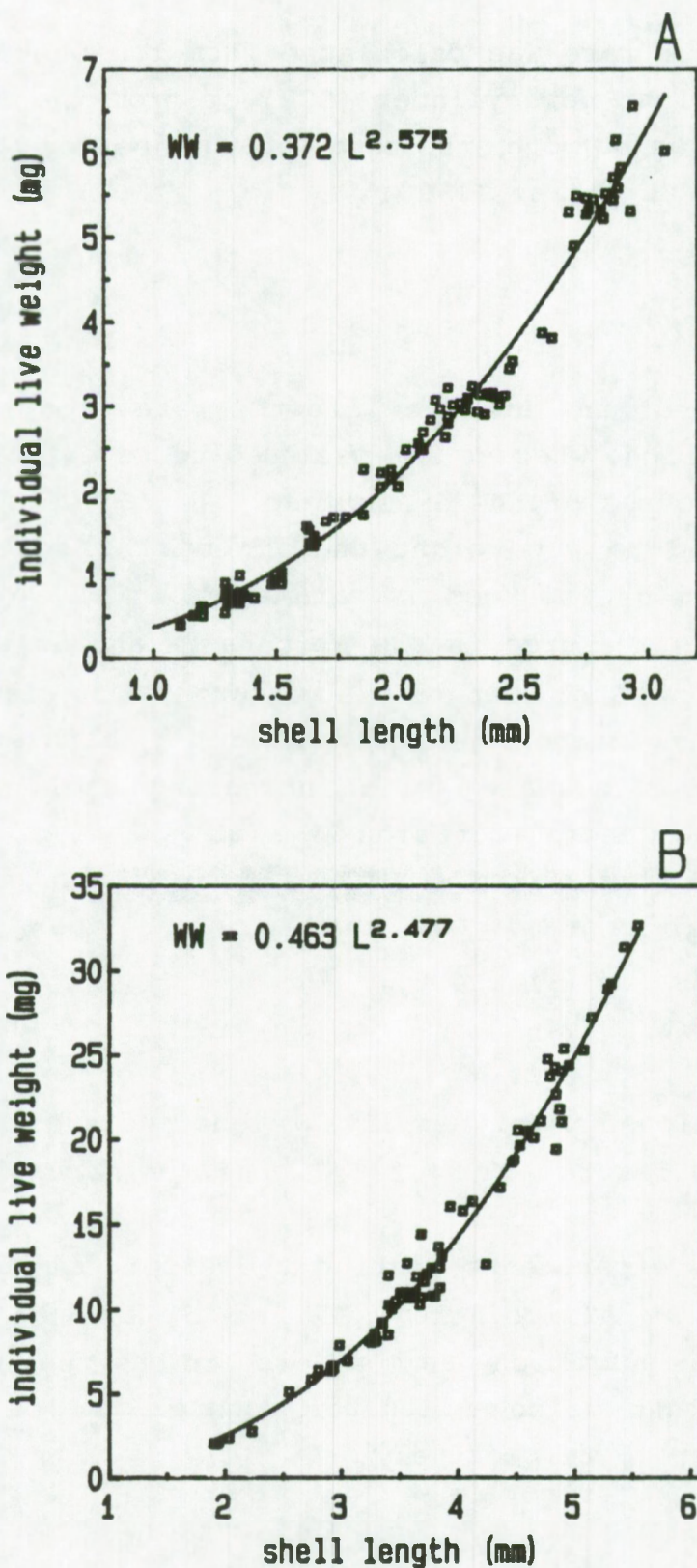


Fig. 49: Relationship between live weight (WW) and shell length (L) for *M. mercenaria* (A) and *T. philippinarum* (B). Data represent group means of 30 and 50-300 clams for shell length and live weight, respectively. Equations were fitted by non-linear regression.

IX.2.8. Data treatment and statistical analysis

Daily growth rates were used to compare the effect of the diets on juvenile growth because this allowed comparisons between experiments that differed in individual live weights of the seed at the start. Furthermore, the expression of the daily growth rates as a percentage of the growth rate achieved for the algal control treatment in each experiment made a comparison possible between trials with various bivalve species and under different culture conditions, such as quality and quantity of the algal control diet (Laing & Millican, 1986).

Statistical analysis of the growth data included analysis of variance and Tukey HSD multiple range tests (Sokal & Rohlf, 1981). The homogeneity of the variances of means for each experiment was checked by Cochran's C-test and Hartley's test. Because of the limited number of replicates, normality was tested on the deviations $Y_{ij} - Y_i$, which were computed separately for each treatment and pooled per experiment, by means of the Kolmogorov-Smirnov test (Sokal & Rohlf, 1981). Departures from the assumptions of analysis of variance could be rectified in most cases by logarithmic transformation of the data. Inherently heteroscedastic data (Cochran's C-test or Hartley's test, $P < 0.05$, even after transformation) were indicated in the tables of the results with "H.D." and were analyzed using an approximate test of equality of means assuming heterogeneity of variances (MCHETV) or, when only two means were to be tested, an approximate t-test (Sokal & Rohlf, 1981).

IX.3. PRELIMINARY TEST WITH *TAPES PHILIPPINARUM* AT TINAMENOR S.A, SPAIN ¹

IX.3.1. Rationale and experimental design

This first experiment was a preliminary evaluation of the use of yeast diets as a substitute for live algae in the culture of juveniles of the Manila clam *Tapes philippinarum*. It was performed in a commercial hatchery which provided the same quality of seawater and algal food as that routinely used for the indoor nursery rearing of clams and oysters. The nutritional value of diets composed of varying proportions of the yeast product Y1 (see IX.2.5., Table 42) and the algal control diet, which consisted of a mixture of five algal species, was compared with that of four single algal diets. In addition, the effect of incorporating an extract of macro-algae in the yeast diet was evaluated (yeast diet Y3; Table 42).

For a detailed description of the methodology of the growth experiment we refer to IX.2.

IX.3.2. Results

Daily growth rate was calculated for the various treatments based on the final individual live weight after four weeks of culture (Table 43). *S. costatum* and *T. suecica* supported significantly lower growth than the mixture of five species, whereas this was not the case for *I. galbana* and *C. gracilis*. A diet consisting solely of *T. suecica* resulted in a growth rate which did not differ significantly from that measured for the 20% mixed algal diet.

The mixed algal ration could be substituted up to 80% by either yeast diet Y1 or Y3 without a significant decrease in growth rate. However, clams fed solely on yeast product Y1 exhibited a strongly reduced growth.

¹parts of this chapter have been published in Albentosa et al. (1989)

Table 43 (Experiment TM): Final live weight and derived daily growth rate of juvenile *T. philippinarum* after 28 days of culture. Data represent mean and standard deviation from four replicates. Unlike superscripts denote significant differences (ANOVA, Tukey HSD test, $P \leq 0.05$).

TREATMENT [§]	final live weight (mg)	daily growth rate (% day ⁻¹)	Z*
unfed control [†]	1.56 ± 0.07	1.24 ± 0.37	11
100% SAR	27.32 ± 4.34 ^a	11.45 ± 0.65 ^a	100
20% SAR	4.97 ± 0.88 ^{cd}	4.86 ± 0.67 ^c	42
50% SAR + 50% Y1	21.86 ± 3.02 ^{ab}	10.58 ± 0.58 ^{ab}	92
20% SAR + 80% Y1	16.16 ± 3.95 ^b	9.34 ± 0.91 ^{ab}	82
100% Y1	3.92 ± 0.94 ^d	4.01 ± 0.88 ^c	35
20% SAR + 80% Y3	22.28 ± 4.79 ^{ab}	10.61 ± 0.94 ^{ab}	93
100% Isog	18.65 ± 2.26 ^{ab}	9.96 ± 0.49 ^{ab}	87
100% Skel	13.72 ± 2.33 ^{bc}	8.73 ± 0.71 ^b	76
100% Chaeg	18.17 ± 3.80 ^{ab}	9.81 ± 0.89 ^{ab}	86
100% Tetra	5.75 ± 2.02 ^{cd}	5.28 ± 1.35 ^c	46

§: standard algal ration: 100% SAR = Isog/Skel/Thal/Chaeg/Tetra (25:25:22.5:22.5:5 mixture on DW) fed at 4% DW WW⁻¹ day⁻¹; with Isog=*Isochrysis galbana*, Skel=*Skeletonema costatum*, Thal=*Thalassiosira pseudonana*, Chaeg=*Chaetoceros gracilis*, Tetra=*Tetraselmis suecica*

Y1, Y3: yeast diets (Table 42)

†: data obtained after 14 days of culture, whereupon negative growth was observed and this treatment was discontinued

*: DGRΣ expressed as percentage of that observed for the 100% SAR treatment initial seed: WW = 1.30 mg ind⁻¹

IX.3.3. Discussion

It is well accepted that larvae as well as juvenile bivalves fed on a diet consisting of several algal species generally grow faster than those fed only one species (reviewed by Epifanio, 1983; Webb & Chu, 1983; Brown *et al.*, 1989). This has been attributed to a mutual compensation of limiting nutrients present in single algal diets (Webb & Chu, 1983) as well as to an improvement of digestibility of refractory algae when fed in combination with easily digestible species (Epifanio, 1983). The

present data confirm this for *T. philippinarum* fed a monodiet of either *T. suecica* or *S. costatum*. The inferior nutritional value of *T. suecica* compared to the other algal species tested was also found for juvenile *C. virginica* (Epifanio, 1979b) and *O. edulis* (Laing & Millican, 1986), and has been related to the poor digestion and absorption of this alga by the oyster (Romberger & Epifanio, 1981). The high nutritional value of *C. gracilis* is corroborated by Enright et al. (1986a) who showed that this alga supported best growth of *O. edulis* spat compared to 15 other phytoplankton species.

The results of this preliminary trial appeared promising with regard to the partial substitution of the standard algal regime by manipulated yeasts. The 20/80% diet of the algal mixture and the yeast product enriched with the algal extract supported clam growth of more than 90% of that of the full algal ration. This relative growth rate, calculated over a period of four weeks and compared to an algal control which is considered quantitatively and qualitatively optimal, is the best to date for juvenile bivalves reared on a 20/80% diet of algae and yeast (see IX.5.2.2.) or any other artificial diet (see III.4.). Laing & Verdugo (1991) reported a significant growth improvement in juveniles of the Manila clam fed spray-dried *T. suecica* by the addition of 20% live *Chaetoceros calcitrans*, though their data do not allow the comparison of growth with a control diet composed of more than one alga. Only recently, Laing (1991) reported comparable growth rates in *T. philippinarum* fed either a mixture of 90% dried *T. suecica* and only 10% live *S. costatum*, or a mixture of live algae (30% *S. costatum*/70% *T. suecica*).

The growth promoting effect of the extract from macro-algae, although not significant in the 80% replacement treatments, is rather important when considering the fact that it constituted only 1% of the diet's dry weight. Chu et al. (1987) improved growth of oyster larvae fed a microencapsulated diet through the inclusion of a lipid extract from micro-algae, and suggested that the latter may play a role as "attractant or phagostimulans" in the artificial diet. The determination of the possible role of

the algal extract in the present experiments requires further investigation. It may have provided micro-nutrients that are lacking in the yeast diet, either directly through uptake by the seed or indirectly by promoting algal and/or bacterial growth in the seawater.

The low growth of clams fed a monodiet of yeast suggests that this diet is nutritionally incomplete. However, the yeast diet still resulted in a growth rate of 76% of that observed when feeding the sole diet of *T. suecica*. From this, it is clear that the nutritional quality of the algal control diet plays a major role in the relative expression of the nutritional value of an artificial diet. Growth rate of juvenile clams fed spray-dried, heterotrophically grown *T. suecica* may thus be similar to that of clams fed live *T. suecica*, but is inferior to that for animals grown on *C. gracilis* or a mixture of live *T. suecica* and *C. gracilis* (Laing & Verdugo, 1991).

IX.4. LABORATORY EXPERIMENTS WITH *TAPES PHILIPPINARUM*²

IX.4.1. Rationale and experimental design

This series of experiments was designed to develop a standardized growth test for rearing seed of *T. philippinarum* on various mixtures of algae and artificial diets. A standardized growth test would then be used to verify the results that were obtained in the hatchery trials in Spain (IX.3.) and to further improve the composition of the yeast-based diet. For a detailed description of the methodology and conditions of the growth experiments we refer to IX.2.

Chaetoceros gracilis, which has proven to be among the best algal diets for *O. edulis* juveniles (Enright et al., 1986a) and is extensively used in several commercial bivalve hatchery and nursery operations (Bayes, Dravers, Gutierrez, Manzi, pers. comm., 1990), was selected as the algal control diet. In a first series of tests (Experiments ARC 1-4), *C. gracilis* was separated from the culture medium by centrifugation and stored as a concentrated stock suspension in the refrigerator in order to simplify the feeding procedure (Winter & Langton, 1976; see IX.2.4.). A preliminary test demonstrated that the feeding regime applied in the Tinamenor hatchery, i.e. 4% DW WW⁻¹ day⁻¹, resulted in a considerable over-dosing of the algae under the present laboratory conditions. As a result, some of the experiments were run to determine the quantitative requirements of juvenile *T. philippinarum* fed the various algal diets used in this study. The experimental designs of the various tests (ARC 1-7) is shortly described below, and is detailed in Table 44 (ARC 5) and the results section (Tables 45, 47, 48, 49, 50; Fig. 63).

A first experiment (ARC 1) attempted to determine the effect of the ration size ranging from 0.1% to 1.5% day⁻¹ for *T. philippinarum* fed the concentrated *C. gracilis*. The cell concentration was frequently measured by means of a Coulter counter (model Zf) in two replicate cultures of each treatment.

²parts of this chapter have been published in Coutteau et al. (1990)

From the counts performed at 2-6 h intervals, an average rate of removal was calculated by means of the formulas described in IX.2.6.1. Because the production of pseudofaeces was not quantified in this study, the weight-specific removal of algae per unit time should be referred to as intake rate, rather than ingestion rate (see III.2.3.). Furthermore, the change of clearance and intake rate in *T. philippinarum* as a function of *Chaetoceros* concentration was studied in a short-term grazing experiment (see IX.2.6.2.). This allowed the determination of the maximal clearance (CR_{max}) and intake (ir_{max}) rates for clams of the same size as those used for the growth tests. The incipient limiting concentration was calculated from the ratio ir_{max}/CR_{max} (Sprung & Rose, 1988) or determined graphically.

In a second experiment (ARC 2), the growth of the clams fed an insufficient algal ration (50% or 20% of the optimal *Chaetoceros* ration) supplemented with different amounts of yeast diet Y1 was examined in order to determine if possible nutritional deficiencies in the artificial diet could be overcome by increasing its dosage. An additional treatment, fed 150% algae, was run to verify the adequacy of the 100% algal control diet.

The substitution of 50% and 80% of the *Chaetoceros* ration by yeast diet Y1 was repeated in experiment three (ARC 3) and compared with the algal replacement by diet Y3. The latter contained 1% (on DW basis) of a seaweed extract, which appeared to promote clam growth during the trials in Spain (IX.3.).

In experiment four (ARC 4), various components were incorporated into the yeast diet in order to improve its nutritional value as a partial algal substitute. Kaolinite (Y5) and rice starch (Y4) were added because of the reported beneficial effect of these compounds on growth of *C. virginica* juveniles fed algae/yeast mixtures (Urban et al., 1983). Furthermore, the effect of increased levels of the fat-soluble vitamins A, D, E, and K (Y2) on clam growth was investigated.

Since the results of the first series of experiments were clearly inferior to those obtained in the trials at Tinamenor, the influence of various methodological aspects of the culture

test on the performance of the yeast diet was examined in a second series of experiments (Experiments ARC 5-7). In the fifth experiment (ARC 5), growth of clams fed a complete as well as a partially substituted ration of the concentrated *C. gracilis* was compared with that of clams fed algae drained from the cultures immediately prior to feeding (Table 44). In addition, the effect of better water quality was examined by increasing the frequency of the water change. The performance of yeast Y5 as an 80% replacement diet for *C. gracilis* was compared with that of spray-dried *T. suecica* (IX.2.5.).

Table 44: Experimental design of experiment ARC 5.

	TREATMENT [§]	<i>C. gracilis</i> [†]	frequency water change
1	100% Chg	concentrate	3 week ⁻¹
2	100% Chg	concentrate	1 day ⁻¹
3	150% Chg*	culture	3 week ⁻¹
4	100% Chg	culture	3 week ⁻¹
5	20% Chg + 80% Y5	concentrate	3 week ⁻¹
6	20% Chg + 80% Y5	concentrate	1 day ⁻¹
7	20% Chg + 80% Y5	culture	3 week ⁻¹
8	20% Chg + 80% dTs	concentrate	3 week ⁻¹
9	Chg "on demand"	week 1: concentrate week 2&3: culture	3 week ⁻¹

§: standard algal ration: 100% Chg = *C. gracilis* fed at a daily ration of 1% day⁻¹

Y5 = yeast diet (Table 42), dTs = spray-dried *Tetraselmis suecica*

*: no data available for the first week

†: concentrate = separated from the culture medium by centrifugation and stored as a concentrated suspension in the refrigerator for a period of maximal three days

culture = drained from the algal culture and counted prior to feeding

During this fifth experiment, the interrelationships between ration size, intake rate and growth was studied for the various treatments fed on algae alone. In addition to the 100% algal control treatments fed a 1% daily ration of either the *C. gracilis* concentrate or culture, one treatment was provided a ration of 1.5% algal culture day⁻¹. Furthermore, an "on demand" treatment was run for which feeding was based on the rate of food clearance and aimed at maintaining the *Chaetoceros* concentration above 20 cells μl^{-1} . The measurement of cell concentration before and after each feeding by means of the Coulter counter allowed

the calculation of an average daily removal of cells (IX.2.6.1.), which in turn could be converted to a weight-specific ration of material removed by the clams from the suspension. From the latter and from the growth rate of the clams in the corresponding treatment, the gross growth efficiency could be estimated as the ratio live weight increase/dry weight of food removed. Moreover, the variation of the average intake rate, computed for each time interval between two consecutive feedings, could be related to the adequacy of the food ration.

In experiment 6 (ARC 6) various yeast diets were evaluated as an 80% replacement of *C. gracilis* drained directly from the culture. Besides some of the yeast diets tried in experiment ARC 3 and 4 (Y1, Y3), the addition of kaolinite and the seaweed extract was combined in one diet (Y6). Also, the effect on clam growth of increasing the level of the seaweed extract in the yeast diet from 1% to 5% of total DW was evaluated (Y7). In order to verify whether the incorporation of silt into the diet affected the yeast during the production process or acted directly on the clams in the cultures, the same amount of kaolinite was fed either as a component of the yeast diet (Y6) or as a separate supplement (Y3+K).

In the final experiment (ARC 7), the influence of the composition of the algal control diet on the relative performance of the yeast diet Y6 as a partial substitute was examined. For this, clams were cultured on a diet consisting of *C. gracilis*, *I. galbana* (clone T-Iso), or a 50/50 mixture (on DW basis) of both species. The growth improvement achieved by supplementing the yeast diet was determined by feeding clams a 20% ration of the various algal diets with or without the yeast supplement.

Table 45 (Experiment ARC 1): Daily growth rate (DGR), final live (WW) and dry (DW) weight, and shell length (L) of *T. philippinarum* fed various daily rations of *C. gracilis*. Data represent mean and standard deviation from four replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD, $P \leq 0.05$, unless stated otherwise).

TREATMENT [§]	WEEK 1	WEEK 2	WEEK 3			
	DGR (% day ⁻¹)	DGR (% day ⁻¹)	DGR (% day ⁻¹)	WW (mg ind ⁻¹)	DW (mg ind ⁻¹)	L (mm)
1) unfed control	2.36 ± 0.30 ^b	0.31 ± 0.50	0.48 ± 0.36 ^c	6.38 ± 0.37 ^c	4.05 ± 0.21 ^c	2.84 ± 0.08 ^c
2) 0.1% Chg	2.91 ± 0.25 ^b	2.05 ± 0.17	1.33 ± 0.26 ^c	8.23 ± 0.28 ^c	5.16 ± 0.17 ^c	3.23 ± 0.15 ^c
3) 0.5% Chg	5.80 ± 0.21 ^a	6.52 ± 0.11	5.74 ± 0.37 ^b	17.99 ± 1.50 ^b	10.82 ± 0.84 ^b	4.37 ± 0.16 ^b
4) 1.0% Chg	6.71 ± 0.67 ^a	9.77 ± 0.09	8.67 ± 0.43 ^a	29.59 ± 3.26 ^a	17.14 ± 1.89 ^a	5.34 ± 0.17 ^a
5) 1.5% Chg	5.84 ± 0.68 ^a	7.85 ± 0.71	9.21 ± 0.53 ^a	25.65 ± 3.27 ^a	14.92 ± 1.89 ^a	5.07 ± 0.18 ^a
ANOVA, F ₅	69.0	H.D. [♦]	407.3	88.8	84.4	157.5

§: Chg = *Chaetoceros gracilis*, rations expressed as initial daily rations (DW WW⁻¹ day⁻¹)
initial seed: 5.13 ± 0.58 mg ind⁻¹ (m ± SD, n=3)

♦: heteroscedastic data, significantly different means separated by / (MCHETV, $P \leq 0.05$):
1/2,3,4,5; 2/3,4,5; 3/4

IX.4.2. Results

IX.4.2.1. Experiment ARC 1: Effect of ration size on growth of *T. philippinarum* fed *Chaetoceros gracilis*

Clam growth increased with increasing daily ration up to a ration of 1% of live weight (Table 45). Further increase of the ration to 1.5% did not result in a significant difference in growth or final size of the clams. The feeding regime was adapted daily to the growth of the clams in the various treatments based on an assumed daily growth rate for each week of the experiment (see IX.2.3.). The actual rations, computed from the feeding regime and the observed growth rates, deviated from the initial ration in the course of each week depending on the accuracy of the assumed growth rate (Fig. 50). To obtain a better estimate of the effective weight-specific ration fed to the clams, the arithmetic mean of the actual daily ration was determined for each week of the experiment (Fig. 50). Daily growth rate showed a saturation response around an effective ration of 1%, though fluctuated between 6.7 and 9.8% day⁻¹ according to the week of the test (Fig. 51). Growth of the starved clams declined from more than 2% day⁻¹ during the first week to less than 0.5% day⁻¹ during the rest of the experiment (Table 45).

The fluctuation of algal concentration showed a similar pattern in all replicates of each treatment and is represented for one replicate system per treatment in Fig. 52. By distributing a weight-specific ration of 1% day⁻¹ over two feedings, a peak concentration was attained twice daily of 30-40 *Chaetoceros* µl⁻¹, which was cleared to a level of 5-10 cells µl⁻¹ in the subsequent 5-7 h period (Fig. 52C). The highest ration regularly resulted in an accumulation of the algae between the periodic renewing of the seawater up to concentrations of 40 (week 3) or even 90 algal cells µl⁻¹ (week 2, Fig. 52D). In the cultures fed the lowest rations, the algae were cleared to levels below 1 to 2 *Chaetoceros* µl⁻¹ (Fig. 52A,B).

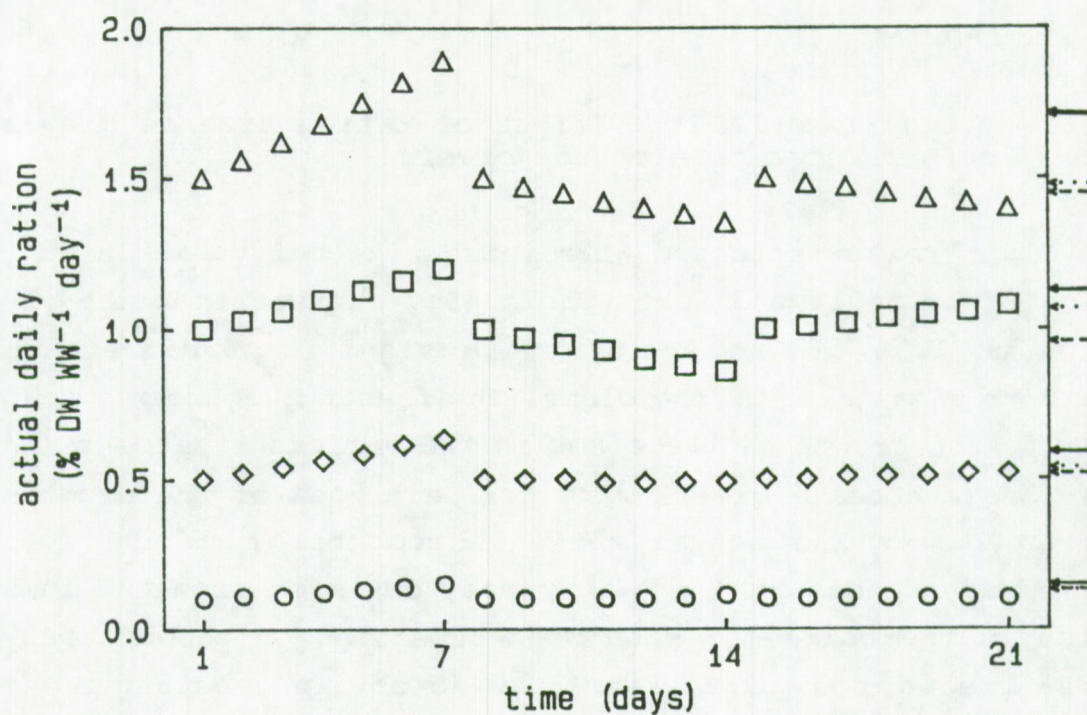


Fig. 50 (Experiment ARC 1): Change in actual daily ration over the course of each week of the experiment for *T. philippinarum* fed *C. gracilis* at an initial daily ration of either 1.5% (Δ), 1% (\square), 0.5% (\diamond), or 0.1% (\circ). The horizontal arrows indicate the effective ration for the various treatments during week one (solid), two (dashed), and three (dotted) of the experiment.

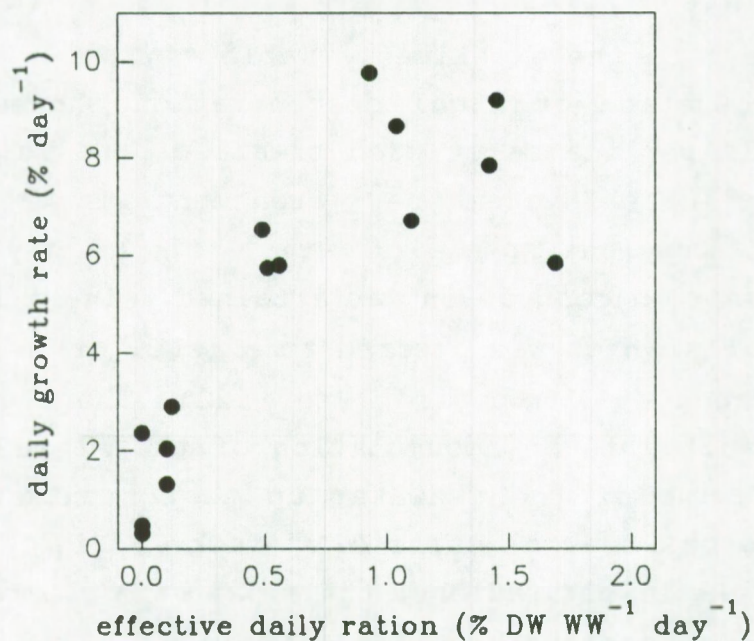


Fig. 51 (Experiment ARC 1): Relationship between the effective daily ration and the daily growth rate of *T. philippinarum* fed *C. gracilis*.

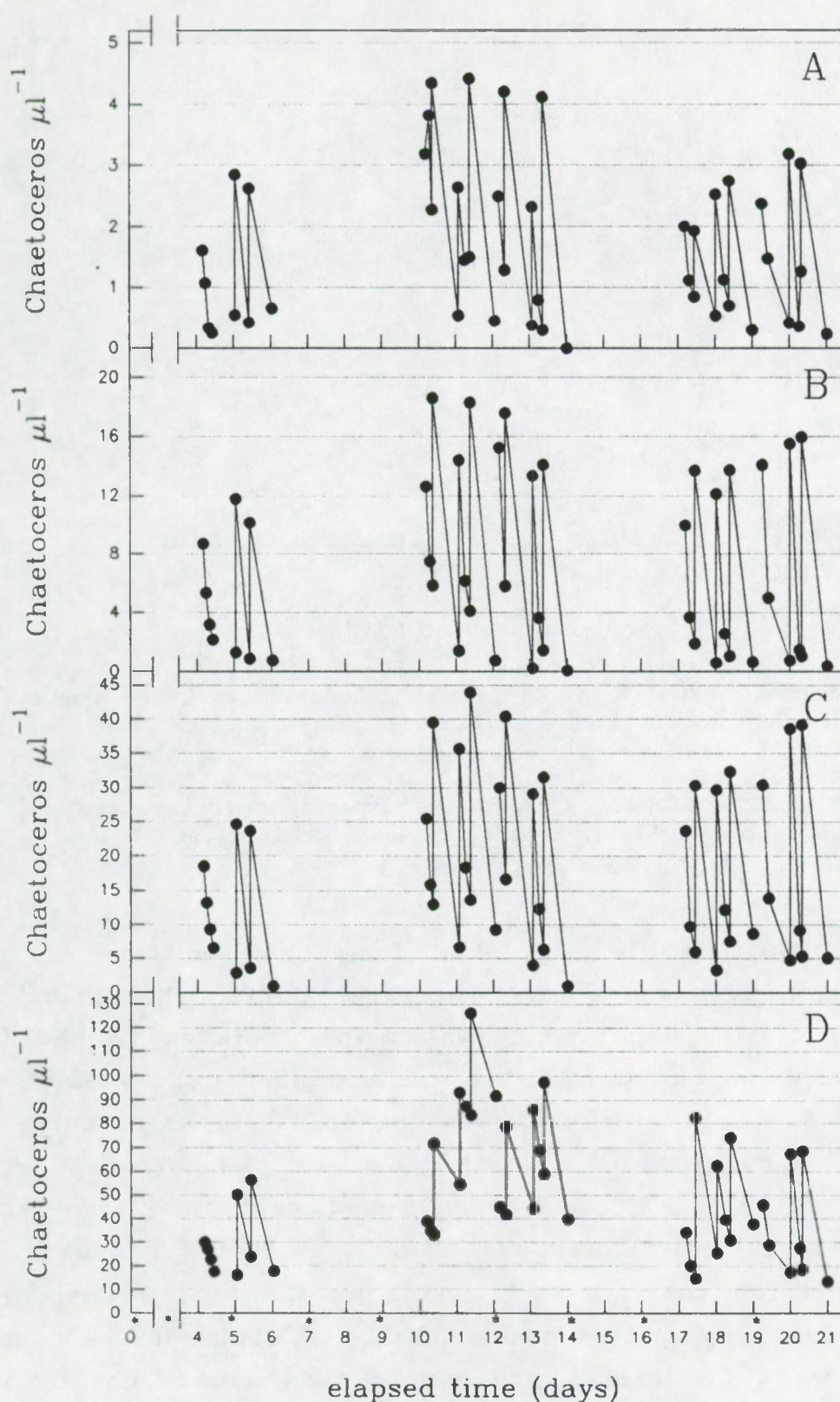


Fig. 52 (Experiment ARC 1): Fluctuation of food concentration in one replicate culture of *T. philippinarum* fed *C. gracilis* at a daily ration of either 0.1% (A), 0.5% (B), 1.0% (C), or 1.5% (D). Cell densities measured before and after feeding are connected with a vertical line. Data points are not connected when measurements were discontinued. The periodic renewal of the seawater is indicated on the time axis (*).

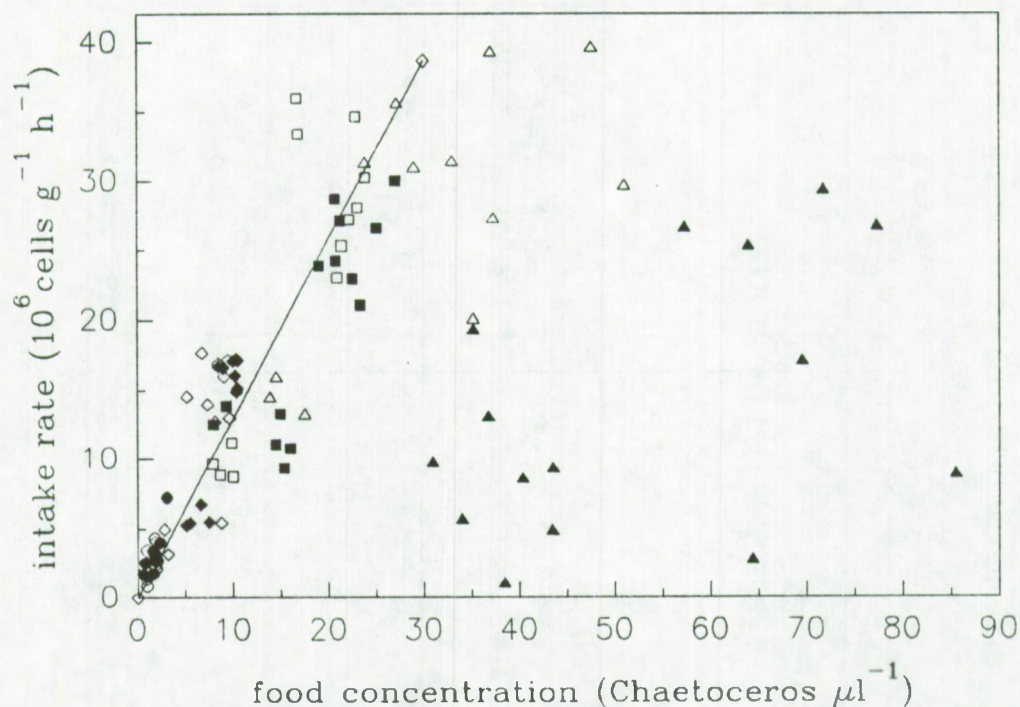


Fig. 53 (Experiment ARC 1): Intake rate as a function of food concentration in *T. philippinarum*. Data are calculated from the decrease of food concentration measured over 2 to 6 h time intervals in the cultures fed various daily rations of *C. gracilis* (0.1%: ○, 0.5%: ◇, 1.0%: □, 1.5%: △) during the second and third week of the experiment (filled and unfilled symbols, respectively). Linear regression equation is given by:

$$ir [10^6 \text{ cells g}^{-1} \text{ h}^{-1}] = 1.229 C [\text{cells } \mu\text{l}^{-1}] \quad (r^2=0.81).$$

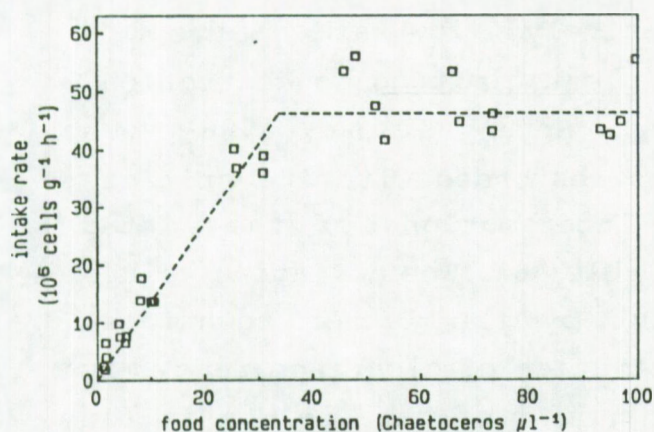
The intake rate, calculated from the decrease of cell concentration over short time intervals during the second and third week of the experiment, was mainly related to the food level present in the culture at the moment of the measurement (Fig. 53). Fluctuation of cell concentration in the cultures fed 1% day⁻¹ thus resulted in a variation of intake rate between 35 and 8.10⁶ cells g⁻¹ h⁻¹, and even lower values may have been observed at the concentrations below 10 *Chaetoceros* μl⁻¹. The rate with which the clams removed the algae from suspension increased linearly up to a concentration of about 30 *Chaetoceros* μl⁻¹. Estimates of intake rate beyond this algal density were mainly derived from the cultures fed 1.5% DW WW⁻¹ day⁻¹ during the second week of the test and fluctuated strongly irrespective of food concentration. Possibly, the strongly depressed intake rates observed at the high food concentrations were artefacts due to the calculation of intake rates from relatively small decreases of cell concentration. Also, the impact of algal growth, which

was not taken into account for the computation of the intake rate, may have been relatively more important at the high algal loads.

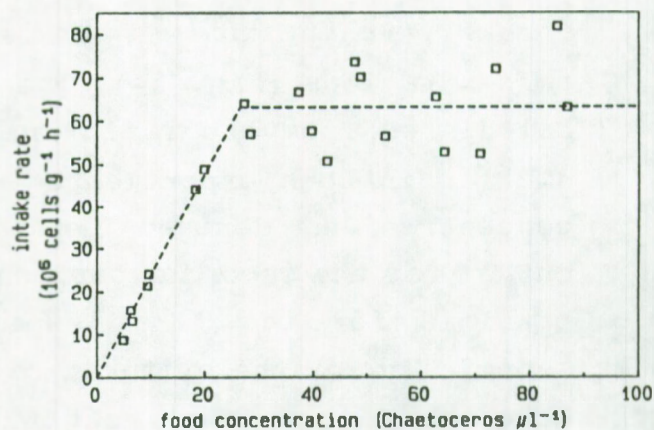
T. philippinarum maintained maximum clearance (CR_{max}) and intake (ir_{max}) rates at, respectively, low and high food concentrations (Fig. 54). The incipient limiting concentration, calculated from the ratio ir_{max}/CR_{max} (Sprung & Rose, 1988), was similar in both experiments and corresponded with the critical concentrations derived from the intersections of the fitted curves for the second experiment (Table 46). The deviating values obtained for the first experiment by the latter technique, especially for the critical concentration of clearance rate, may have been due to the insufficient number of data points in the proximity of the incipient limiting concentration. Maximum weight-specific rates of filtration and feeding were about 30% higher during the second test than in the first experiment.

Table 46: Maximal clearance (CR_{max}) and intake (ir_{max}) rate, and their critical concentrations for juvenile *T. philippinarum* (20 mg live weight) fed *C. gracilis* in two independent experiments. Data derived from Fig. 54A,B.

	test A	test B
ir_{max} (10^6 cells g^{-1} h^{-1})	46.2	63.2
minimum concentration for $ir=ir_{max}$ (cells μl^{-1})	33.7	26.6
CR_{max} (ml g^{-1} h^{-1})	1771	2301
minimum concentration for $CR<CR_{max}$ (cells μl^{-1})	15.2	24.1
incipient limiting concentration [ir_{max}/CR_{max}] (cells μl^{-1})	26.1	27.4



A



B

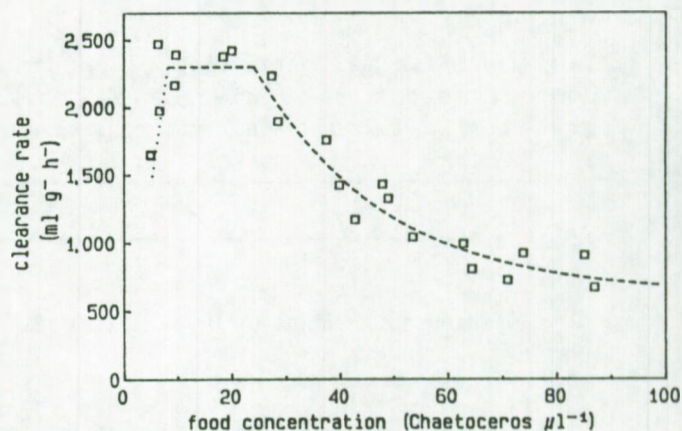
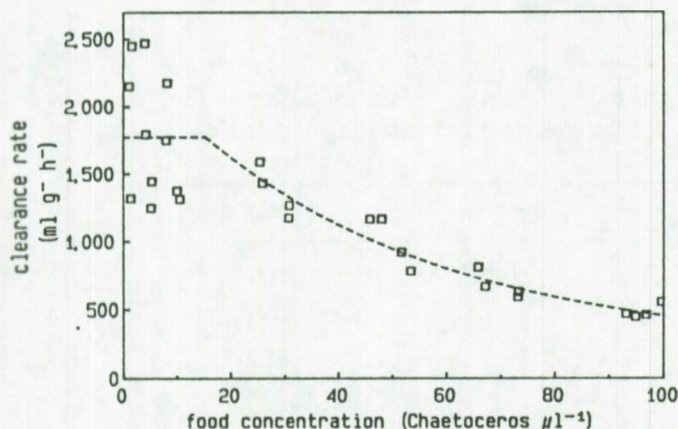


Fig. 54: Clearance and intake rates as a function of food density in *T. philippinarum* (20 mg live weight) fed *C. gracilis*. Maximum clearance and intake rates are calculated from the mean of the data points below and above the critical concentrations, respectively. A and B represent the results of two independent experiments. Curves were fitted to the data points for clearance and intake rate, respectively, above and below the incipient limiting level through, respectively, non-linear ($y=Ae^{-Bx}+Ce^{-Dx}+E$) and linear ($y=Ax$) regression. Derived parameters are presented in Table 46.

IX.4.2.2. Experiment ARC 2: Supplementing varying amounts of the yeast diet Y1

Growth of clams fed a daily ration of 1.5% *C. gracilis* did not differ from or was even significantly lower (week 1) than that observed for the algal control fed 1% day⁻¹, which confirmed the findings of the first experiment. Also, growth rate of the algae-fed controls varied in the course of the experiment (Table 47).

Table 47 (Experiment ARC 2): Daily growth rate (DGR), final live (WW) and dry (DW) weight of *T. philippinarum* fed on *C. gracilis* with or without various amounts of yeast diet Y1. Data represent mean and standard deviation from three replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD, $P \leq 0.05$; unless stated otherwise).

TREATMENT [§]	WEEK 1	WEEK 2	WEEK 3		
	DGR (% day ⁻¹)	DGR (% day ⁻¹)	DGR (% day ⁻¹)	WW (mg ind ⁻¹)	DW (mg ind ⁻¹)
1) unfed control	1.14 ± 0.11 ^a	0.17 ± 0.14 ^a	0.48 ± 0.16	6.90 ± 0.41 ^a	4.13 ± 0.31 ^a
2) 100% SAR	5.23 ± 0.53 ^{ab}	7.98 ± 0.57 ^a	5.57 ± 0.11	25.16 ± 1.00 ^a	14.45 ± 0.51 ^a
3) 150% SAR	3.82 ± 0.20 ^{cd}	7.96 ± 0.60 ^a	5.68 ± 2.09	22.90 ± 3.06 ^a	13.07 ± 0.78 ^a
4) 50% SAR + 50 Y1	5.75 ± 0.23 ^a	6.28 ± 0.06 ^b	4.90 ± 0.28	21.27 ± 1.64 ^a	12.43 ± 1.07 ^a
5) 50% SAR + 100 Y1	4.64 ± 0.37 ^{bc}	5.25 ± 0.27 ^{bc}	4.09 ± 0.47	16.19 ± 1.36 ^b	9.61 ± 0.72 ^b
6) 50% SAR + 150 Y1	3.66 ± 0.50 ^d	4.91 ± 0.22 ^c	4.02 ± 0.77	15.53 ± 0.86 ^{bc}	9.26 ± 0.46 ^{bc}
7) 20% SAR + 80 Y1	4.29 ± 0.26 ^{bcd}	2.76 ± 0.63 ^d	2.31 ± 0.24	11.42 ± 1.34 ^{cd}	6.83 ± 0.87 ^d
8) 20% SAR + 160 Y1	3.54 ± 0.15 ^d	2.59 ± 0.17 ^d	1.88 ± 1.03	11.22 ± 0.65 ^d	6.80 ± 0.47 ^d
9) 20% SAR + 240 Y1	3.45 ± 0.36 ^d	2.99 ± 0.60 ^d	2.55 ± 0.20	12.25 ± 1.26 ^{bcd}	7.27 ± 0.77 ^{cd}
ANOVA, F ₈	46.1	115.8	H.V. [♦]	51.6	67.9

§: standard algal ration: 100% SAR= *Chaetoceros gracilis*, fed at 1% DW WW⁻¹ day⁻¹

Y1: yeast diet (Table 42)

initial seed: 5.65 ± 0.41 mg ind⁻¹ (m ± SD, n=3)

♦: heteroscedastic data, significantly different means separated by / (MCHETV, $P \leq 0.05$):

1/2,4,5,7,9; 7/2,4; 9/2,4

Substituting 50% of the algal ration for an equivalent dry weight of yeast product yielded on average a growth rate which did not differ significantly from that of the clams fed the full *Chaetoceros* ration (Fig. 55), and ranged each week between 110% and 79% of the latter (Table 47). Replacing 50% of the *Chaetoceros* diet by more than the equivalent dry weight dosage of yeast depressed clam growth significantly (Fig. 55).

The replacement of 80% of the algal ration by yeast resulted in an important decrease in growth relative to that observed for the algal control, from 80% during the first week to less than 40% during the subsequent period (Table 47). As a result, clams fed 20% algae in combination with yeast grew significantly slower than those fed the 50/50 algae/yeast diet (Fig. 55). Furthermore, the amount of yeast added to the 20% algal ration did not significantly affect clam growth.

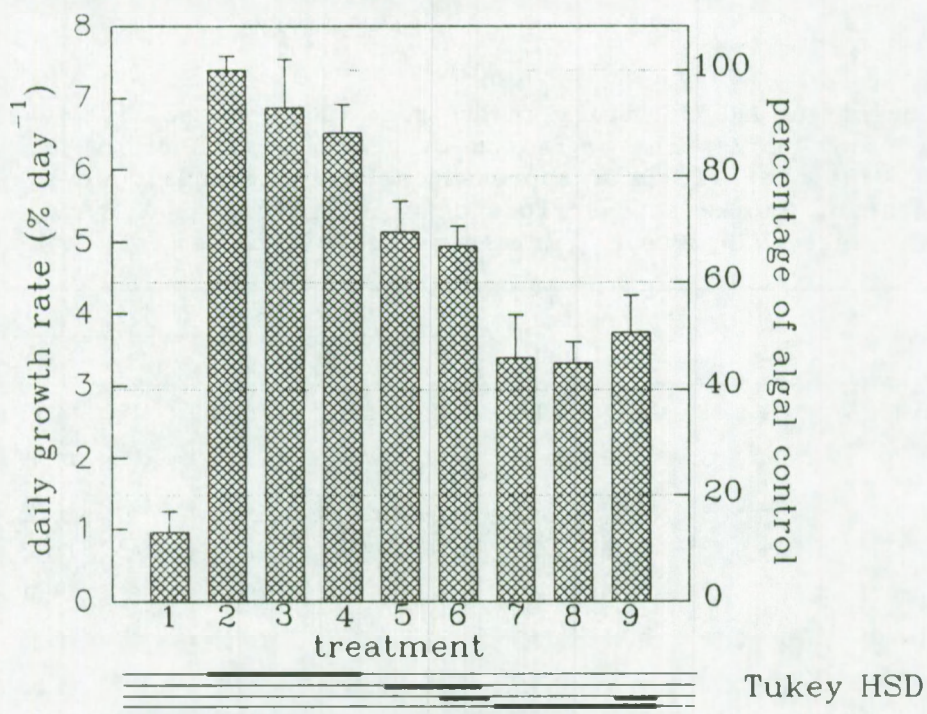


Fig. 55 (Experiment ARC 2): Growth of juvenile *T. philippinarum* fed various mixtures of *C. gracilis* and yeast diet Y1.

Data represent daily growth rate (DGRΣ, mean ± SD, n=3) calculated from the increase of individual live weight over the three week experimental period, and expressed either absolutely (% day⁻¹) or relatively compared to the DGRΣ obtained for the algae-fed controls (%). The various treatments are explained in Table 47 (____) denotes statistically similar groups (Tukey HSD test, P≤0.05).

IX.4.2.3. Experiment ARC 3: Addition of an extract from seaweeds to the yeast diet

During the third experiment growth rates were elevated in all treatments during the first week and leveled off in the rest

of the test to values which were comparable with those achieved previously (Table 48). The replacement of 50% of the *Chaetoceros* ration by either of the yeast products did not result in a significant decline of growth rate calculated each week from the increase of biomass in the system (Table 48). However, compared to the growth of clams fed the 50% algal ration alone, a significant growth improvement was only obtained during the second week of the test. As a result, final weight and average growth rate of the clams fed the 50/50% algae/yeast diets were significantly lower than those of the algal-fed controls (Table 48, Fig. 56).

Growth of clams fed the 20% algal ration was significantly improved by the supplementation of yeast, but remained inferior to the growth of clams fed on the 50% algal ration (Fig. 56). During the last week of the experiment, feeding the 20/80% algae/yeast diets yielded lower growth compared to the algal controls than in the first two weeks (Table 48).

No significant difference in growth was observed between *T. philippinarum* fed either of the yeast diets as a partial substitute for *C. gracilis* (Fig. 56, Table 48).

Table 48 (Experiment ARC 3): Daily growth rate (DGR), final live (WW) and dry (DW) weight of *T. philippinarum* fed on *C. gracilis* with or without supplements of either yeast diet Y1 or Y3. Data represent mean and standard deviation from three replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD, $P \leq 0.05$).

TREATMENT [§]	WEEK 1	WEEK 2	WEEK 3		
	DGR (% day ⁻¹)	DGR (% day ⁻¹)	DGR (% day ⁻¹)	WW (mg ind ⁻¹)	DW (mg ind ⁻¹)
1) 100% SAR	12.38 ± 1.02 ^a	7.38 ± 0.40 ^a	6.78 ± 1.20 ^a	36.48 ± 3.93 ^a	21.39 ± 2.05 ^a
2) 50% SAR	9.84 ± 0.37 ^{bc}	5.48 ± 0.30 ^b	4.90 ± 0.08 ^b	24.27 ± 0.79 ^b	14.22 ± 0.38 ^b
3) 20% SAR	6.30 ± 0.38 ^c	2.71 ± 0.10 ^d	2.00 ± 0.18 ^d	12.03 ± 0.55 ^c	7.08 ± 0.35 ^d
4) 50% SAR + 50% Y1	10.99 ± 0.05 ^{ab}	7.00 ± 0.30 ^a	5.69 ± 0.61 ^{ab}	27.81 ± 1.52 ^b	16.61 ± 0.83 ^b
5) 50% SAR + 50% Y3	10.79 ± 0.17 ^{ab}	6.62 ± 0.46 ^a	5.49 ± 0.24 ^{ab}	26.59 ± 3.48 ^b	15.92 ± 1.90 ^b
6) 20% SAR + 80% Y1	7.97 ± 0.50 ^d	4.42 ± 0.13 ^c	2.98 ± 0.36 ^c	17.86 ± 1.56 ^c	10.76 ± 0.96 ^c
7) 20% SAR + 80% Y3	8.97 ± 0.30 ^{cd}	3.74 ± 0.49 ^c	2.97 ± 0.27 ^c	16.88 ± 1.58 ^c	10.17 ± 0.99 ^{cd}
ANOVA, F _s	63.3	80.0	54.2	39.5	45.3

§: standard algal ration: 100% SAR= *Chaetoceros gracilis*, fed at 1% DW WW⁻¹ day⁻¹

Y1, Y3: yeast diets (Table 42)

initial seed: 5.63 ± 0.11 mg ind⁻¹ (m ± SD, n=3)

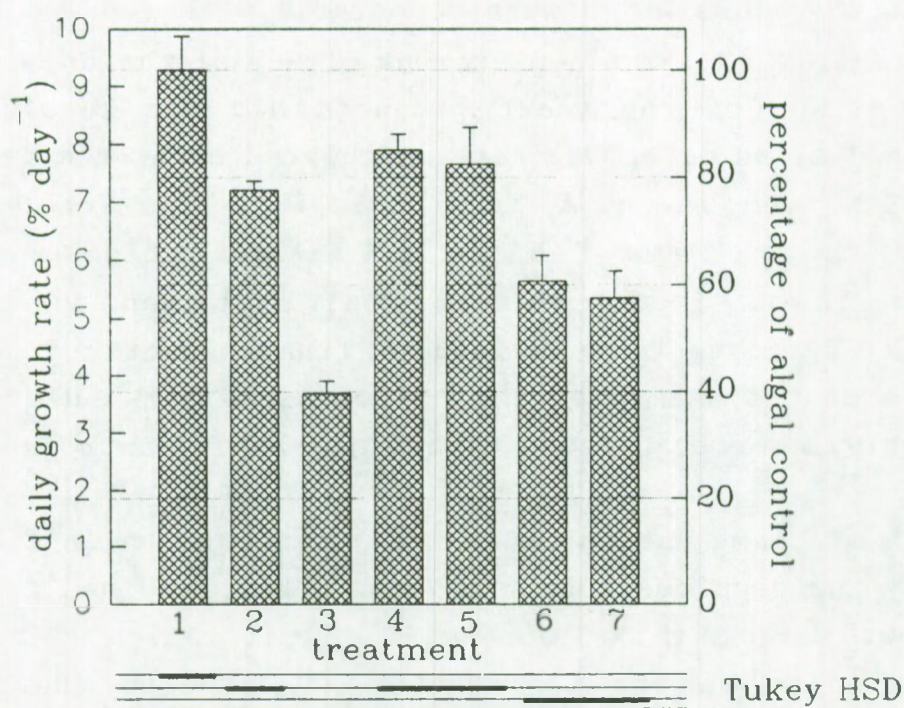


Fig. 56 (Experiment ARC 3): Growth of juvenile *T. philippinarum* fed various mixtures of *C. gracilis* and yeast diets Y1 or Y3. Data represent daily growth rate (DGR Σ , mean \pm SD, $n=3$) calculated from the increase of individual live weight over the three week experimental period, and expressed either absolutely (% day⁻¹) or relatively compared to the DGR Σ obtained for the algae-fed controls (%). The various treatments are explained in Table 48. (____) denotes statistically similar groups (Tukey HSD test, $P \leq 0.05$).

IX.4.2.4. Experiment ARC 4: Addition of kaolinite, rice starch, or fat-soluble vitamins to the yeast diet

The addition of either kaolinite, rice starch, or fat-soluble vitamins to the yeast diet did not significantly improve growth of clams fed a 20/80% algae/yeast diet, at least when considering each week of the test separately (Table 49). However, the incorporation of kaolinite into the yeast product resulted in a significantly higher average growth rate over the total culture period compared to that of clams fed yeast diet Y1 as an 80% replacement (Fig. 57). The yeast supplement could only significantly enhance growth of clams fed on 20% of the *Chaetoceros* ration when either kaolinite, rice starch, or a 50/50 mixture of both was added to the diet (Fig. 57). Growth was

significantly depressed by doubling the amount of yeast diet Y2 fed to the 80% algal replacement treatment. Substituting 50% of the algal ration with yeast diet Y2 yielded a significantly, *i.e.* about 25%, lower growth rate compared to that of algae-fed controls.

Table 49 (Experiment ARC 4): Daily growth rate (DGR), final live (WW) and dry (DW) weight of *T. philippinarum* fed on *C. gracilis* with or without supplements of various yeast diets. Data represent mean and standard deviation from three replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD, $P \leq 0.05$).

TREATMENT ^a	WEEK 1	WEEK 2	WEEK 3		
	DGR (% day ⁻¹)	DGR (% day ⁻¹)	DGR (% day ⁻¹)	WW (mg ind ⁻¹)	DW (mg ind ⁻¹)
1) 100% SAR	7.69 ± 0.52 ^a	5.47 ± 0.14 ^a	8.06 ± 0.28 ^a	5.25 ± 0.13 ^a	2.95 ± 0.05 ^a
2) 20% SAR	3.96 ± 0.36 ^{ef}	2.25 ± 0.38 ^{cd}	3.07 ± 0.16 ^e	2.41 ± 0.08 ^a	1.36 ± 0.05 ^f
3) 20% SAR + 80% Y1	4.69 ± 0.22 ^{cde}	2.41 ± 0.77 ^{cd}	3.91 ± 0.35 ^{cde}	2.64 ± 0.14 ^{de}	1.55 ± 0.08 ^a
4) 20% SAR + 80% Y5	5.60 ± 0.20 ^{bc}	2.76 ± 0.71 ^c	4.09 ± 0.50 ^{cd}	3.22 ± 0.35 ^c	1.76 ± 0.01 ^c
5) 20% SAR + 80% Y4	5.26 ± 0.22 ^{cd}	2.42 ± 0.42 ^{cd}	4.28 ± 0.13 ^c	2.86 ± 0.07 ^{cd}	1.69 ± 0.03 ^{cd}
6) 20% SAR + 40% Y4 + 40% Y5	5.60 ± 0.28 ^{bc}	2.51 ± 0.21 ^c	4.29 ± 0.58 ^c	2.88 ± 0.10 ^{cd}	1.70 ± 0.05 ^{cd}
7) 20% SAR + 80% Y2	4.60 ± 0.04 ^{de}	2.51 ± 0.02 ^c	3.98 ± 0.16 ^{cd}	2.65 ± 0.03 ^{de}	1.57 ± 0.01 ^{de}
8) 20% SAR + 160% Y2	3.62 ± 0.49 ^f	1.26 ± 0.05 ^d	3.37 ± 0.09 ^{de}	2.25 ± 0.04 ^a	1.33 ± 0.02 ^f
9) 50% SAR + 50% Y2	6.20 ± 0.26 ^b	4.20 ± 0.06 ^b	5.84 ± 0.22 ^b	3.74 ± 0.08 ^b	2.17 ± 0.05 ^b
ANOVA, F _s	44.4	27.6	75.4	118.8	372.4

^a: standard algal ration: 100% SAR= *Chaetoceros gracilis*, fed at 1% DW WW⁻¹ day⁻¹

Y1, Y2, Y4, Y5: yeast diets (Table 42)

initial seed: 1.31 ± 0.03 mg ind⁻¹ (m ± SD, n=3)

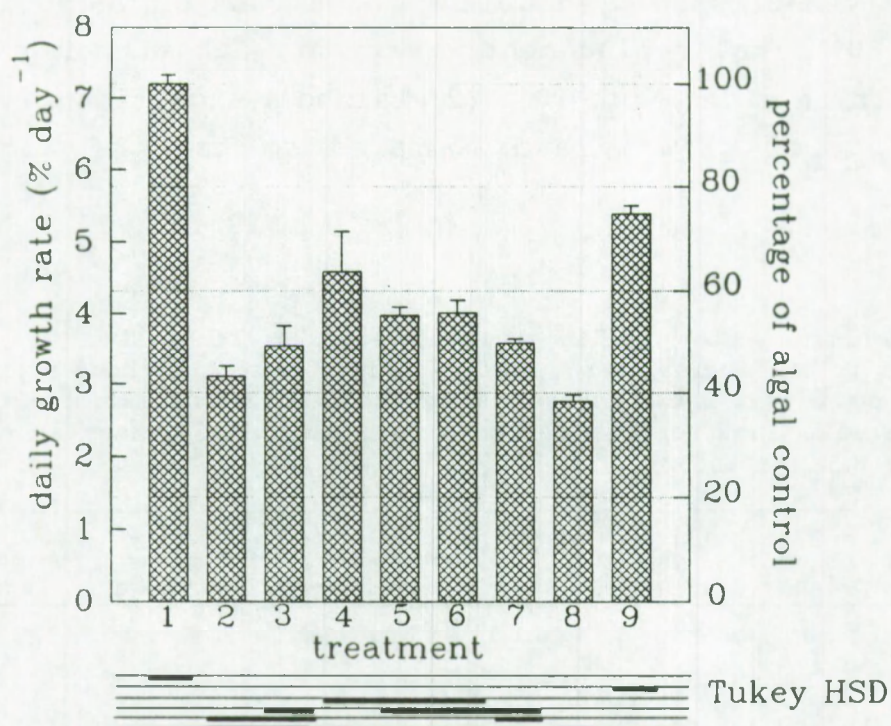


Fig. 57 (Experiment ARC 4): Growth of juvenile *T. philippinarum* fed various mixtures of *C. gracilis* and yeast diets Y1, Y2, Y4, and Y5. Data represent daily growth rate (DGR Σ , mean \pm SD, $n=3$) calculated from the increase of individual live weight over the three week experimental period, and expressed either absolutely (% day⁻¹) or relatively compared to the DGR Σ obtained for the algae-fed controls (%). The various treatments are explained in Table 49. (___) denotes statistically similar groups (Tukey HSD test, $P \leq 0.05$).

IX.4.2.5. Experiment ARC 5: Effect of methodological aspects on the performance of the algal control diet and the yeast diet as a partial substitute

During the first week of the fifth test the seed exhibited a generally depressed growth, which was less pronounced for clams fed the algae derived directly from the culture. Clams fed the concentrated algae grew at a rate of about 30% of that observed during the subsequent weeks, whereas this was about 60% for seed fed *Chaetoceros* cultures (Fig. 58).

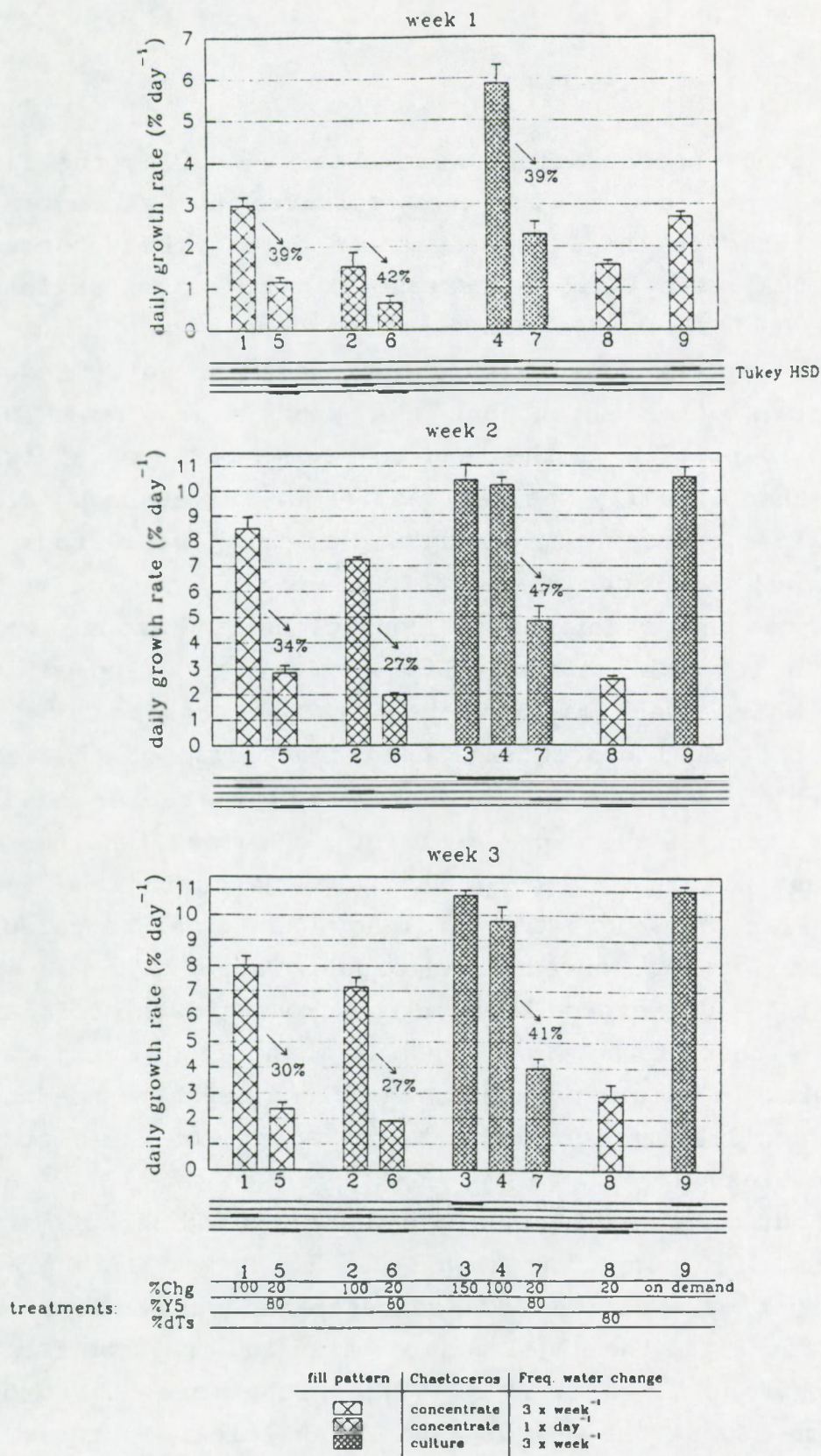


Fig. 58 (Experiment ARC 5): Growth of juvenile *T. philippinarum* fed *C. gracilis* with or without partial replacement by yeast diet Y5 or dried *T. suecica* under various experimental conditions (see also Table 44 for experimental design).

100% algae corresponds with a daily ration of 1% DW WW⁻¹ day⁻¹. Data represent daily growth rate (DGR, mean \pm SD, n=3) calculated from the increase of total biomass per replicate during each week. Percentages above bars represent the growth rate, expressed as a percentage of the DGR obtained for the corresponding algae-fed controls. Initial mean individual live weight = 1.35 \pm 0.05 mg. (—) denotes statistically similar groups (Tukey HSD test, $P \leq 0.05$).

Higher food concentrations were observed during the first week of the experiment in the clam cultures fed concentrated algae as compared to the levels measured during the subsequent weeks (Fig. 59A). The limited decrease of food concentration in this treatment indicated a depressed feeding rate during the initial three to four days, although the average daily removal of algae was similar throughout the experiment (Table 50). However, the adaptation of the feeding regime based on a higher growth rate than actually was realized (*i.e.* 10% assumed *versus* 3% day⁻¹ realized) caused an over-dosing of the algae. Probably, the low growth was due to difficulties of the clams in acclimating to the experimental conditions and food, which resulted in a low utilization efficiency of the filtered food (Table 50). This effect may have been further enhanced by the build-up of the algal concentration in the course of the first week. Feeding *Chaetoceros* cultures resulted in higher feeding rates at the start of the experiment (Fig. 59C) and, whereas the average amount of algae removed by the clams during the first week was similar, gross growth efficiency was higher compared to that of clams fed the concentrated algae (Table 50). It is of interest that the difference between the gross growth efficiency of clams fed either of the algal types is much reduced during the last two weeks. In this experiment, the clams may have needed an additional acclimatization period to adapt to a diet of concentrated algae.

Throughout the experiment, algae that were fed directly from the culture medium supported significantly better clam growth than those that were stored as a concentrated suspension, both in the treatments fed the algal control diet and in those fed the 20/80% algae/yeast mixtures (Fig. 58). Furthermore, the growth rate of clams fed on the 20/80% algae/yeast diet, expressed as a percentage of that of the corresponding algae-fed control, was slightly higher when feeding *Chaetoceros* cultures (39-47%) than when concentrated algae were fed (30-39%).

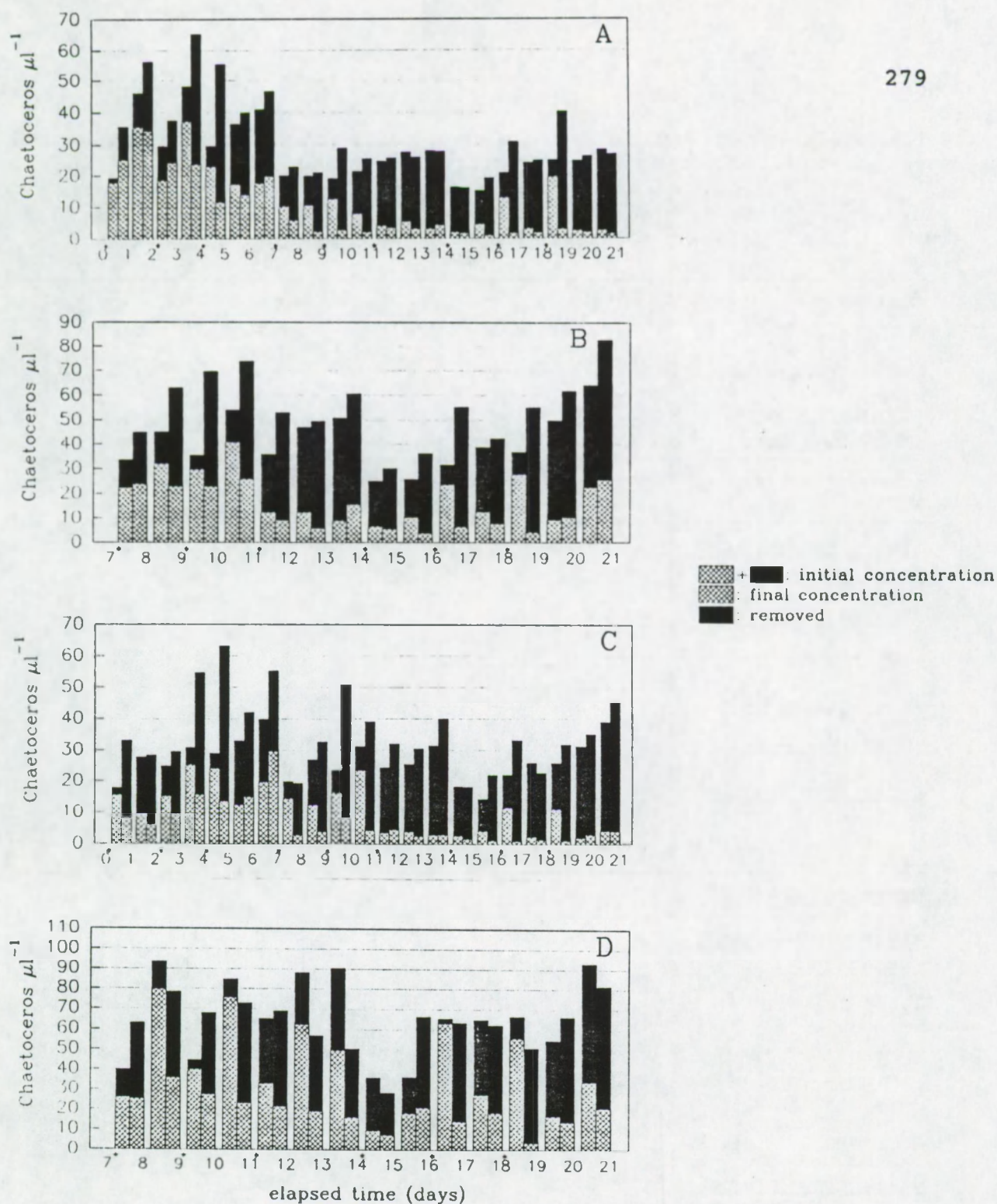


Fig. 59 (Experiment ARC 5): Change of food concentration in cultures of *T. philippinarum* fed various daily rations of *C. gracilis* derived either directly from the culture or from a concentrated stock suspension. Total and lower bar represent algal concentration before and after feeding, respectively. Top bars represent decreases in cell concentration during each time interval between two consecutive feedings. Feeding occurred twice daily ("on demand" treatment occasionally once daily when the food concentration was found to be sufficiently high before feeding).

A: Chg concentrate 1.0%

C: Chg-culture 1.0%

B: Chg culture 1.5%

D: Chg-culture "on demand"

Table 50 (Experiment ARC 5): Mean number of cells removed from suspension and gross growth efficiency in *T. philippinarum* fed various rations of *C. gracilis* (Chg). Data represent mean and standard deviation for week one, and weeks two and three. Unlike superscripts denote significant differences among means (week 2&3: ANOVA, Tukey HSD test, $P \leq 0.05$).

TREATMENT -	Chg conc. 100%	Chg culture 100%	Chg culture 150%	Chg culture "on demand"
FEEDING REGIME - (% DW WW ⁻¹ day ⁻¹)	1%	1%	1.5%	1.73% (average)
<hr/> WEEK 1 <hr/>				
<u>algae removed from suspension</u>				
1. (10 ⁶ g ⁻¹ day ⁻¹)				
mean ± SD (n=7)	346 ± 131	346 ± 61		
min. - max. value	121 - 481	268 - 434		
CV (%)	38	18		
2. (% DW WW ⁻¹ day ⁻¹) [§]	0.82	0.82		
<u>daily growth rate</u> (% day ⁻¹)	2.98	5.88		
<u>GGE</u> [†]	3.6	7.2		
<hr/> WEEKS 2&3 <hr/>				
<u>algae removed from suspension</u>				
1. (10 ⁶ g ⁻¹ day ⁻¹)				
mean ± SD (n=14)	301 ± 25 ^a	342 ± 56 ^a	457 ± 55 ^b	486 ± 87 ^b
min. - max. value	263 - 353	219 - 435	327 - 552	370 - 640
CV (%)	8	16	12	18
2. (% DW WW ⁻¹ day ⁻¹) [§]	0.72	0.81	1.09	1.16
<u>daily growth rate</u> (% day ⁻¹) [†]	8.25	9.98	10.60	10.77
<u>GGE</u> [‡]	11.5	12.3	9.7	9.3

§: *C. gracilis*: 23.8 pg DW cell⁻¹ (Table 41)

†: Daily growth rate for "weeks 2&3" is the average of the mean DGRs for the second and third week of each treatment.

‡: GGE = gross growth efficiency = live weight increase per dry weight of food cleared, estimated as DGR (%WW day⁻¹)/algae removed (%DW day⁻¹)

Increasing the frequency of the water renewal from 3 times week⁻¹ to 1 day⁻¹ yielded decreased growth in the algae-fed controls as well as the 20/80% algae/yeast diets, although this was only significant for the former. This may have been due to the daily handling of the animals and the loss of part of the ration during the water change (Fig. 58).

Replacing 80% of the algal ration by either yeast diet Y5 or dried *T. suecica* did not yield a significantly different growth rate (Fig. 58).

Feeding *Chaetoceros* cultures at daily rations of 1.5% (150% Chg) or 1.73% (effective daily ration for "on demand" fed treatment) resulted in significantly higher growth compared to the controls fed 1% during the last week of the test. This suggests that the ration for maximal growth may be higher than for the concentrated algae (Fig. 58). This was corroborated by the measurements of cell concentration before and after feeding in one replicate of the algae-fed treatments (Fig. 59). The decrease of the food concentration over the time interval between two feedings mainly depended on the duration of the interval and the total biomass present in the system. In this way, the number of cells removed from suspension per unit live weight increased linearly with the time elapsed between two consecutive feedings, as demonstrated in Figure 60 for treatment 3 (150% Chg). However, the average rate of removal per unit time, further referred to as intake rate, was mostly lower during the first time interval (typically 9:00-18:00) than during the second feeding period for the clams fed a daily ration of 1% (Fig. 61A). This was probably due to the depletion of the food during the latter, relatively longer period (Fig. 59C). Feeding a daily ration of 1.5% maintained the food level above 5-10 *Chaetoceros* μl^{-1} throughout the experiment (Fig. 59B) and resulted in higher and more consistent average intake rates (Fig. 61B). As a result, clams fed *Chaetoceros* at a daily ration of 1.5% or more, removed daily a significantly larger amount of algae from suspension than those fed 1% (Table 50). However, the higher filtered ration was less efficiently converted into clam biomass (Table 50) and resulted only during the last week in a significant growth improvement.

The high food loads observed in the treatment fed "on demand", i.e. up to 90 *Chaetoceros* μl^{-1} , affected gross growth efficiency only to a small degree, which further indicates that the low growth observed during the first week was not primarily caused by the accumulation of food during the initial days.

Clams fed the highest ration (1.73% day^{-1} , treatment fed "on demand") removed the largest weight-specific ration of 1.16% DW $\text{WW}^{-1} \text{day}^{-1}$ (Table 50), which was equivalent to 67% of the offered ration. Feeding a daily ration of 1% resulted in a clearance of more than 80% of the food offered.

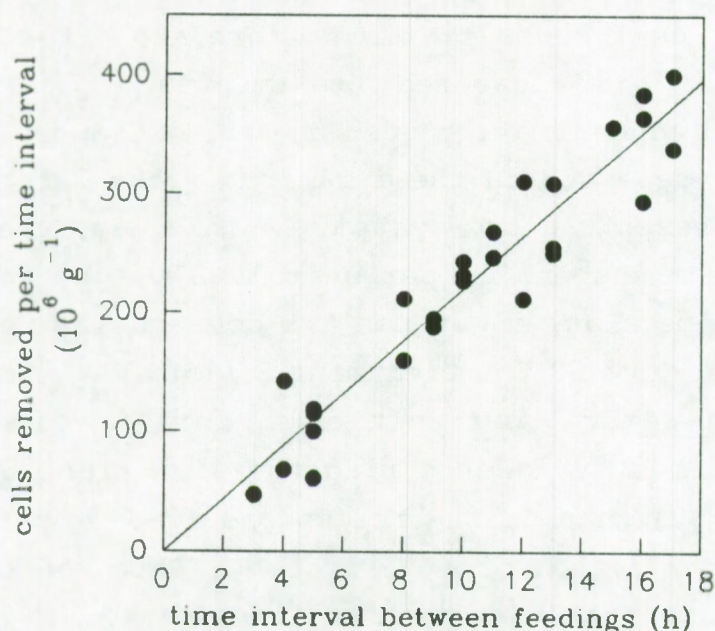


Fig. 60 (Experiment ARC 5): Number of cells cleared per unit live weight during each time interval between two feedings as a function of the elapsed time for *T. philippinarum* fed a daily ration of 1.5% *C. gracilis* culture. Linear regression equation is given by:

$$y [10^6 \text{ cells g}^{-1}] = 22.0 t [\text{h}] \quad (r^2=0.91)$$

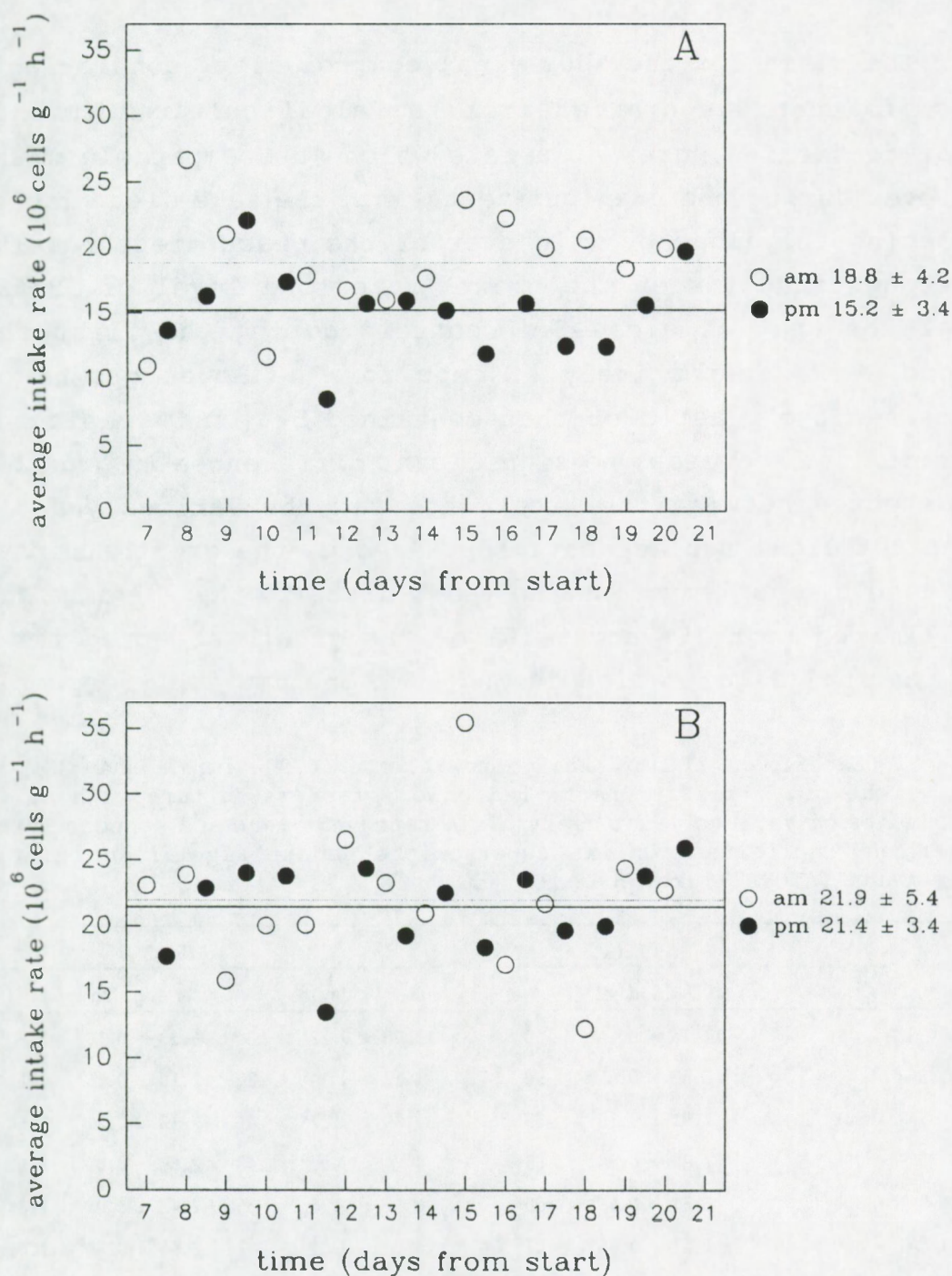


Fig. 61 (Experiment ARC 5): Change of average intake rate for each time interval between two feedings in the course of the second and third week for *T. philippinarum* fed *C. gracilis* culture at a daily ration of 1.0% (A) or 1.5% (B). Lines represent overall mean intake rates (mean \pm SD indicated) for the am (...) and pm (—) feeding periods.

IX.4.2.6. Experiment ARC 6: Various yeast diets as a partial substitute for *C. gracilis* derived directly from the culture

The clams fed the 100% algal control diet, consisting of *C. gracilis* that was drained from the algal cultures immediately prior to feeding, grew at a rate which was comparable with that achieved during the previous tests with the same diet (Table 51). Replacing the algae by whichever of the yeast diets resulted in a strong reduction of the growth rate to a level of 59-69% and 35-42% of that of algae-fed controls during the first and the second week, respectively. Except for the lower growth of the clams fed the yeast diet that contained 5% (on DW basis) of the extract from seaweeds, no significant differences in growth could be detected between the treatments fed the various yeast diets as an 80% algal replacement. Furthermore, the growth improvement achieved by supplementing the yeast diets to the 20% algal ration was limited to 9-19% and 9-16% of the growth observed for clams fed the full algal ration during week one and two, respectively.

Table 51 (Experiment ARC 6): Daily growth rate (DGR), final live (WW) and dry (DW) weight of *T. philippinarum* fed on *C. gracilis* culture with or without supplements of various yeast diets. Data represent mean and standard deviation from three replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD, $P \leq 0.05$).

TREATMENT [§]	WEEK 1		WEEK 2			
	DGR (% day ⁻¹)	%*	DGR (% day ⁻¹)	%*	WW (mg ind ⁻¹)	DW (mg ind ⁻¹)
1) 100% SAR	11.16 ± 0.14 ^a	100	11.86 ± 0.22 ^a	100	5.33 ± 0.10 ^a	2.80 ± 0.10 ^a
2) 20% SAR	5.55 ± 0.55 ^d	50	3.12 ± 0.14 ^c	26	2.10 ± 0.15 ^d	1.14 ± 0.05 ^d
3) 20% SAR + 80% Y1	7.01 ± 0.18 ^{bc}	63	4.89 ± 0.37 ^b	41	2.70 ± 0.27 ^{bc}	1.54 ± 0.18 ^{bc}
4) 20% SAR + 80% Y3	7.34 ± 0.34 ^{bc}	66	4.74 ± 0.30 ^b	40	2.68 ± 0.09 ^{bc}	1.50 ± 0.05 ^{bc}
5) 20% SAR + 80% Y7	6.61 ± 0.15 ^c	59	4.11 ± 0.26 ^b	35	2.42 ± 0.04 ^{cd}	1.34 ± 0.02 ^{cd}
6) 20% SAR + 80% Y6	7.51 ± 0.28 ^b	67	4.90 ± 0.33 ^b	41	2.64 ± 0.04 ^{bc}	1.48 ± 0.04 ^{bc}
7) 20% SAR + 80% [Y3 + K]	7.74 ± 0.18 ^b	69	4.94 ± 0.43 ^b	42	3.05 ± 0.25 ^b	1.71 ± 0.11 ^b
ANOVA, F ₆	106.3		266.3		134.8	88.1

§: standard algal ration: 100% SAR= *Chaetoceros gracilis*, fed at 1% DW WW⁻¹ day⁻¹

Y1, Y3, Y6, Y7: yeast diet (Table 42), K: kaolinite supplemented at same rate as kaolinite component in Y6, i.e. 30% of yeast DW

initial seed: 1.32 ± 0.02 mg ind⁻¹ (m ± SD, n=3)

*: DGR expressed as a percentage of the DGR obtained for the 100% SAR treatment

IX.4.2.7. Experiment ARC 7: Partial replacement of single and mixed diets of *C. gracilis* and *I. galbana* (clone T-iso)

The preliminary series of experiments demonstrated a similar growth response to increasing ration size for *T. philippinarum* (live weight 1.7-6.9 mg) fed either *C. gracilis*, *I. galbana* (clone T-Iso), or a 50/50 mixture (on DW basis) of both species. Growth increased sharply up to a daily ration of 1%, reached a maximum value at 1.3%, and then decreased with a further increase of algal ration (Fig. 62). Because the experiments were performed with clams of different initial size and previous history, the data do not allow a comparison of the nutritional value of the three algal diets.

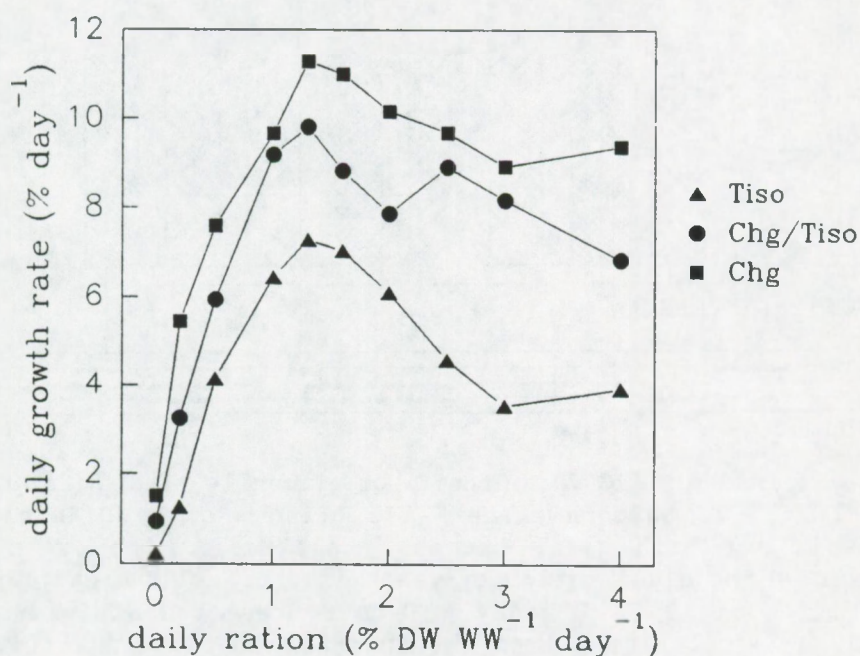


Fig. 62: Daily growth rate as a function of daily ration for *T. philippinarum* fed *C. gracilis* (Chg), *I. galbana* clone T-Iso (Tiso), or a 50/50 mixture (on dry weight basis) of both algal species (Chg/Tiso). Data sets were obtained from independent, one week experiments with one replicate culture per ration. Initial mean individual live weight = 1.73 ± 0.03 mg (Chg), 6.94 ± 0.62 mg (Tiso), 3.92 ± 0.15 mg (Chg/Tiso).

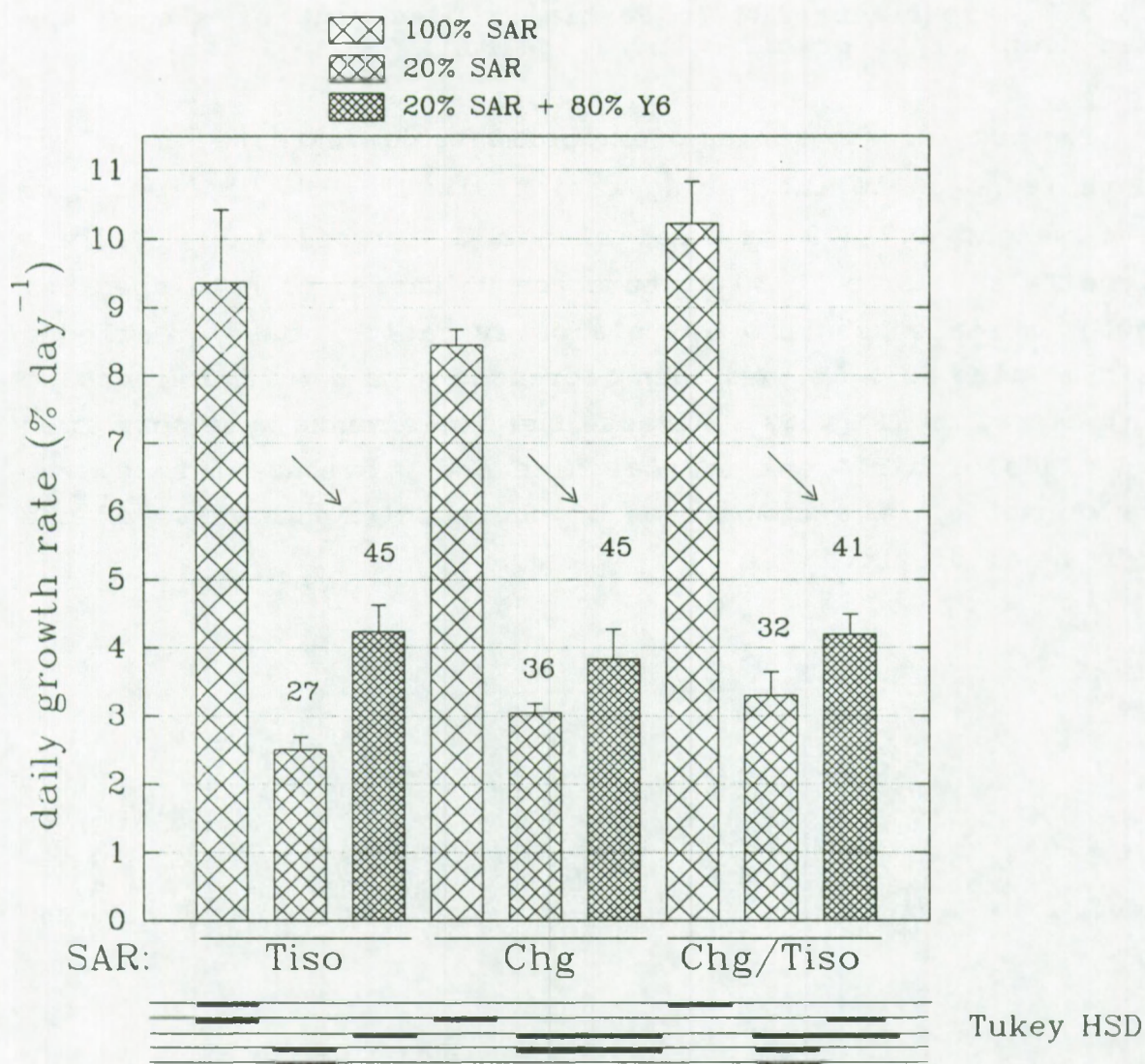


Fig. 63 (Experiment ARC 7): Growth of juvenile *T. philippinarum* fed *C. gracilis* (Chg), *I. galbana* clone T-Iso (Tiso), or a 50/50 mixture (on dry weight basis) of both algal species (Chg/Tiso), with or without partial replacement of the algal ration by yeast diet Y6. 100% algae corresponds with a daily ration of 1% DW WW⁻¹ day⁻¹. Data represent daily growth rate (DGRΣ, mean ± SD, n=3) calculated from the increase of individual live weight over the three week experimental period, and expressed either absolutely (% day⁻¹) or relatively compared to the DGRΣ obtained for the algae-fed controls (%). Initial mean individual live weight = 4.91 ± 0.09 mg. (____) denotes statistically similar groups (Tukey HSD test, P≤0.05).

For the evaluation of the nutritional value of the yeast diet, a ration of 1% day⁻¹ was adopted for the three algal control diets consisting of *C. gracilis* and/or *I. galbana* (clone T-Iso). Clam growth obtained by feeding the algal mixture was significantly higher compared to that of clams fed a sole diet

of *C. gracilis*, but similar to that of the *Isochrysis*-fed controls (Fig. 63). The two single algal diets did not yield a significantly different growth of the clam juveniles. Also, no differences could be found between the clams fed a 20% ration of the different algal diets. The growth improvement due to supplementing the yeast diet to the latter was limited to 9-18% of the growth of the corresponding algae-fed controls, and was only significant for the animals fed *Isochrysis*.

IX.4.3. Discussion

The evaluation of artificial diets as a partial algal substitute for rearing bivalves requires a well-founded knowledge of their quantitative requirements for live algae. One could indeed easily replace part of the algal ration when the full ration is set at too high a level. The present series of experiments demonstrated that the optimal ration of *C. gracilis* for maximal growth of juvenile *T. philippinarum* in the size range of 1 to 40 mg live weight is situated between 1 and 1.5% DW WW⁻¹ day⁻¹. Growth of clams showed a linear response to increasing rations up to 1% day⁻¹, was not significantly affected by rations ranging from 1 to 1.5%, and eventually decreased with a further increase of the ration (Experiments ARC 1,2,5,7). The preliminary test of experiment 7 indicated that growth attained a maximum for *T. philippinarum* fed either *C. gracilis*, *I. galbana* (clone T-Iso), or a mixture of both species, at a daily ration of 1.3%.

In clam cultures fed 1% *Chaetoceros* day⁻¹, the cell concentration was declining rapidly after feeding and was mostly below the incipient limiting level, i.e. about 25 *Chaetoceros* µl⁻¹ (ARC 1). As a result, the clams exhibited fluctuating intake rates in the course of the experiment (Figs. 53, 61A). The higher average food concentration occurring in the cultures that were provided daily rations exceeding 1%, allowed the clams to feed more continuously and maximize their daily food uptake, but also resulted in a lower efficiency of food utilization (ARC 5, Table 50). This is in agreement with the observations for other bivalves showing that growth rate is maximized at higher rations

than gross growth efficiency (Thompson & Bayne, 1974; Goldstein & Roels, 1980; Urban *et al.*, 1983; see III.2.4.4.).

Interesting is that clams fed the concentrated *C. gracilis* at a higher ration than 1% day⁻¹ showed a tendency towards depressed growth (ARC 1 & 2), whereas the animals fed the same alga directly from its culture tended to further increase growth (ARC 5). Apparently, the optimal ration for maximal growth is shifted to lower values for the concentrated algae. This is supported by the lower gross growth efficiency observed for the latter (Table 50).

The periodic feeding activity of the clams fed *Chaetoceros* at 1% day⁻¹ appeared to be mainly imposed by the discontinuous feeding regime which resulted twice daily in a depletion of the food. However, the latter feeding strategy yielded a higher gross growth efficiency and similar growth compared to that of clams which filtered the food more continuously and at higher rates (ARC 5). From this it would appear appropriate to apply discontinuous feeding regimes in aquaculture systems in order to maximize the utilization efficiency of the algal food. In the same way, Epifanio & Ewart (1977) observed a discontinuous feeding activity for *C. virginica* in continuously replenished suspensions of algae and proposed to offer algae in pulses rather than maintaining constant food concentrations. This is further supported by the findings of Langton & McKay (1976) who reported better growth of *C. gigas* spat fed discontinuously than when feeding the same amount of food continuously.

The maximal daily amount of *C. gracilis* dry weight removed from suspension averaged 1.16% of the clam's live weight (Table 50). This is comparable with the weight-specific daily ration removed by *C. virginica* (15 g live weight) which ranged from 0.4% for *T. pseudonana* to 1.5% for *I. galbana*. Nevertheless, it appears difficult to explain why the optimal ration for juvenile *T. philippinarum* was as low as 1 to 1.5% DW WW⁻¹ day⁻¹ in the present laboratory experiments, whereas the standard regime in the trials at the Tinamenor hatchery for seed of the same size and under comparable conditions of temperature and salinity, consisted of 4% DW WW⁻¹ day⁻¹. Also, the scarce literature data

with regard to the effect of ration size on bivalve growth indicate higher values than those observed in the present study (Urban *et al.*, 1983; Enright *et al.*, 1986ab). However, optimal ration differs according to the algal species (Enright *et al.*, 1986a) and may be affected by the algal culture conditions (Enright *et al.*, 1986b). Our data indicate that the optimal ration increases with increasing nutritional value of the algal diet (in ascending order: *Chaetoceros* concentrate, *Chaetoceros* culture, mixed algal diet used in Tinamenor). Furthermore, various methodological factors may affect the value of the estimated daily ration, such as the accuracy of the algal dry weight analysis, the adaptation of the feeding regime to growth during the experiment, the natural food present in the seawater, and the settling and/or growth of the algae in the culture system.

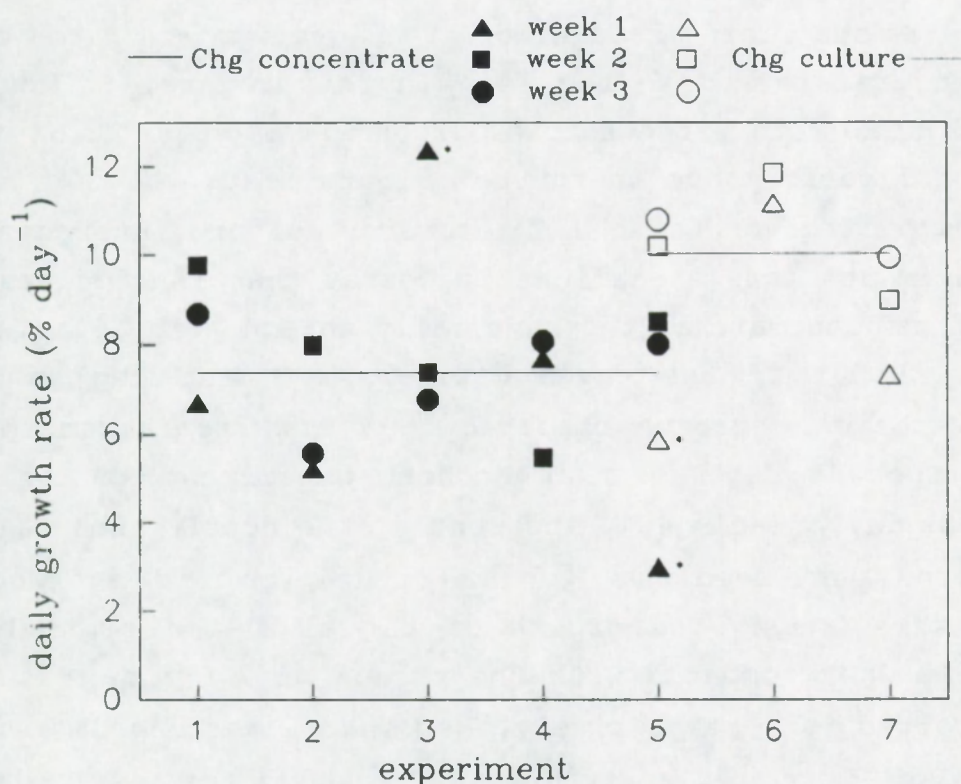


Fig. 64: Daily growth rate of *T. philippinarum* fed *C. gracilis* at a daily ration of 1% in seven independent experiments. Each data point represents the mean daily growth rate of three (four: Experiment ARC 1) replicate cultures for each of the 1-week periods. Lines indicate mean daily growth rate obtained with the concentrate ($7.4 \pm 1.4 \text{ \% day}^{-1}$) and the culture ($10.0 \pm 1.5 \text{ \% day}^{-1}$) of *Chaetoceros*, respectively (mean \pm SD, outliers (*) excluded).

Mean daily growth rate of clams obtained during the various weeks of the seven experiments for clams fed *Chaetoceros* at a daily ration of 1% is presented in Fig. 64. For the five experiments where the concentrated *Chaetoceros* was used, the daily growth rate did not vary significantly according to the week of the experiment (ANOVA, $F_{2,5}=1.204$, $P=0.37$) nor between experiments (ANOVA, $F_{4,10}=0.780$, $P=0.56$). The outlying values observed during the first week of experiments three and five, may have been due to the relatively short acclimatization period prior to the start of the experiment. In this way, a bad initial condition of the spat may have caused difficulties to adapt to the experimental food, resulting in reduced feeding rate and gross growth efficiency (ARC 5). Alternatively, animals that originate from a well-fed population may benefit from their food reserves and initially maintain higher growth rates (ARC 3). Laing & Millican (1986) thus found that greater lipid reserves in *O. edulis* spat were associated with higher growth rates of the seed when transferred to the sea. In this regard, it would be interesting to relate the initial biochemical composition of the seed to its performance in future culture tests.

Growth of clams fed the *Chaetoceros* culture, averaged over all experiments, was significantly better than that of spat fed the algal concentrate at the same daily ration (Fig. 64; t-test: $P<0.05$, $P<0.001$ if the three outliers are excluded) and was matching the mean growth observed in *T. philippinarum* fed the same alga at 4% day⁻¹ in the hatchery experiment at Tinamenor (i.e. 9.8% day⁻¹, Table 43). Apparently, the nutritional value of *Chaetoceros* decreased due to centrifugation and storage for maximal three days. The growth of the algal-fed controls was acceptable when compared with the values of 7.6 % day⁻¹ and 14% day⁻¹ reported for, respectively, *Mercenaria mercenaria* (initial live weight 25 mg) fed a mixture of *T. pseudonana* and *I. galbana* (Urban & Pruder, in press), and *O. edulis* (initial live weight 1.14 mg) fed *C. calcitrans* (Laing & Millican, 1986).

The performance of the yeast diets as a partial substitute in the laboratory cultures was clearly inferior to the results

observed previously in the preliminary test at the Tinamenor hatchery. Replacing 50% of the *Chaetoceros* ration by yeast resulted in a growth rate of 75% to 88% of that of the algae-fed controls (ARC 2, 3, 4), whereas the best relative growth obtained with an 80% replacement amounted to only 64% (ARC 4). This poor result was due to a limited growth improvement resulting from the supplementation of yeast to the insufficient algal rations (e.g. 15 to 20% of the growth of the algae-fed controls) and to a decline of the growth rate in the course of the experiment when feeding mixed algae/yeast diets (e.g. ARC 2, 6). Doubling the amount of the yeast component in the 20/80% and 50/50% algae/yeast diets either did not affect or depressed clam growth (ARC 2, 4). Urban & Langdon (1984) also reported decreased growth of *C. virginica* fed more than the dry weight equivalent of the yeast *Candida utilis* as an 80% replacement, but found best growth for the 50% substitution when doubling the amount of yeast.

The nutritional value of the yeast diet could only be significantly improved by the addition of kaolinite (ARC 4), although this could not be confirmed when *Chaetoceros* was fed directly from its culture (ARC 7). The supplementation of rice starch, fat-soluble vitamins or the seaweed extract did not significantly improve clam growth. By contrast, Urban & Langdon (1984) enhanced growth of oysters fed a 50/50 algae/yeast diet through the addition of kaolinite and any of various non-algal compounds, including rice starch. The incorporation of the extract from seaweeds at a level of 5% of the diet's dry weight resulted in significantly lower growth. Possibly, feeding the extract at high concentrations may have affected the water quality as it is mainly water-soluble and may contain toxic compounds.

Ameliorating the nutritional value of the algal control diet by switching from the *Chaetoceros* concentrate to either the *Chaetoceros* culture or a mixture of the latter and *I. galbana* (clone T-Iso), did not improve relative growth of the clams fed the 20/80% algae/yeast diet (ARC 6, 7). Furthermore, replacing 80% of the *Chaetoceros* culture by spray-dried *T. suecica*, which was proven to be a valuable partial replacement diet for live

algae in the culture of various bivalve species (Laing & Verdugo, 1991; Laing, 1991), yielded similar growth as the yeast diets (ARC 5).

In conclusion, the feeding of algal diets during the present laboratory experiments with *T. philippinarum* yielded growth which was in accordance with earlier findings (IX.3.) and with data reported in literature. However, conflicting results were generated with regard to the value of algal substitution diets. The low nutritional value of yeast diets, irrespective of the supplementation with various non-algal compounds (in contrast with IX.3, 5, & 6; Urban & Langdon, 1984), and of the dried *Tetraselmis* (as opposed to Laing & Verdugo, 1991) suggests that certain methodological aspects of the growth experiment selectively affected the nutritional value of the artificial diets. Various experimental factors were indeed difficult to control and may differ according to the experimental location, such as the biochemical composition of the micro-algae (e.g. effect of culture conditions on HUFA content), the quality of the seawater (e.g. natural food supply, silt load), and the condition of the seed (e.g. derived from an outdoor nursery versus hatchery). Further research is needed to estimate the importance of the latter factors in the performance of artificial diets as an algal substitute for rearing bivalves.

IX.5. LABORATORY EXPERIMENTS WITH *MERCENARIA MERCENARIA* ³

IX.5.1. Rationale and experimental design

For a detailed description of the set-up and methodology used for the growth experiment we refer to IX.2.

The algal control diet consisted of a 50/50% mixture of *Chaetoceros gracilis* and *Isochrysis galbana* (clone T-Iso). During a first experiment (SC 1), the effect of algal ration size, ranging from 0.2 to 1.5% DW WW⁻¹ day⁻¹, on the growth of *M. mercenaria* spat was evaluated. On the basis of the results of the first week, food rations were doubled in all treatments and the unfed treatment was replaced by a 200% SAR treatment during the second week, resulting in a range of 0.4-4% DW WW⁻¹ day⁻¹. For the adjustment of feeding to growth, a growth rate of 10% and 14% (=highest DGR obtained during first week) was assumed for all treatments during the first and second week, respectively. An additional algal control treatment (SAR "on demand") was fed based on fluorescence measurements in order to maintain algal density above a minimal level of 30-40 fluorescence units (for aperture 30x, Turner Fluorometer), which was equivalent to 10-15 *I. galbana* cells µl⁻¹. The latter treatment corresponded to the routinely applied feeding strategy for rearing hard clam juveniles in recirculating nursery systems (Hadley, pers. comm.). Furthermore, two artificial diets (Y1, Y5) were evaluated as partial substitutes of the algal control diet.

The second and third experiment (SC 1 & 2) aimed at confirming the results of the first experiment with regard to, respectively, the optimal algal ration and the substitution of the latter by various yeast-based diets. The diets containing 23% of kaolinite (Y5) or rice starch (Y3) were fed at 1.3 times higher rations in order to feed the additional component as a supplement compared to the Y1 diet.

³parts of this chapter have been published in Coutteau *et al.* (1991)

IX.5.2. Results and discussion

The results and statistical analysis of the three experiments are presented in Tables 52, 53, and 54. The small size of the spat made them very sensitive to drying during the harvesting and weighing. This may explain why the individual live weight data, based on a subsample of the total biomass per tray, occasionally conflicted with data on daily growth rate, which were calculated from the change in weight of the total biomass per seed cylinder. Also, DGR proved to be more sensitive to diet effects than shell length, as demonstrated by the higher F-ratios obtained from one-way analysis of variance for the former parameter (Tables 52, 53, and 54). This is due to the lower specific rate of growth expressed in linear shell dimensions, compared to that based on weight measurements. Thus, clams averaging a daily growth rate of 9.6% WW day⁻¹ increased their shell length only 3.5% day⁻¹ in the same period (Table 54: 100% SAR treatment). Further analysis is mainly based on the daily growth rate data.

IX.5.2.1. Optimal algal ration for growth

The feeding regime was adapted to the growth of the clams by daily increasing the amount of food based on an assumed DGR (see IX.2.3.). For experiment one, the actual weight-specific daily ration was *a posteriori* calculated on the basis of the feeding regime and the realized DGR. This actual daily ration deviated in the course of the week from the target value as a result of the difference between realized and assumed DGR (Fig. 65). For example the 0.2% algal ration increased during the first week gradually to a 0.29% ration due to a realized DGR of only 3.2% day⁻¹ instead of the assumed value of 10% day⁻¹. Nevertheless, the deviations of the weight-specific ration from the initial values were far less than those encountered in the literature for similar experiments. The actual ration of *Tetraselmis suecica* fed to *O. edulis* decreased from 35 to 2% of oyster live weight over a 3-week period (Walne & Spencer, 1974).

Urban *et al.* (1983) observed a decrease of weight-specific ration from 4.6 to 1.9% during each week of their growth experiments with *Crassostrea virginica* juveniles.

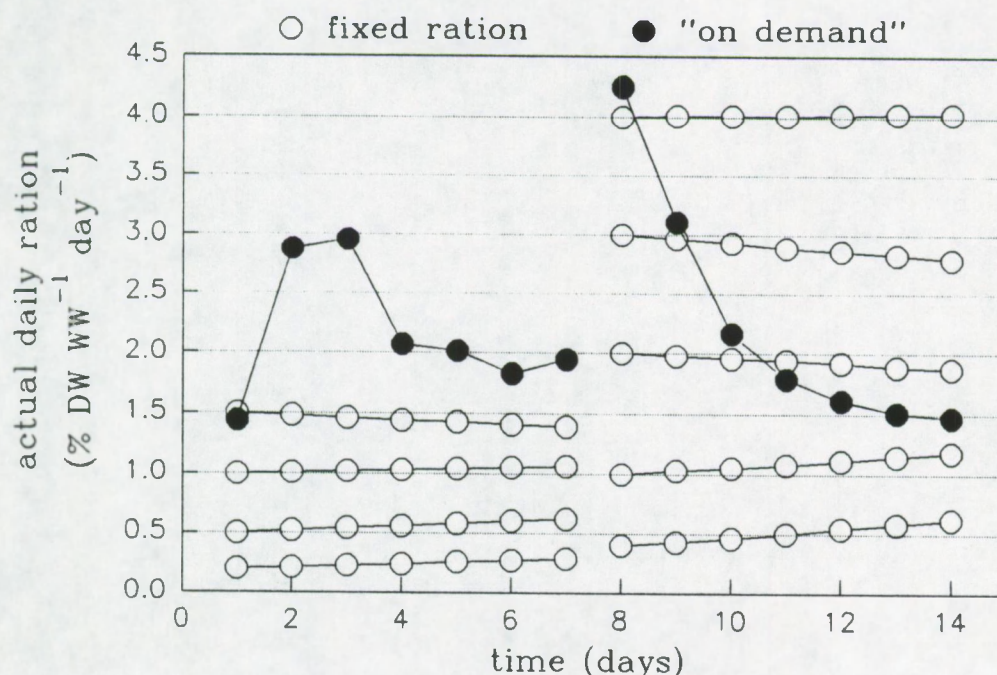


Fig. 65 (Experiment SC 1): Change in actual daily ration over the course of each week of the experiment for *M. mercenaria* fed initial daily rations ranging from 0.2 to 2% and from 0.4 to 4% during the first and second week of the experiment, respectively.

The effective daily ration, which is the arithmetic mean of the actual daily ration for each week of the experiment, was very close to the initially targeted % ration (Fig. 65). During an initial evaluation period, the feeding "on demand" resulted in over-feeding and stabilized around a daily ration of 2% and 1.5% DW WW⁻¹ day⁻¹ during the first and second week, respectively (Fig. 65).

During the first experiment (Table 52) it was shown that spat fed 1% DW WW⁻¹ day⁻¹ (100% SAR, week 1; 50% SAR, week 2) grew significantly slower than those fed higher rations. However, feeding rations higher than 2% DW WW⁻¹ day⁻¹ did not further increase growth rate (150% SAR, week 2) or even resulted in significantly depressed growth rate (200% SAR, week 2). Furthermore, no significant difference in growth rate was found between the seed receiving 2% DW WW⁻¹ day⁻¹ and that fed "on

demand".

Table 52 (Experiment SC 1): Daily growth rate (DGR), individual live weight (WW), and shell length (L) of *M. mercenaria* fed various algal rations with or without yeast supplementation. Feeding regime was increased after first week (see footnote). Data represent mean and standard deviation from three replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD test, $P \leq 0.05$, unless stated otherwise).

TREATMENT [§]	WEEK 1 [†]				WEEK 2 [†]			
	DGR (% day ⁻¹)	% [‡]	WW (mg ind ⁻¹)	L (mm)	DGR (% day ⁻¹)	% [‡]	WW (mg ind ⁻¹)	L (mm)
1) unfed control	1.05 ± 0.29 ^h	12	0.39 ± 0.01	1.11 ± 0.01 ^d	NA	NA	NA	NA
2) SAR "on demand"	13.66 ± 0.16 ^a	154	0.97 ± 0.04	1.51 ± 0.02 ^a	15.80 ± 0.80 ^a	106	3.07 ± 0.18 ^a	2.22 ± 0.04 ^a
3) 200% SAR	NA	NA	NA	NA	13.55 ± 0.09 ^{cd}	91	2.90 ± 0.08 ^a	2.14 ± 0.02 ^{ab}
4) 150% SAR	11.35 ± 0.10 ^b	128	0.88 ± 0.01	1.50 ± 0.03 ^a	15.08 ± 0.68 ^{ab}	101	2.58 ± 0.10 ^b	2.08 ± 0.01 ^{bc}
5) 100% SAR	8.87 ± 0.19 ^c	100	0.77 ± 0.01	1.33 ± 0.03 ^b	14.94 ± 0.21 ^{ab}	100	2.23 ± 0.02 ^c	1.91 ± 0.05 ^d
6) 50% SAR	6.06 ± 0.03 ^f	68	0.61 ± 0.01	1.20 ± 0.01 ^c	10.66 ± 0.40 ^e	71	1.36 ± 0.03 ^f	1.65 ± 0.01 ^e
7) 20% SAR	3.26 ± 0.30 ^g	37	0.51 ± 0.01	1.17 ± 0.02 ^c	5.57 ± 0.39 ^f	37	0.89 ± 0.08 ^g	1.31 ± 0.03 ^f
8) 50% SAR + 50% Y1	7.12 ± 0.27 ^e	80	0.64 ± 0.09	1.32 ± 0.02 ^b	12.58 ± 0.49 ^d	84	1.67 ± 0.03 ^d	1.74 ± 0.04 ^e
9) 50% SAR + 50% Y5	9.21 ± 0.49 ^c	104	0.73 ± 0.01	1.37 ± 0.01 ^b	13.97 ± 0.13 ^{bc}	94	2.08 ± 0.06 ^c	1.96 ± 0.04 ^{cd}
10) 20% SAR + 80% Y1	6.75 ± 0.15 ^e	76	0.65 ± 0.01	1.32 ± 0.03 ^b	10.42 ± 0.07 ^e	70	1.41 ± 0.04 ^{ef}	1.64 ± 0.01 ^e
11) 20% SAR + 80% Y5	8.10 ± 0.28 ^d	91	0.73 ± 0.01	1.36 ± 0.06 ^b	11.14 ± 0.64 ^e	75	1.61 ± 0.09 ^{de}	1.70 ± 0.13 ^e
ANOVA, F_s	531.8		H.D. [♦]	56.6	124.4		196.1	76.8

§: standard algal ration: SAR= *Chaetoceros gracilis*/*Isochrysis galbana*, clone T-Iso (50/50 on DW basis)

Y1, Y5: yeast diets (Table 9.3)

200% SAR treatment was started during second week with seed derived from the SAR "on demand" treatment

†: 100% SAR = 1 % DW algae/WW clams/day

‡: 100% SAR = 2 % DW algae/WW clams/day

*: DGR expressed as percentage of DGR obtained for the 100% SAR treatment

initial seed: WW = 0.398 ± 0.005 mg ind⁻¹, L = 1.13 ± 0.01 mm (group mean ± SD, n=3)

♦: heteroscedastic data, significantly different means separated by / (MCHETV, $P \leq 0.05$): 1/2,3,4,8,9,10; 4/3; 5/2,3,4,8,9,10; 6/2,3,4,5,8,9,10; 8/3; 9/3,4,8; 10/3

Table 53 (Experiment SC 2): Daily growth rate (DGR), individual live weight (WW), and shell length (L) of *M. mercenaria* fed various algal rations. Data represent mean and standard deviation from three replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD test, $P \leq 0.05$).

TREATMENT [§]	WEEK 1			WEEK 2			WEEK 3			
	DGR (% day ⁻¹)	% [*]	WW (mg ind ⁻¹)	DGR (% day ⁻¹)	% [*]	WW (mg ind ⁻¹)	DGR (% day ⁻¹)	% [*]	WW (mg ind ⁻¹)	L (mm)
SAR "on demand"	6.66 ± 0.05 ^a	82	0.68 ± 0.02 ^{ab}	11.24 ± 0.11 ^b	83	1.59 ± 0.12 ^{ab}	12.01 ± 0.65 ^b	85	6.96 ± 1.45 ^a	2.92 ± 0.10 ^a
100% SAR	8.13 ± 1.07 ^a	100	0.71 ± 0.06 ^a	13.58 ± 0.67 ^a	100	1.98 ± 0.29 ^a	14.20 ± 0.49 ^a	100	5.64 ± 0.28 ^{ab}	3.05 ± 0.32 ^a
50% SAR	4.92 ± 0.62 ^b	61	0.61 ± 0.01 ^b	9.03 ± 0.20 ^c	66	1.36 ± 0.15 ^b	10.71 ± 0.42 ^c	75	3.58 ± 1.03 ^b	2.27 ± 0.09 ^b
20% SAR	2.23 ± 0.27 ^c	27	0.72 ± 0.03 ^a	4.45 ± 0.02 ^d	33	0.77 ± 0.05 ^c	5.48 ± 0.26 ^d	39	1.27 ± 0.04 ^c	1.60 ± 0.11 ^c
ANOVA, F _s	83		5.5	911.9		24.0	180.6		49.6	56.2

§: standard algal ration: 100% SAR = *Chaetoceros gracilis*/*Isochrysis galbana*, clone T-Iso (50/50 on DW basis), fed at 2 % DW algae/WW clams/day

*: DGR expressed as percentage of DGR obtained for the 100% SAR treatment

initial seed: WW = 0.500 ± 0.010 mg ind⁻¹, L = 1.16 ± 0.04 mm (group mean ± SD, n=3)

Table 54 (Experiment SC 3): Daily growth rate (DGR), individual live weight (WW), and shell length (L) of *M. mercenaria* fed various algal rations with or without yeast supplementation. Data represent mean and standard deviation from three replicates. Unlike superscripts denote significant differences among means (ANOVA, Tukey HSD test, $P \leq 0.05$).

TREATMENT [§]	WEEK 1				WEEK 2			
	DGR (% day ⁻¹)	% [*]	WW (mg ind ⁻¹)	L (mm)	DGR (% day ⁻¹)	% [*]	WW (mg ind ⁻¹)	L (mm)
100% SAR	11.48 ± 0.34 ^a	100	3.12 ± 0.11 ^a	2.22 ± 0.08 ^{bc}	10.22 ± 0.50 ^a	100	6.24 ± 0.28 ^a	2.96 ± 0.10 ^a
20% SAR	7.43 ± 0.80 ^b	65	2.53 ± 0.08 ^b	2.09 ± 0.08 ^c	5.33 ± 0.32 ^c	52	3.74 ± 0.18 ^c	2.55 ± 0.08 ^c
20% SAR + 80% Y1	10.73 ± 0.36 ^a	93	2.93 ± 0.03 ^a	2.30 ± 0.04 ^{ab}	8.23 ± 0.18 ^b	81	5.17 ± 0.24 ^b	2.71 ± 0.05 ^{bc}
20% SAR + 80% Y2	9.81 ± 0.52 ^a	85	3.21 ± 0.22 ^a	2.39 ± 0.05 ^a	8.46 ± 0.16 ^b	83	5.42 ± 0.09 ^b	2.77 ± 0.05 ^b
20% SAR + 80% Y3	10.84 ± 0.43 ^a	94	3.12 ± 0.01 ^a	2.39 ± 0.03 ^a	8.97 ± 0.20 ^{ab}	88	5.47 ± 0.25 ^b	2.81 ± 0.05 ^{ab}
20% SAR + 80% Y4	10.01 ± 0.58 ^a	87	3.13 ± 0.05 ^a	2.32 ± 0.05 ^{ab}	8.92 ± 0.22 ^{ab}	87	5.56 ± 0.28 ^b	2.84 ± 0.08 ^{ab}
20% SAR + 80% Y5	10.68 ± 0.96 ^a	93	2.92 ± 0.12 ^a	2.22 ± 0.04 ^{bc}	8.63 ± 0.83 ^b	84	5.42 ± 0.11 ^b	2.87 ± 0.05 ^{ab}
ANOVA, F _s	13.9		15.6	11.4	51.7		37.3	11.1

§: standard algal ration: 100% SAR = *Chaetoceros gracilis*/*Isochrysis galbana*, clone T-Iso (50/50 on DW basis), fed at 2 % DW algae/WW clams/day

Y1, Y2, Y3, Y4, Y5: yeast diets (Table 9.3)

*: DGR expressed as percentage of DGR obtained for the 100% SAR treatment

initial seed: WW = 1.741 mg ind⁻¹, L = 1.82 ± 0.20 mm

The fluorescence measurements performed on the culture medium (Fig. 66) confirmed these findings. Feeding more than 2 % DW WW⁻¹ day⁻¹ led to an accumulation of algae in the cultures between the two feedings each day, although the total amount of algae removed by the clams seemed to be higher. Part of the filtered algae may have been rejected as pseudofaeces due to the exposure of the clams to peak concentrations (e.g. up to 6 µg DW/ml for the 200% SAR treatment). Epifanio & Ewart (1977) observed the production of large amounts of pseudofaeces in *Crassostrea virginica* fed algal concentrations exceeding 10 µg ml⁻¹. The optimal feeding regime of 2% DW WW⁻¹ day⁻¹ resulted twice daily in a decrease of food concentration from a peak concentration to a minimal level, which were equivalent to, respectively, 100-130 and 15-25 *I. galbana* cells µl⁻¹ (Fig. 66). The latter ground level approximates that targeted by the "on demand" feeding strategy.

The second experiment was characterized by a general slow growth during the first week (Table 53). Fluorescence measurements revealed that the seed was not capable of efficiently clearing an algal ration of 2% DW WW⁻¹ day⁻¹ during the initial seven to eight days, while during the next two weeks algae were cleared to a similar ground level as in test one. From the second week onwards, growth rate in the 100% SAR treatment did not significantly differ from that found for the equivalent treatment of experiment one, although this was only attained for the 50% and 20% ration during the third week (Table 55). Probably, initial difficulties in adapting to the experimental conditions resulted in depressed feeding rates and consequently reduced growth of the spat.

+ ■ : initial concentration
 ■ : final concentration
 ■ : removed

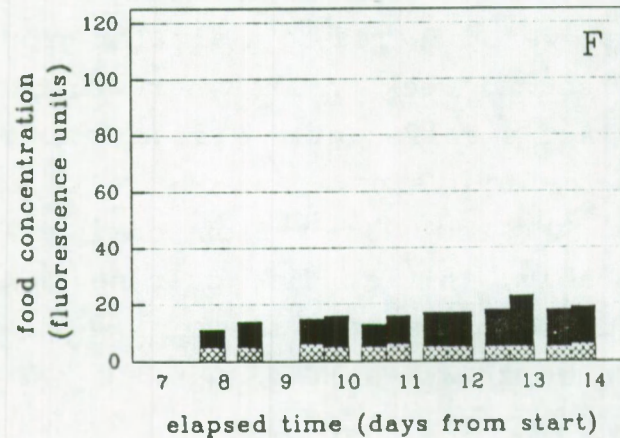
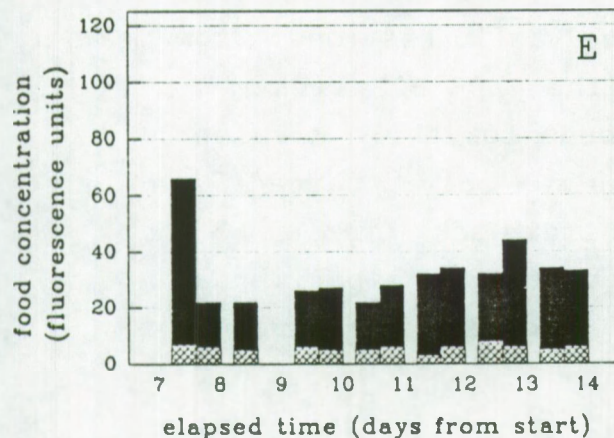
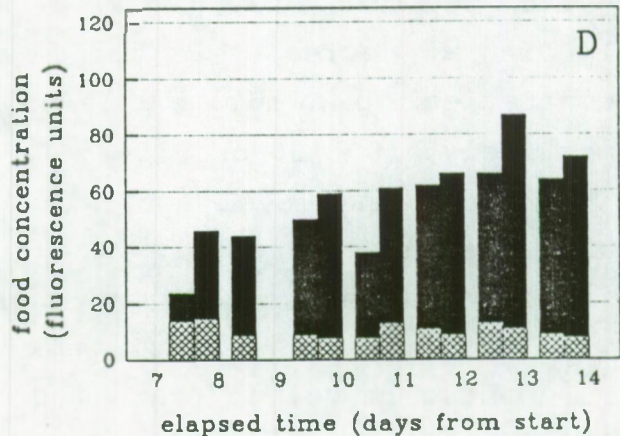
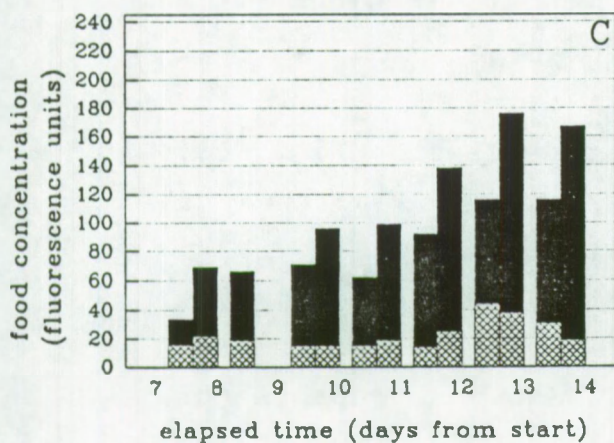
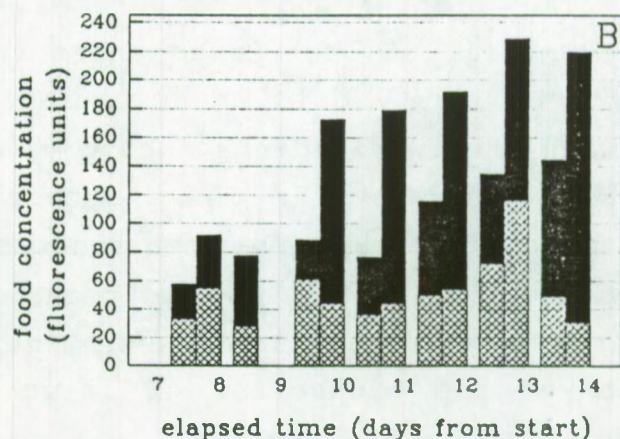
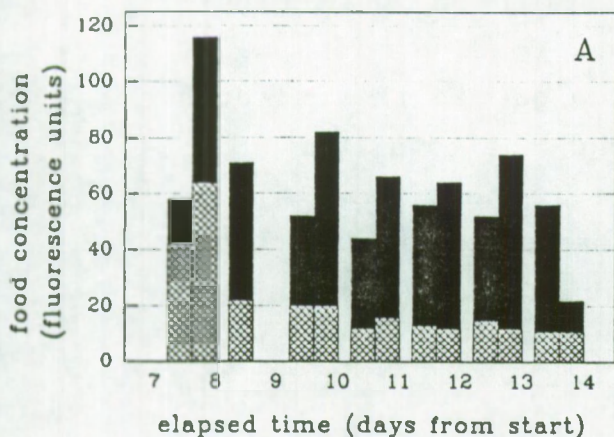


Fig. 66 (Experiment SC 1): Fluctuation of fluorescence during the second week in the cultures of *M. mercenaria* fed various rations of the algal control diet (SAR). Data represent Turner Fluorometer units measured at the 10x aperture or calculated equivalent values when measurement was performed at another aperture. Initial and final concentration of each feeding period are represented in the total bar and the lower bar, respectively. Data are missing when no bar is present.

A: SAR "on demand"

B: 200% SAR (= 4% DW WW⁻¹ day⁻¹)

C: 150% SAR (= 3% DW WW⁻¹ day⁻¹)

D: 100% SAR (= 2% DW WW⁻¹ day⁻¹)

E: 50% SAR (= 1% DW WW⁻¹ day⁻¹)

F: 20% SAR (= 0.4% DW WW⁻¹ day⁻¹)

Table 55: Comparison of growth rates of *M. mercenaria* fed identical algal rations in experiments SC 1 and 2. Unlike superscripts denote significant different means (Tukey HSD test, $P \leq 0.05$).

diet	experiment SC 1	experiment SC 2			ANOVA
	week 2	week 1	week 2	week 3	$F_{3,8}$
100% SAR	14.94 ^a	8.13 ^b	13.58 ^a	14.20 ^a	61.3 ^{***}
50% SAR	10.66 ^a	4.92 ^c	9.03 ^b	10.71 ^a	116.6 ^{***}
20% SAR	5.57 ^a	2.23 ^c	4.45 ^b	5.48 ^a	96.6 ^{***}

***: $P \leq 0.001$

The daily rations reported in this study represent offered rations which do not correspond to ingested rations in the overfed treatments, where part of the algae was discarded during the daily water renewal. Nevertheless, the relationship between the effective daily ration and the daily growth rate of *M. mercenaria* (Fig. 67) was in agreement with the model proposed by Thompson & Bayne (1974, see Chapter III, Fig. 10) relating scope for growth to ingested ration, except that growth was always positive even when no algae were fed to the clams. Live weight increases of unfed oysters and clams have also been observed by several other authors (e.g. Urban *et al.*, 1983; Langdon & Bolton, 1984; Urban & Pruder, in press) and probably result from the build-up of shell material at the expense of stored reserves (Urban, pers. comm., 1991) and the natural food supply in the seawater.

As a result of the high weight-specific growth rates of small seed, it is not unlikely that the ration requirements decrease in the course of the growth experiment. This is corroborated by the shift of the final ration in the treatment fed "on demand" from 2 to 1.5% during experiment one. Furthermore, growth of the clams fed the suboptimal algal rations (50% SAR, 20% SAR) increased relatively compared to that obtained for the seed fed the full ration as the clam live weight at the start of the week was larger (e.g. week 2 versus week 3 of experiment 2, experiment 3 versus experiment 1 and 2).

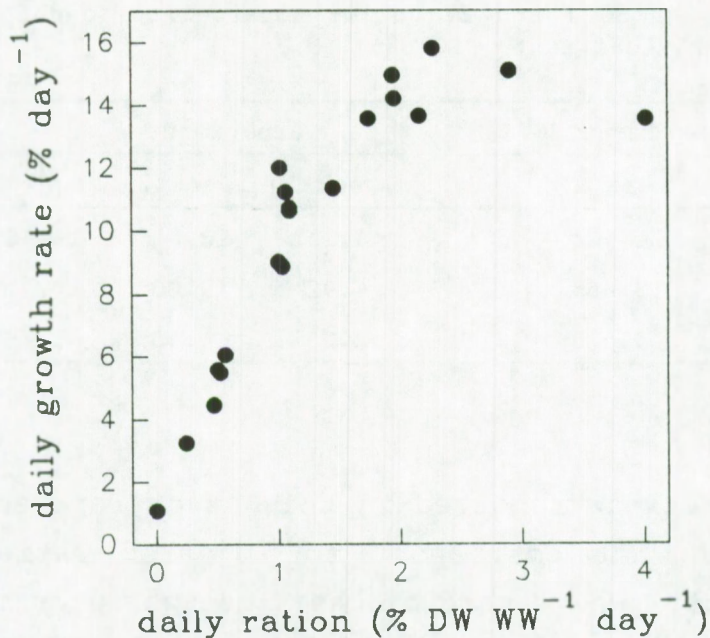


Fig. 67: Relationship between the effective daily ration and the daily growth rate of *M. mercenaria*. Data are derived from the SAR treatments of experiments SC 1 and 2. The aberrant data of the first week of test two are excluded.

Empirical studies of the relationship between ration size and growth of bivalves weighing less than 1 g are very scarce. In particular, information on the quantitative food requirements of postset up to a size of 5 mg live weight is completely lacking, although the latter juvenile stages are the largest consumers of intensively cultured micro-algae in most commercial hatchery operations (Claus, 1981; Manzi & Castagna, 1989; Helm, 1990a). Ease of handling has prompted most researchers to use larger juveniles for nutritional studies and, moreover, empirical work has been mainly restricted to oysters. Several equations have been described relating ration size of *C. virginica* to oyster weight (see III.2.4.3.). However, these formulas are derived from measurements of maximum filtration rates for oysters mainly in the size range of 10-100 g and predict unreasonably high weight-specific rations for oysters weighing less than 10 mg (see Chapter III, Fig. 8). Enright *et al.* (1986b) reported saturation of the growth rate of *O. edulis* (initial live weight 5-25 mg) at a daily adjusted ration between 2.5 and 4.9% DW of

Chaetoceros gracilis. Maximum growth of *C. virginica* (initial live weight 11 mg) occurred when the oysters were fed the highest initial daily ration of 4.6% which was equivalent to an effective daily ration of 2.8% of oyster weight (Urban *et al.*, 1983). The experimental data reported by Urban & Pruder (*in press*) showed a linear growth response in *M. mercenaria* juveniles (initial WW 25 mg) to increased ration from 0 to 1% initial ration. The latter authors did not provide the actual daily rations, which should have been lower because feeding was adjusted only on a weekly basis.

The optimal daily ration of 1.5 to 2% DW WW⁻¹ day⁻¹ for *M. mercenaria* seed (in the size range of 0.4 to 7 mg live weight) fed a mixture of *C. gracilis* and *I. galbana* (clone T-Iso), is close to the value found for *T. philippinarum* fed on the same algal species either solely or as a mixture, *i.e.* 1.3% (see IX.4.2.7.), but lower than most of the values reported in the literature. This may be due to feeding constant weight-specific rations throughout the experiment, which allowed an accurate estimation of the effective daily ration. Alternatively, the optimal ration size might be affected by culture conditions influencing the efficiency with which the food is utilized, such as concentration and quality of the algae, and clam stocking density and condition (see also IX.4.3.).

IX.5.2.2. Yeast-based diets as a partial algal substitute

Results

Supplementing the yeast products always significantly improved the growth rate of *M. mercenaria* fed insufficient algal rations (Tables 52 and 54). During the first experiment, the results obtained for the Y5 product were significantly better than those obtained with the Y1 diet, except for the 80% substitution treatment during the second week. Replacing 50% of the algal ration by Y5 did not result in a significant drop in growth rate relative to the algae-fed controls, and yielded at the end of the experiment clams of the equivalent live weight and

shell length as the algal control treatment. However, the growth rate decreased significantly when 80% of the algae were substituted by the yeast diet. Furthermore, the 20/80% algae/Y5 diet showed a decrease of growth rate during the experiment, relative to the algal control, from 91% in the first week to 75% in the second week (Table 52).

Experiment three did not reveal any significant differences in nutritional value of the various yeast diets which were fed as an 80% algal replacement. During the first week, growth rate of the clams fed the 20/80% algae/yeast diets ranged from 85 to 94% of that obtained for the algae-fed controls and did not differ significantly from the latter. In the subsequent week, growth rates were between 81 and 88% of that measured in the 100% SAR treatment, which was significantly lower for three out of five 20/80% algae/yeast diets. The 12 to 19% difference in growth rate resulted at the end of the experiment in clams with a significantly smaller live weight compared to the seed fed the full algal ration (Table 54).

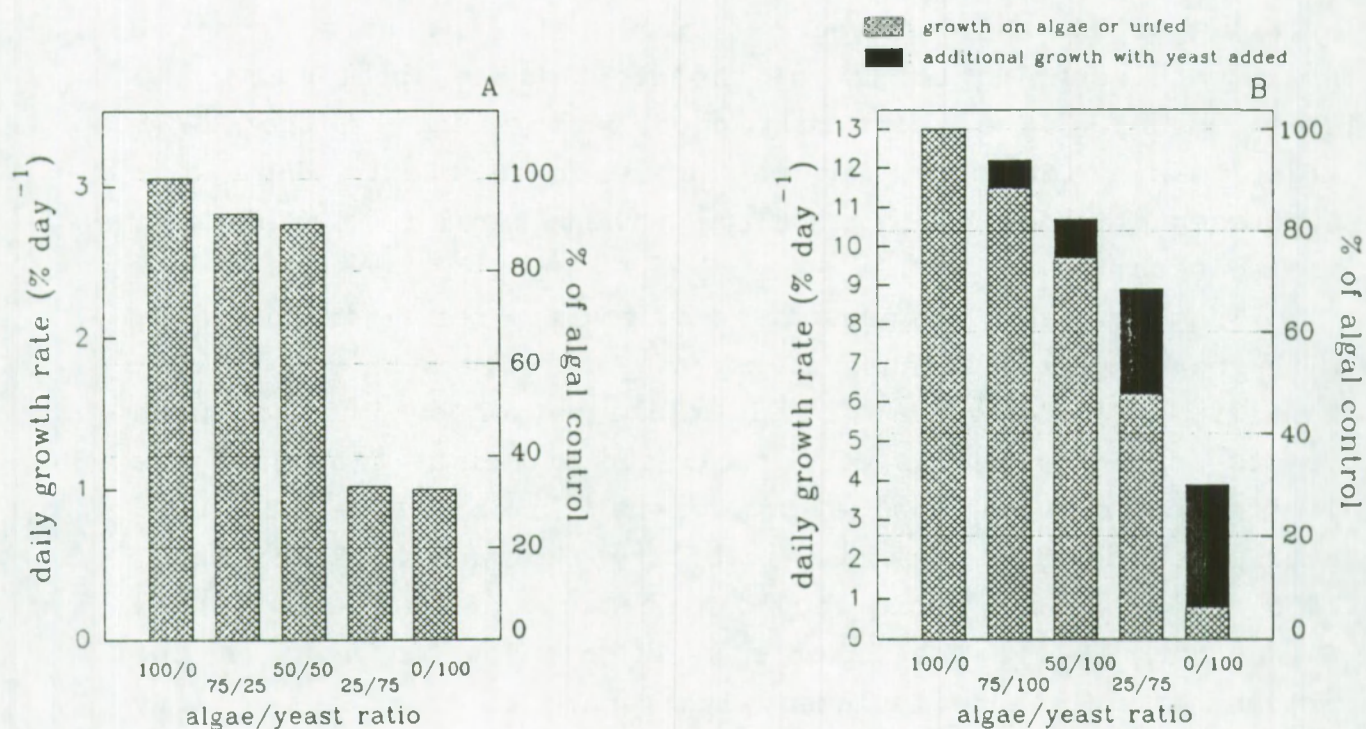
Discussion

Few studies have been published on the use of yeasts as algal substitutes for feeding bivalves (see III.4.4.). About a decade ago research at the University of Delaware (USA) demonstrated for several bivalves that up to 50% of the algal ration could be substituted by spray-dried feed yeast (*Candida utilis*).

Epifanio (1979a) found that a diet consisting of 50% *C. utilis* and 50% algae supported growth comparable to a 100% algal ration when fed to either juvenile *Argopecten irradians*, *Mercenaria mercenaria*, or *Mytilus edulis*, but was deficient for the American oyster *Crassostrea virginica*. The latter was confirmed by Urban & Langdon (1984), who could improve growth in *C. virginica* fed the 50/50% algae/yeast diet by supplementing large amounts of rice starch and kaolinite to the cultures (on DW basis equivalent to, respectively, 2x and 15x the 50% algal ration). Conversely, Alatalo (1980) could not detect a

significant difference in the increase of dry tissue weight between juvenile *C. virginica* fed either a sole diet of *Isochrysis galbana* or a 50/50 mixture of this alga with yeast. Nevertheless, the above studies showed a strongly reduced growth of bivalve seed when more than 50% of the algae were substituted by the yeast (Epifanio, 1979a; Alatalo, 1980; Urban & Langdon, 1984; Fig. 68AB). Only recently, Urban & Pruder (in press) reported for *M. mercenaria* a growth rate of up to 82% of that obtained for the algal control when replacing 75% of the algal ration with *Torula* yeast. However, the latter authors observed over a period of four weeks a growth in the unfed control treatment amounting to 37% of the growth rate obtained in the algal control. The relatively good performance of the 25/75 algae/yeast diet thus may be at least partially due to a supplementation of nutrients through the natural food present in the seawater.

The results of the present study confirm the literature data with regard to the successful use of yeast diets as a 50% algal substitute, but showed a considerable better performance with yeasts at higher levels of partial substitution (Fig. 68). The limited nutritional value of yeasts for bivalves has been attributed to their low digestibility (Epifanio, 1979a; Nell, 1985) as well as a deficiency or imbalance of nutrients (Urban & Langdon, 1984). The baker's yeast preparation used in the present study was manipulated with regard to its digestibility by means of a chemical treatment which improved the yeast's digestibility for the brine shrimp *Artemia* (see Chapters IV & VI). The bivalve's stomach and digestive diverticula are well equipped for the digestion of algal carbohydrates by the presence of various carbohydrases, including chitinase and laminarinase (Reid, 1983). However, the latter enzymes are not necessarily appropriate for an efficient digestion of the polysaccharides composing the cell wall of intact yeast cells (see IV.3.1.). This is further supported by Portères (1988) who observed through transmission electron microscopy that yeast is preferentially lysed in the digestive system of *T. philippinarum* juveniles when in multiplication phase, and found that yeast was assimilated at an efficiency as low as 30%.



1- Fig. 68: Comparison of the results from the present study with reported data on the use of yeast as an algal substitute for rearing bivalve juveniles. Data represent daily growth rates calculated from the increase of individual live weight (B, C, D) or dry meat weight (A) over the total experimental period. Growth rates are expressed as a percentage of the growth rate observed in the algal control treatment (100/0% algae/yeast).

A: (modified from Epifanio, 1979a)

M. mercenaria (10 mg initial dry meat weight), alga= *Thalassiosira pseudonana*, clone 3H, yeast= *Candida utilis*, 28 days.

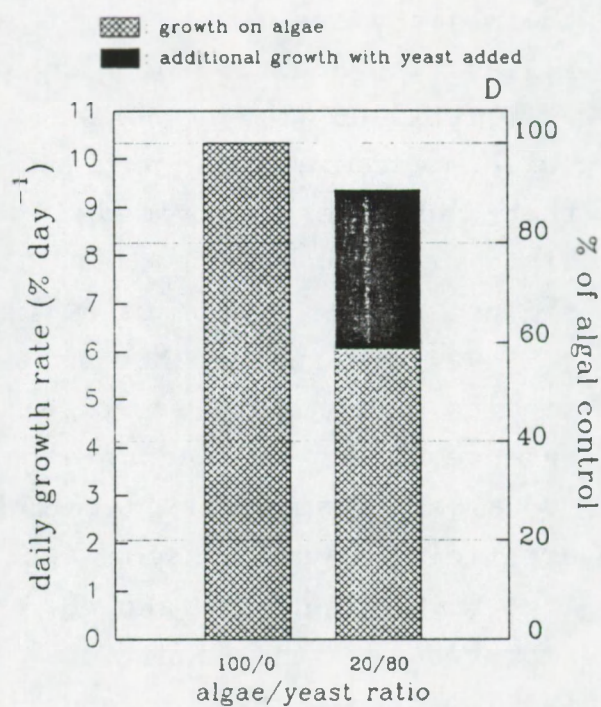
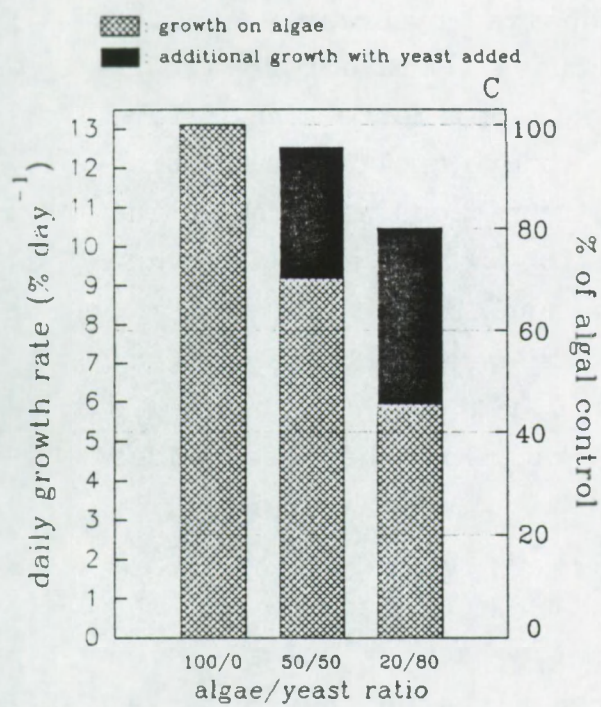
B: (modified from Urban & Langdon, 1984; selection of algae/yeast ratios supporting best growth)

Crassostrea virginica (11.3 mg initial live weight), 100% algae= 2.3% DW WW⁻¹ day⁻¹ *Thalassiosira pseudonana*, clone 3H/ *Isochrysis galbana*, clone T-Iso (50/50 DW), yeast= *Candida utilis*, 21 days.

C, D: best results obtained in the present study

C: experiment 1: yeast=Y5

D: experiment 2: yeast=Y4



The general lack of information on nutritional requirements in bivalves (see III.3.2.) makes it difficult to evaluate the nutritional quality of yeast. The gross composition of yeast does not seem to offer an explanation for its low nutritional value (Epifanio, 1979a). In the present study, (n-3) HUFAs, which are considered essential for *C. gigas* juveniles (Langdon & Waldock, 1981), were present in high levels in the yeast diets and may have enhanced growth on the mixed diets of algae and yeast. Furthermore, the high dietary lipid content of the yeast diets may have compensated for the deficiency in available calories of the algae/yeast diets, which was hypothesized by Urban & Langdon (1984). The addition of rice starch, however, did not result in a significant growth increase. Also, the supplementation of fat-soluble vitamins did not significantly improve growth, suggesting that these were provided in satisfactory amounts through the lipid fraction of the diet. The inclusion of the algal extract did not affect growth of *M. mercenaria* juveniles.

Various authors have shown that suspended silt may affect feeding and growth in bivalves (see III.2.6.). In order to enhance growth in feeding experiments, kaolinite has been added to algal suspensions (Enright *et al.*, 1986a) as well as artificial diets (Langdon & Bolton, 1984, Urban & Langdon, 1984). The latter authors showed that growth of *C. virginica* was improved by the addition of kaolinite to an algal diet supplemented with yeast and rice starch, although they observed the contrary when the silt was added to the algae/yeast diet alone. The significant improvement of growth in the first experiment by the addition of kaolinite to the yeast diet could not be reproduced in experiment three. Possibly, the expression of a positive effect from the kaolinite on growth depended on experimental parameters which were difficult to control, such as the initial silt load of the seawater.

IX.6. VERIFICATION EXPERIMENTS WITH *TAPES PHILIPPINARUM* AND *CRASSOSTREA GIGAS* AT GUERNSEY SEA FARMS LTD., UK

The limited success obtained with the replacement of live algae by artificial diets in the laboratory tests with *T. philippinarum* urged another set of experiments under conditions which resemble more those encountered in a hatchery. In addition to recirculation experiments with a similar set-up as the previous tests, a preliminary test was performed with a flow-through culture system.

IX.6.1. Recirculation tests with *T. philippinarum* and *C. gigas*

IX.6.1.1. Experimental design

For a detailed description of set-up and methodology used in the growth experiment we refer to IX.2. The algal control diet consisted of a 50/50 mixture (on DW basis) of *Tetraselmis suecica* and *Skeletonema costatum* (experiment GSF 1: *T. philippinarum*) or a monodiet of the latter species (experiment GSF 2: *C. gigas*). Based on the standard feeding regime that was applied at Guernsey Sea Farms for hatchery rearing of clam and oyster spat, the initial daily ration was set to 2% organic dry weight (ODW) per seed live weight. The weight per seed cylinder was determined and adjusted to the starting weight every 7 to 9 days (GSF 1: 8+8+9 days, GSF 2: 7+8+9 days). These periods are referred to as weeks one to three.

In the clam experiment (GSF 1), 80% of the algal ration was replaced by either an equivalent ODW of yeast diet Y6 (see Table 42) or twice the latter amount, and by spray-dried *Tetraselmis suecica*. The second test (GSF 2) compared growth of oyster seed fed a diet containing 20% live *S. costatum* and either 80% yeast diet Y6 or dried *Cyclotella cryptica* to that of algae-fed controls.

The number of tanks limited the size of the test. Because the experiment was performed with only two replicates per treatment, the data were not treated statistically.

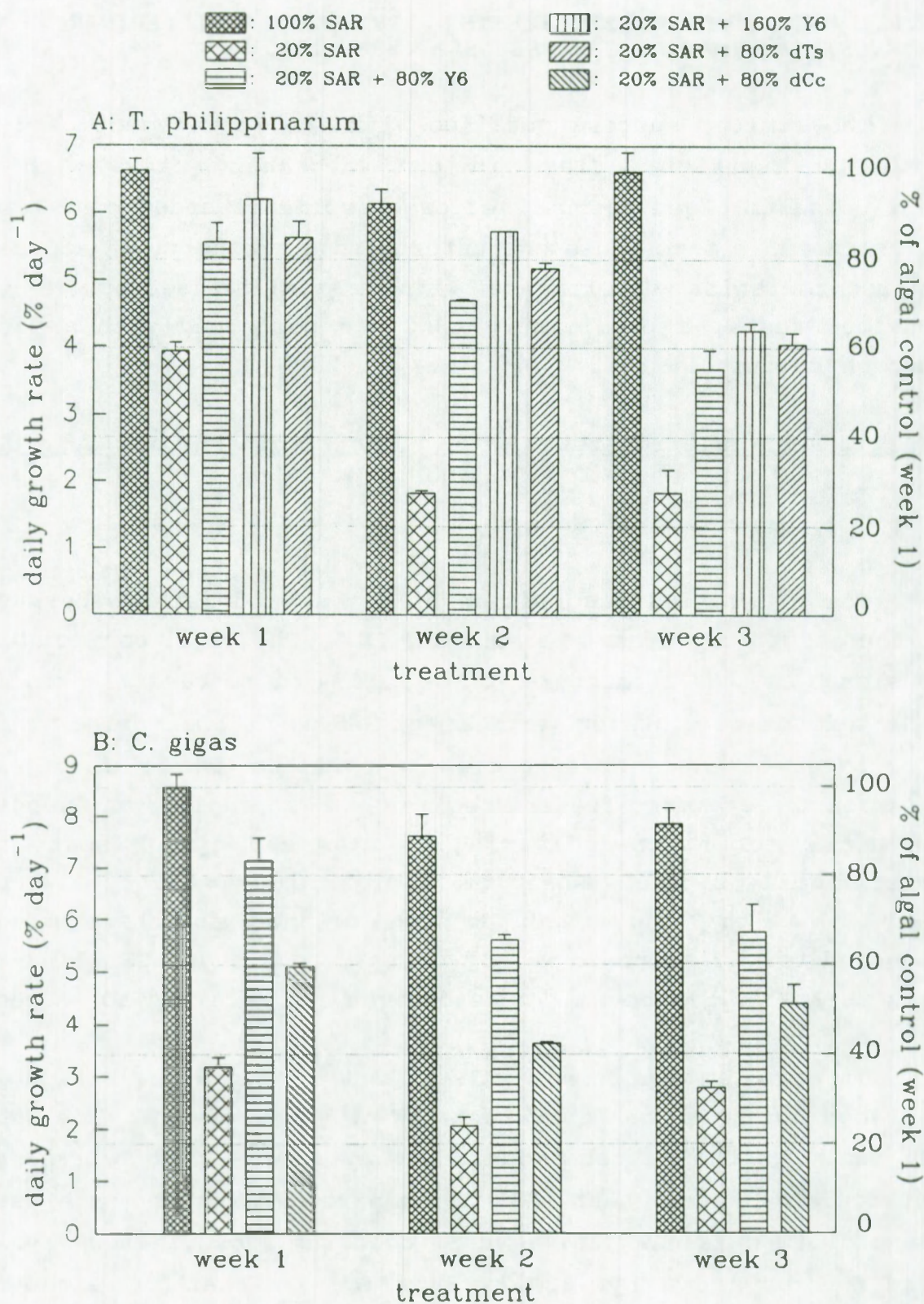


Fig. 69 (Experiments GSF 1&2): Daily growth rate of *T. philippinarum* (A) and *C. gigas* (B) calculated from the weekly determination of total biomass per seed cylinder. Data represent mean and standard deviation of 2 replicates.

IX.6.1.2. Results

The daily growth rate was calculated from the weekly determination of total wet weight per seed cylinder (Fig. 69 AB). At the end of the clam experiment, live and dry weight was determined, which allowed the computation of a global daily growth rate (DGRE) from the increase of individual live weight over the total culture period (Table 56).

The clam seed contained about 5% dead shells at the start of the experiment. Because mortality did not increase during the test (Table 56), it had probably occurred in an earlier stage of the seed production.

Table 56 (Experiment GSF 1): Individual wet (WW) and dry (DW) weight, and daily growth rate (DGRE) calculated from initial and final individual wet weight of *T. philippinarum* after 25 days of culture. Data represent mean and standard deviation of two replicates.

TREATMENT [§]	WW (mg ind ⁻¹)	DW (mg ind ⁻¹)	mortality (%)	DGRE (% day ⁻¹)	%*
100% SAR	15.81 ± 1.10	8.98 ± 0.58	2.0 ± 0.4	7.23 ± 0.30	100
20% SAR	6.14 ± 0.39	3.64 ± 0.26	4.1 ± 1.1	3.25 ± 0.26	45
20% SAR + 80% Y6	10.22 ± 0.01	6.02 ± 0.01	3.0 ± 0.3	5.37 ± 0.01	74
20% SAR + 160% Y6	11.20 ± 0.03	6.58 ± 0.08	3.2 ± 0.9	5.76 ± 0.01	80
20% SAR + 80% dTs	10.77 ± 1.03	6.19 ± 0.65	2.0 ± 0.9	5.59 ± 0.40	77

§: standard algal ration: 100% SAR = *T. suecica*/*S. costatum* (50/50 on ODW) fed initially at 2% ODW WW⁻¹ day⁻¹

Y6: Yeast diet (Table 42), dTs: spray-dried *T. suecica*

initial seed: WW= 2.76 ± 0.15 mg ind⁻¹ (mean ± SD, n=3)

*: DGRE expressed as a percentage of that observed for the 100% SAR treatment

The growth of the clam and oyster seed fed the full algal ration proved to be nearly constant throughout the experimental period. By contrast, clam growth decreased after the first week in the 20% SAR treatment from 60% to 30% of the growth measured in the 100% algal control. The growth of the clams fed the 80% replacement diets, although constant during the first 15 days, showed a similar relative decrease during the final week of the test. Replacing 80% of the algae with more than the ODW equivalent of yeast supported consistently better growth than replacement on ODW basis by either yeast or dried *T. suecica* (Fig. 69A). Nevertheless, the final live and dry weight, and the

derived DGRΣ did not differ between clams fed either of the three supplements (Table 56).

The *C. gigas* spat fed the 20/80% algae/yeast diet showed after the first week a less pronounced drop in growth rate than the clam seed. The yeast diet supported better oyster growth as an 80% replacement of *S. costatum* than the dried *Cyclotella cryptica*, which may be due to the fast settling of the latter food particles (Fig. 69B).

IX.6.1.3. Discussion

The present results demonstrate that retarded growth due to low algal rations could be partially compensated by supplementing with artificial diets. Supplementing yeast diet Y6 improved growth of juvenile clams and oysters fed 20% of the algal ration from 30-40% to 70-80% of that observed for the 100% algae-fed controls over a period of three weeks. This relative growth increase was comparable with that achieved for *M. mercenaria* (IX.5.), and higher than the 15-20% observed during the laboratory experiments with *T. philippinarum* conducted at the Laboratory of Aquaculture (IX.4.). The present results, obtained in a similar set-up as that used for the ARC experiments with *T. philippinarum*, support the hypothesis that culture conditions affect the relative performance of artificial diets as an algal substitute for bivalves.

The performance of the yeast diet Y6 as an 80% algal substitute was at least comparable with that of dried *T. suecica* and considerably better than that of dried *C. cryptica*. Spray-dried *T. suecica* has been reported as a useful substitute for live algae for a range of bivalve species (Laing, 1989; Helm & Hancock, 1990; Helm, 1990a; Laing & Verdugo, 1991), whereas only recently dried *C. cryptica* has been used to supplement live feeds in the culture of *T. philippinarum* spat (Laing, 1991). The above authors showed that growth of bivalve seed fed solely on dried *T. suecica* is comparable to that of spat fed live, light-grown *Tetraselmis*, although growth is greatly improved by a supplement

of certain species of live algae, such as *Chaetoceros calcitrans* (Laing & Verdugo, 1991) and *S. costatum* (Laing, 1991). In this regard, our results conflict with those of Laing (1991), who found that a mixture of 90% dried *T. suecica* and only 10% live *S. costatum* provided similar growth of Manila clam juveniles as a mixture of live algae (*T. suecica*/*S. costatum*, 70:30). Possibly, this is due to differences in the methodology of the growth experiment, in particular with regard to the feeding strategy. In the present tests, a fixed ration was fed throughout the experiment, resulting in an initial weight-specific daily ration of 2% (ODW food WW^{-1}). Furthermore, as a result of the weekly adjustment of seed biomass, the actual weight-specific ration decreased during each week of the experiment (e.g. to 1.2% in the clam test). Conversely, Laing and co-workers provided food based on monitoring daily cell clearance and fed spat so that between 25% and 75% of the algae remained uneaten after 24 h (Laing & Millican, 1986; Laing & Verdugo, 1991). The latter feeding strategy may cause a saturated growth response in the algal control treatment and thus result in a hidden overdosing of the live algae in the treatments fed the replacement diets.

The decline of growth rate in *T. philippinarum* after 16 days of feeding on the 20/80% algae/yeast diet needs to be further investigated. To a lesser extent, this has also been observed with *C. gigas* and in some of the laboratory tests with *T. philippinarum* (IX.4.) and *M. mercenaria* (IX.5.). It may indicate that a 20/80% algae/yeast diet can maintain an acceptable growth only for a limited time period, after which nutritional deficiencies become growth limiting. The expression of these deficiencies may depend on experimental conditions, such as the initial condition of the seed, the natural food supply in the seawater and the composition of the algae. This would explain the varying degree with which the time-dependent decrease of growth occurred in the different experiments (e.g. negligible in the test at Tinamenor).

IX.6.2. Preliminary flow-through test with *C. gigas*

IX.6.2.1. Experimental design

The purpose of this preliminary experiment (GSF 3) was to evaluate the nutritional value of algal substitutes in a flow-through culture system that is routinely used in commercial indoor rearing of clams and oysters up to a size of about 2 mm. The experiment was designed (see IX.2.) in such a way that the normal continuous flow conditions were simulated for a relatively small quantity of seed, i.e. 70 g per bottle.

Seed of the Pacific oyster *Crassostrea gigas* originated from the winter stocks of GSF's nursery. At the start of the experiment water temperature in the outdoor nursery was 8-9 °C and food concentration was extremely low. The seed (10 mg unit wet weight, mortality < 3%) were acclimatized to the experimental conditions for three days in an upwelling table (per ± 0.5 kg of seed: flow rate of 1-2 l min⁻¹, fed *ad libitum*, 20 °C).

Five treatments, corresponding with those of the recirculation test with *T. philippinarum*, were run with two replicates each (Table 57). The algal control was fed a mixture of *T. suecica* and *S. costatum* at a constant concentration of 50 *I. galbana* (clone T-iso) equivalents µl⁻¹ (Table 58), and flow rate of 1 l min⁻¹. The algae were replaced by the artificial diets on an equivalent organic dry weight (ODW) basis, except for treatment 20/160% SAR/Y6 treatment which received a double dose of the yeast diet. Difficulties with the peristaltic pumps resulted in considerable fluctuations of the food dosing at the inflow and, although the mean actual daily rations approximated the targeted values for the replacement diets, the live algae were about 20% overdosed (Table 57). The total biomass was weighed and reduced to 70 g per bottle after 6 days of culture and the experiment was terminated after 13 days.

Table 57 (Experiment GSF 3): Experimental design for the flow-through test with *C. gigas*.

Treatment	Target food concentration in the water inflow (μl^{-1})	Daily ration (g ODW ⁻¹ bottle ⁻¹ day ⁻¹)					
		Algae		Artificial diets		Total	
		Target	Actual*	Target	Actual*	Target	Actual*
100% SAR	50 Tiso-EQ	1.07	1.31 \pm 0.22			1.07	1.31
20% SAR	10 Tiso-EQ	0.21	0.26 \pm 0.04			0.21	0.26
20% SAR + 80% Y6	10 Tiso-EQ + 0.78 10^{-9} g Y6	0.21	0.26 \pm 0.04	0.86	0.76 \pm 0.22	1.07	1.02
20% SAR + 80% dTs	10 Tiso-EQ + 0.66 10^{-9} g dTs	0.21	0.26 \pm 0.04	0.86	0.84 \pm 0.40	1.07	1.10
20% SAR + 160% Y6	10 Tiso-EQ + 1.56 10^{-9} g Y6	0.21	0.26 \pm 0.04	1.71	1.96 \pm 0.53	1.92	2.22

*: mean actual daily ration calculated from frequent measurements of food flow rates during the experiment (mean \pm SD from 17 observations)

Table 58 (Experiment GSF 3): Composition of the algal control for the flow-through test with *C. gigas*.

Algal species	Tiso-EQ [†] μl^{-1}	cells μl^{-1}	mg ODW day ⁻¹ [‡]	% of total ODW
<i>T. suecica</i>	25	2.50	720	67
<i>S. costatum</i>	25	8.33	348	33
TOTAL	50	10.83	1068*	100

†: Tiso-EQ = empirical equivalent of one *Isochrysis galbana* (clone T-Iso) cell: *T. suecica*= 10, *S. costatum*= 3

‡: based on algal ODW data from Helm (1990b) (pg cell⁻¹): *T. suecica*= 200, *S. costatum*= 29; and assuming constant flow rate of 1 l min⁻¹.

*: equivalent to initial weight-specific ration of 1.5 % ODW algae WW⁻¹ day⁻¹

IX.6.2.2. Results and discussion

This first flow-through experiment evaluating the use of artificial diets was hampered by some practical problems and the results should be interpreted with caution. The malfunctioning of the peristaltic pumps resulted in a fluctuating food supply during the experiment and an overdosing of the live algae (see Table 57). This may have caused the relatively high growth of the oysters fed the 20% SAR diet (Table 59) and overfeeding of the 20/80% algae/yeast treatments (see below). Furthermore, the size and condition of the oyster seed, which was derived from an

outdoor stock, was certainly not optimal for the purpose of the experiment. However, the test revealed some methodological problems that arise from the flow-through conditions and allowed a preliminary evaluation of the algal replacement diets.

Table 59 (Experiment GSF 3): Daily growth rate (A) of *C. gigas* calculated from the increase of the total biomass per bottle (DGR) or the individual wet weight (DGRΣ) after 6 and 13 days of culture. Live and dry weight, and percentage mortality are given in B. Data represent mean and standard deviation from two replicates.

A

TREATMENT [§]	week 1 (6 days)				week 2 (7 days)				total culture period (DGRΣ)	
	DGR		DGRΣ		DGR		DGRΣ			
	% day ⁻¹	%	% day ⁻¹	%	% day ⁻¹	%	% day ⁻¹	%	% day ⁻¹	%
100% SAR	5.2 ± 0.4	100	5.8 ± 0.7	100	6.2 ± 0.1	100	7.5 ± 2.0	100	6.7 ± 0.8	100
20% SAR	2.9 ± 0.4	56	3.8 ± 0.2	64	4.6 ± 0.4	74	4.4 ± 0.7	59	4.1 ± 0.4	61
20% SAR + 80% Y6	4.8 ± 0.3	92	5.0 ± 0.0	86	4.1 ± 0.4	66	4.9 ± 0.1	66	5.0 ± 0.1	74
20% SAR + 160% Y6	4.5 ± 0.4	87	4.8 ± 1.3	82	2.2 ± 0.5	35	2.6 ± 1.2	34	3.5 ± 0.1	52
20% SAR + 80% dTs	5.4 ± 0.4	105	5.3 ± 0.4	90	5.0 ± 0.5	81	6.6 ± 1.3	88	6.0 ± 0.5	89

B

TREATMENT [§]	week 1 (6 days)			week 2 (7 days)		
	WW (mg ind ⁻¹)	DW (mg ind ⁻¹)	mortality	WW (mg ind ⁻¹)	DW (mg ind ⁻¹)	mortality (%)
100% SAR	14.36 ± 0.55	8.61 ± 0.30	4.3 ± 0.5	23.81 ± 2.18	14.37 ± 1.51	5.3 ± 1.2
20% SAR	12.88 ± 0.11	7.74 ± 0.24	3.2 ± 0.1	17.45 ± 0.91	10.72 ± 0.43	3.7 ± 1.1
20% SAR + 80% Y6	13.76 ± 0.02	7.98 ± 0.09	3.8 ± 1.4	19.28 ± 0.14	11.53 ± 0.08	3.8 ± 0.9
20% SAR + 160% Y6	13.62 ± 0.93	7.81 ± 0.29	2.7 ± 0.9	16.22 ± 0.24	9.65 ± 0.13	3.6 ± 0.5
20% SAR + 80% dTs	13.95 ± 0.30	8.04 ± 0.08	2.3 ± 0.3	21.84 ± 1.36	13.05 ± 0.54	3.6 ± 1.0

§: standard algal ration: 100% SAR: see Table 58; Y6: Yeast diet (Table 42), dTs: spray-dried *T. suecica*
 *: DGR expressed as a percentage of DGR obtained for the 100% SAR treatment.
 initial seed: WW= 10.51 ± 0.14 mg ind⁻¹ (group mean ± SD, n=3)

Table 59A shows that daily growth rates calculated from the increase of total wet weight per bottle (DGR) were mostly lower than those based upon the increase of individual wet weight (DGRΣ) for the same period (up to a difference of 1.3 %/day for

the 100% SAR treatment during week 2). The water content of the samples used for determining the unit live weight was constant (linear regression analysis of DW and WW data of 20 samples: $DW = 0.62 WW - 0.46$, $r^2 > 0.99$). However, determining the live weight of a large sample of oyster seed is more difficult and, in addition to occasional losses of seed during the cleaning, may have caused an underestimation of DGR. Whereas DGR Σ appeared to be a more accurate measure for growth rate, it reflected the high variation between replicates from the unit wet weight determination (Table 59B). Mortality did not differ between the various treatments.

The results of the 80 % algal replacement appeared promising during the first week of the test, resulting for the yeast diet and the dried *T. suecica* in, respectively, a growth rate (DGR Σ) of 86 and 90% of that measured for the algal control (Table 59A). During the second week, supplementing the manipulated yeast to the 20% algal ration provided less growth than the addition of the dried algae. Furthermore, growth declined below the level of the 20% SAR control when a high concentration of the yeast was fed and copious pseudofaeces production was observed. This negative effect of higher yeast concentrations on growth suggested that the 20/80% SAR/Y6 treatment may have been overfed. This would mean that the optimal concentration for the dried algal diet would be higher than the one for yeast. Further research is needed to define optimal food levels of artificial diets in continuous flow cultures.

Artificial diets may have a different nutritional value for bivalves depending on whether they are evaluated either under continuous flow conditions or in batch-fed recirculating systems. The latter expose the diets to prolonged microbial activity and result in higher bacterial concentrations, which in turn may affect bivalve growth positively (*i.e.* through utilization of the bacteria as a food supplement, improving digestibility of the food particles) as well as negatively (*i.e.* by causing degradation and clumping of food particles, production of toxic substances, causing diseases) (see III.3.3.). Langdon & Siegfried (1984) obtained growth rates as high as 73% that of algal-fed

controls when feeding a microparticulate diet to *C. virginica* held in beakers, whereas much poorer relative growth (10-22%) occurred if the oysters were fed on the same diet in a flow-through apparatus. Solid comparisons between the upwelling and recirculation experiments with *C. gigas* are not possible because the initial seed and composition of the algal control differed. However, the growth improvement due to the supplementation of the artificial diets in the upwelling system indicated that these diets served as a direct food source and were digested by the oysters. The better performance of the dried algae compared to the yeast diets in the flow-through system may indicate that the nutritional value of the latter is more dependent on the development of a suitable microflora in the culture.

Chapter X

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Chapter X

THE USE OF ALGAL SUBSTITUTES AND THE REQUIREMENT FOR LIVE ALGAE IN THE HATCHERY AND NURSERY REARING OF BIVALVE MOLLUSCS: AN INTERNATIONAL SURVEY

X.1. INTRODUCTION AND RATIONALE

In the early stages of research in the field of intensive bivalve rearing, the mass culture of micro-algae was identified as the main constraint. An extreme illustration of this can be found in the earlier literature, where it was estimated that one oyster, during its growth from egg to market size, will consume approximately 1.28×10^{12} cells of the alga *Thalassiosira pseudonana* (Pruder et al., 1976), which is equivalent to about 250 liter of dense algal culture. At present, the requirement for live algae in the intensive culture of bivalves is strongly reduced by the transfer of the small spat (1-2 mm) as soon as possible from the hatchery to the nursery. From the latter stage onwards they are fed partially, or in some cases exclusively, natural phytoplankton. Once the seed attains planting size (5-10 mm), they are transferred to grow-out areas, where they reach market size feeding solely on natural food. Since, several authors have recognized the production of large volumes of micro-algae, which is labor-intensive and requires specialized facilities, as the main bottle-neck for the culture of bivalve seed (Persoone & Claus, 1980; Urban & Langdon, 1984; De Pauw & Persoone, 1988; Jones et al., 1991). This has prompted a search for alternatives to on-site algal production and lead to numerous publications concerning the development and evaluation of various non-algal foods for bivalves (see III.4.). More recently, research efforts in laboratories and hatcheries in the United States, Canada, United Kingdom, and Australia resulted in the development of techniques to grow algae heterotrophically (e.g.

Laing *et al.*, 1990; Gladue, 1991), to preserve algal pastes (e.g. Donaldson, 1991), and to prepare simple micro-encapsulated diets (e.g. Southgate *et al.*, in press). Except for the sporadic reports at international meetings (Helm & Hancock, 1990), the extent to which these products have been tried, and rejected or retained, by the hatchery operators is poorly documented. Furthermore, in order to direct future research efforts, it is essential to know the selection criteria of the farmer for an algal substitute which is eventually to be used in the daily practice of bivalve seed production. For example, depending on the bivalve species and the applied production technology, either a cheap bulk feed or a more complete, high quality diet may be preferred.

The actual algal requirement and production cost of the bivalve seed industry is difficult to estimate due to the nearly complete lack of information concerning the quantity of seed or algae produced. Also, the requirement for live algae greatly varies between hatcheries, as it depends on the availability of natural phytoplankton, the size at which the spat leaves the hatchery, and the bivalve species cultured. The great diversity in the algal culture technology hinders the estimation of a standard algal production cost, since it varies with the yield and efficiency of the culture system. As a result, extrapolations based on a case study of one hatchery are of a limited value. For this reason, the present survey aimed at the collection of data concerning the requirement for live algae and the associated costs encountered in several commercial as well as academic hatcheries. Furthermore, the hatchery operators were questioned about their knowledge of and experience with alternatives for live algae, and their intention and requirements to use artificial diets. Finally, this survey offered the opportunity to collect some unique data on the quality and quantity of hatchery-produced bivalve seed, and the employment in this relatively small sector of aquaculture.

X.2. DESIGN, DISTRIBUTION, AND RETURN RATE OF THE QUESTIONNAIRE

The questionnaire consisted of six multiple questions (see Appendix). The first question offered the possibility to protect confidential data that were possibly communicated in the questionnaire. The second question aimed at an evaluation of the profile of the hatchery on the basis of its productivity and the number of employees in 1990. The third and fourth question offered the possibility to detail, respectively, the knowledge of and experience with algal replacement diets. The capacity and nature of the algal production facilities of the hatchery were queried in question five. Finally, the last question consisted of various subquestions concerning the algal production cost (6A, B, C), the extent to which the algal production capacity is a limiting factor for the hatchery and may be expanded in the future (6D, E), the intention to use artificial diets (6F) and the most important characteristics these should comply with (6G).

The survey was announced in several aquaculture magazines and newsletters (including, Fish Farming International, Austasian Aquaculture, AquaRevue, World Aquaculture, Newsletter of the European Aquaculture Society), and through a poster presentation at two international aquaculture meetings (Coutteau & Sorgeloos, 1991ab). Furthermore, thanks to the support of several people who provided useful addresses, a world directory was compiled containing more than 250 addresses of people involved in the practice of bivalve culture.

In total, 265 forms were distributed over 43 countries. The poor representation of some regions in the directory, such as the USSR, Arabic, African and Asian countries (Fig. 70), was due to the difficulties to obtain addresses from these parts of the world. Over 90 people responded to the survey and 50 questionnaires were retained for evaluation. The efficiency with which the distributed forms were returned ranged between 10% (Europe) and 38% (South and Central America). In this way, the contributions of the various regions in the world were well balanced in the survey, with the exception of the exclusion of the USSR, and Arabic and African countries. For the analysis of

the 50 completed forms, a distinction was made between 25 private hatcheries (further referred to as "commercial") and 25 facilities run by research institutes and governments ("academic"). Since about 50% of the commercial and 30% of the academic hatcheries demanded secrecy, all data were treated anonymously.

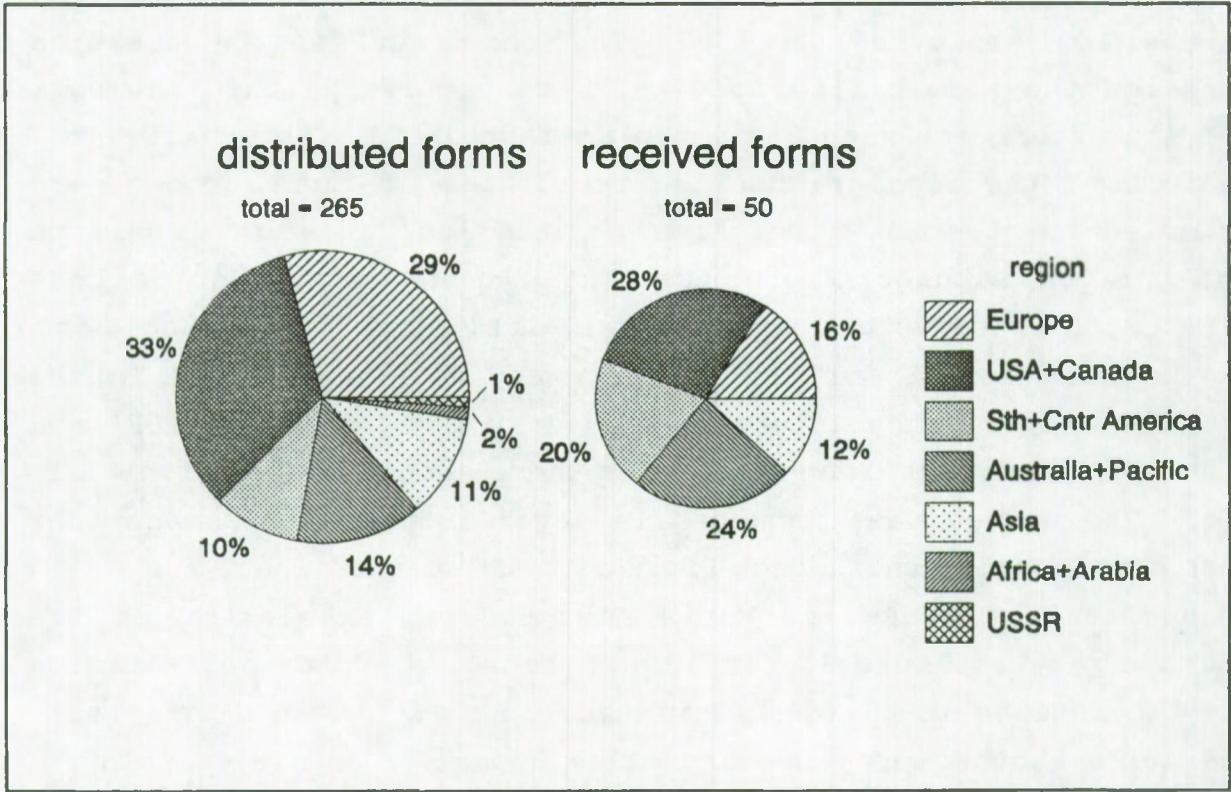


Fig. 70: Contribution of the various regions in the world to the distributed and received questionnaires.

X.3. RESULTS

1) SECRECY CLAUSE

"The answers to this questionnaire are confidential, and no reference may be made to the name and address in all further reports"

reply:	YES	NO	total
academic hatcheries	8	17	25
commercial hatcheries	13	12	25
total	21	29	50

2) PROFILE OF THE FARM

a) production data for 1990

In the present investigation, hatchery production included the rearing of eyed larvae (300-500 μ m) for remote setting as well as small postset (1-2 mm; 1 cm for giant clams). Nursery production consisted of the rearing of juveniles from 2 mm to planting size (4-15 mm: clams; 5-30 mm: oysters and scallops; 15-20 cm: giant clams).

The total hatchery and nursery production reported for 33 different bivalve species, and the relative contribution of the academic and commercial hatcheries is presented in Table 60. The production figures and the number of hatcheries producing each species demonstrated that the commercial hatcheries focus on the production of a few species of oysters (*C. gigas*, *C. virginica*, *S. commercialis*, *O. edulis*), clams (*T. philippinarum*, *M. mercenaria*, *T. decussata*) and scallops (*A. purpuratus*, *P. yessoensis*, *A. irradians*), representing over 98% of the total seed production. The remaining bivalve species were primarily reared in research and state's facilities in relatively low numbers. Furthermore, the hatchery production was dominated by the large amounts of eyed larvae and small postset (< 1 mm) of *Crassostrea gigas*, produced primarily in hatcheries along the west coast of the United States. As a result oysters represented 90% of the recorded hatchery production (Fig. 71). Interesting

was that more than 70% of the larval production of the pacific oyster was due to the efforts of one company. The recorded production of larger clam and oyster seed was equally important, whereas scallops represented only 5% of the nursery production (Fig. 71).

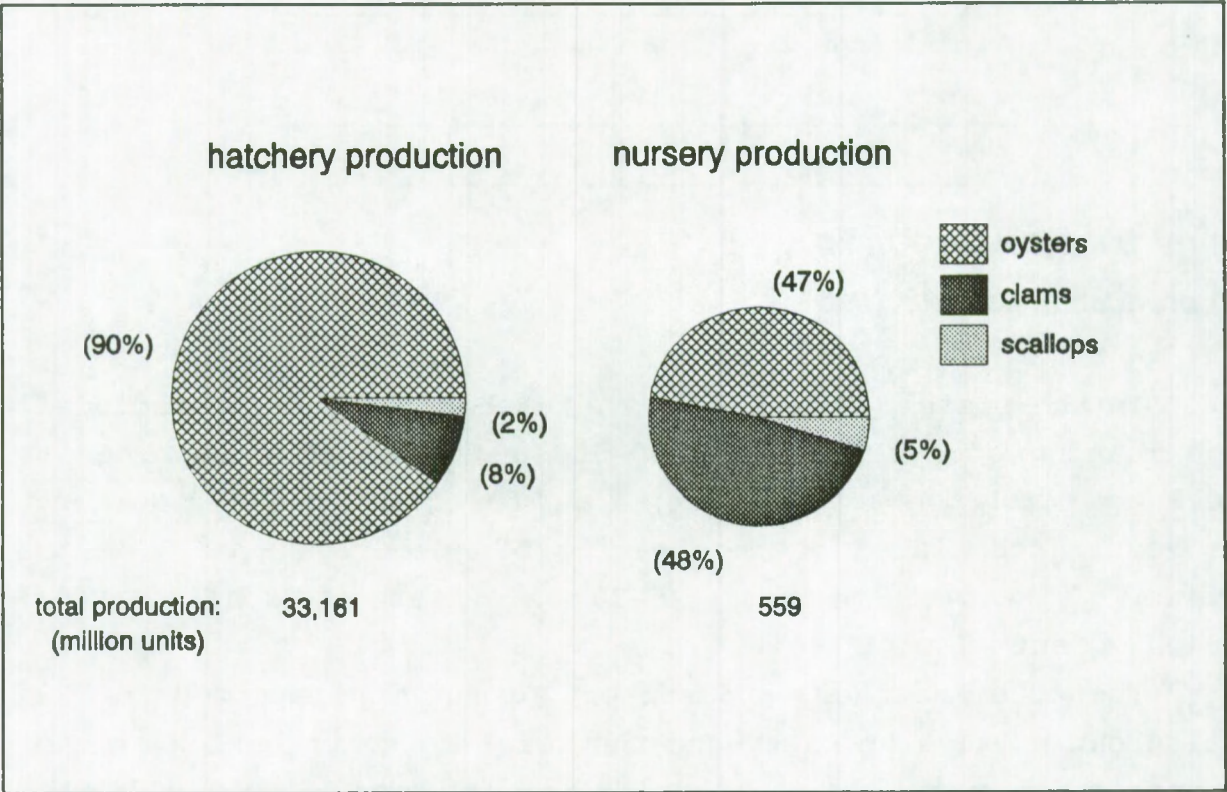


Fig. 71: Hatchery and nursery production of oysters, clams and scallops in 50 commercial and academic operations in 1990. Data for clams include cockles and arkshells.

Table 60: Hatchery and nursery production of various bivalve species in 50 commercial and academic hatcheries in 1990. Species were ranked according to the nursery production. The percentage contribution of commercial operations in the production of each species (% C), and the number of commercial (n_C) and academic (n_A) hatcheries involved are indicated.

Species	HATCHERY (larvae + spat < 2 mm)				NURSERY (spat ≥ 2 mm)			
	Total production (10 ⁶ units)	% C	n_C	n_A	Total production (10 ⁶ units)	% C	n_C	n_A
OYSTERS								
<i>Crassostrea gigas</i>	29661.0	99	10	3	183.0	98	7	2
<i>Crassostrea virginica</i>	336.5	96	4	2	64.5	98	3	2
<i>Saccostrea commercialis</i>	6.0	50	1	2	6.3	79	1	2
<i>Pinctada maxima</i>	11.0	100	2	0	5.2	100	2	0
<i>Crassostrea belcheri</i>	20.3	0	0	3	2.4	0	0	3
<i>Ostrea edulis</i>	53.2	100	4	1	1.1	100	3	0
<i>Crassostrea iredalei</i>	0.5	0	0	1	0.5	0	0	1
<i>Pinctada fucata</i>	0.4	0	0	1	0.3	0	0	1
<i>Crassostrea lugubris</i>	0.5	0	0	1	0.050	0	0	1
<i>Pinctada margaritifera</i>	0.060	0	0	1	0.050	0	0	1
<i>Tiostrea lutaria</i>	0.017	0	0	1	0.007	0	0	1
<i>Saccostrea echinata</i>	2.0	100	1	0	-	-	-	-
number of species	12				11			
total production	30092				263			
CLAMS, COCKLES, and ARKSHHELLS								
<i>Tapes philippinarum</i>	1982.0	85	7	2	155.0	96	6	2
<i>Mercenaria mercenaria</i>	211.0	74	4	3	63.4	84	4	3
<i>Tapes decussata</i>	103.8	100	4	1	25.7	100	4	1
<i>Panopea abrupta</i>	150.0	0	0	1	7.0	0	0	1
<i>Tapes pullastra</i>	1.6	100	1	0	7.0	100	1	0
<i>Spisula solidissima</i>	5.3	57	1	1	3.1	97	1	1
<i>Mya arenaria</i>	7.0	71	1	1	2.5	20	1	1
<i>Mulinia lateralis</i>	5.0	0	0	1	1.0	0	0	1
<i>Tridacna gigas</i>	59.1	0	1	2	0.3	7	1	2
<i>Anomalocardia brasiliana</i>	0.5	0	0	1	0.3	0	0	1
<i>Anadara brouthtoni</i>	0.4	0	0	1	0.3	0	0	1
<i>Tridacna derasa</i>	0.110	0	0	1	0.110	0	0	1
<i>Hippopus hippopus</i>	25.3	0	0	2	0.095	0	0	2
<i>Tridacna maxima</i>	15.0	0	0	1	0.040	0	0	1
<i>Codakia orbicularis</i>	3.0	0	0	1	-	-	-	-
number of species	15				14			
total production	2569				266			
SCALLOPS								
<i>Argopecten purpuratus</i>	110.0	100	3	1	22.5	100	2	1
<i>Patinopecten yessoensis</i>	172.0	99	1	1	0.1	0	0	1
<i>Argopecten irradians</i>	14.0	71	1	1	5.5	27	1	1
<i>Argopecten circularis</i>	202.0	0	0	1	1.0	0	0	1
<i>Pecten ziczac</i>	1.0	0	0	1	0.5	0	0	1
<i>Crassadoma gigantea</i>	0.5	0	0	1	0.025	0	0	1
number of species	6				6			
total production	500				30			

b) Number of employees

The total number of people employed in about 30 bivalve rearing facilities, including hatchery, nursery, and grow-out operations, was less than 500 (Table 61). Most of the private companies engaged two to four people in the hatchery and about the same number in the nursery, whereas a larger staff was involved in the more labor-intensive grow-out operations (Fig. 72).

Table 61: Total number of employees in the various stages of bivalve culture operations.

	hatchery		hatchery + nursery		hatchery + nursery + grow-out	
	total employment	n [†]	total employment	n [†]	total employment	n [†]
commercial hatcheries	77	19	127	20	427	14
academic hatcheries	30	12	95	16	61	14
total	107	31	222	36	488	28

†: number of replies to question 2b from 50 forms

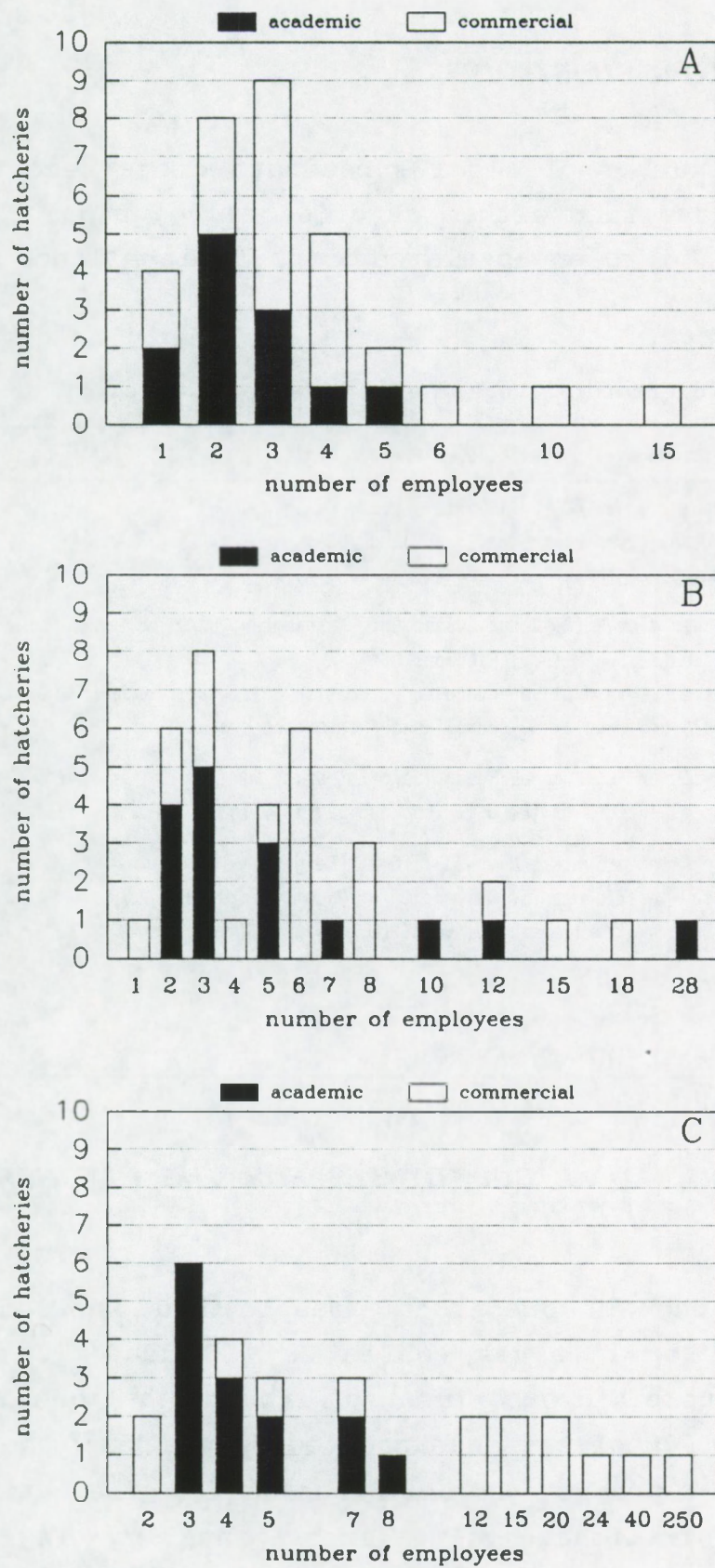


Fig. 72: Employment per operation for hatchery (A, n=31), hatchery + nursery (B, n=36) and hatchery + nursery + grow-out (C, n=28) rearing of bivalves.

3) INVENTORY OF ALGAL SUBSTITUTES

The limited number of algal substitute diets reported in this study was classified either as dried algae, algal pastes, yeast-based diets, micro-encapsulated diets or miscellanea (Table 62).

Table 62: Substitute diets for live algae in bivalve culture.

classification	diet (C = commercially available, E = experimental)
dried algae	- <i>Tetraselmis suecica</i> (C, Cell Systems Ltd., Cambridge UK) - <i>Nitzschia</i> sp. (E, Martek Corp., Maryland, USA) - <i>Spirulina</i> (C, Earthrise Farms, California, USA)
algal pastes	-Coast oyster diet 1 (C, Coast oyster Co., Washington, USA) -algal paste (E, SeaAg Inc., Florida, USA) -algal paste (C, Innovative Aquaculture, British Columbia, Canada) -algal paste (refrigerated, centrifuged from excess production)
microcapsules	-Frippak Booster (C, Frippak Feeds, Sanofi, Paris, F) -micro-encapsulated diet (E, James Cook University, Townsville, Australia)
yeast-based diets	-Topal (C, Artemia Systems N.V.-S.A., Gent, Belgium) -manipulated yeast diets (E, University of Ghent, Gent, Belgium) -various brands of dried baker's yeast (e.g. Mauri, Nauplius)
miscellanea	-cornflour (maizena) -corn starch (source not specified) -fry food (C, Biokyowa, Montana, USA)

4) EXPERIENCE WITH ALGAL SUBSTITUTES FOR THE HATCHERY AND NURSERY CULTURE OF BIVALVES

34 out of the 50 questioned people had knowledge of artificial diets for bivalves, while 28 (15 academic and 13 commercial) operators had experimented with at least one of them (Fig. 73). Nearly 60% of the interrogated people knew the dried *Tetraselmis suecica* product and more than half of the latter had evaluated its nutritional value experimentally. The other alternatives to live algae were relatively less well-known (Fig. 73).

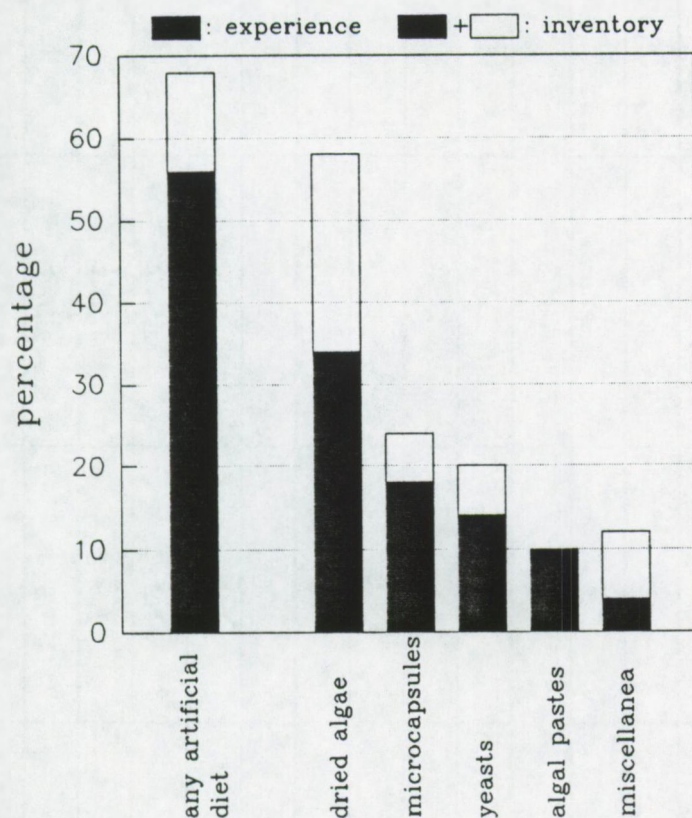


Fig. 73: Percentage of hatchery operators that claimed to have knowledge of and/or experience with various classes of artificial diets as reported in 50 questionnaires.

It should be emphasized that the experimental results reported in this survey could not be verified concerning the dependability and profundity of the applied methodology, and should thus be regarded as preliminary. It was tried to reproduce the data as they were mentioned by the experimenters in the questionnaire. The experience recorded for the various bivalve species, culture phases and substitute diets is summarized in Table 63.

Table 63: Reported experience with the use of various algal substitutes in the culture of different species and stages of bivalves (B= broodstock, L= larvae, S= spat). The results obtained with the various diets experimentally, as backup (†) or in routine culture (‡) are given in the text.

bivalve species	ARTIFICIAL DIETS				
	dried algae	algal pastes	yeasts	microcapsules	miscellaneous
OYSTERS					
<i>Crassostrea gigas</i>	BS † ‡	BS ‡	S	LS	B
<i>Crassostrea virginica</i>	BS †	BS †			
<i>Ostrea edulis</i>	LS				
<i>Saccostrea commercialis</i>				L	
<i>Pinctada margaritifera</i>				L	
CLAMS					
<i>Tapes philippinarum</i>	BLS †		BS	S	
<i>Tapes decussata</i>			BS		
<i>Mercenaria mercenaria</i>	BLS †	BS †	S		
<i>Panopea abrupta</i>	S				
<i>Dosinia dunkerii</i>			L		
<i>Tridacna gigas</i>	LS		LS	LS ‡	
<i>Tridacna maxima</i>	LS ‡		LS ‡	LS ‡	
<i>Tridacna derasa</i>	L ‡		LS ‡	LS ‡	
<i>Tridacna squamosa</i>	L ‡		LS ‡	LS ‡	
<i>Hippopus hippopus</i>	LS			LS ‡	
SCALLOPS					
<i>Patinopecten yessoensis</i>	BS				

The routine application of algal substitutes was reported by only three interviewees. Live algae were routinely replaced for up to 75% by algal paste (Coast oyster Co.) in the rearing of spat and broodstock of *C. gigas* and up to 25% by spray-dried *T. suecica* (Cell Systems Ltd.) in the culture of spat. Furthermore, algal culture was absent in five of the six hatcheries producing giant clam larvae, which were fed dried yeast, dried *T. suecica*, the Frippak micro-encapsulated diet, or a mixture of the latter two.

Several experiments indicated that substitute diets may be used to supplement insufficient rations of live algae. The spray-dried *T. suecica* and algal paste were found to be useful as a backup diet to replace 50% of the live algae in the diet of broodstock and spat of *C. virginica* and *M. mercenaria*. For spat of *T. philippinarum*, a replacement of 20-30% of the algae by dried *T. suecica* was applied in the absence of sufficient amounts of live algae. Dried *T. suecica* was found to be a satisfactory diet for feeding *O. edulis* in the size range of 2-10 cm during disease experiments, although growth was inferior to that in nature.

Despite the extensive efforts to evaluate various diets, the use of artificial diets appeared to be mostly restricted to the experimental stage. Contrary to the previous reports on the use of dried *T. suecica* as a partial algal substitute, various operators found it unsatisfactory either because of its high price (i.e. US \$ 170 per kg) or its poor performance (reported for larvae of *M. mercenaria*, *O. edulis*; spat of *T. philippinarum*, *C. gigas*, *P. yessoensis*; broodstock of *M. mercenaria*). The latter was mostly associated with difficulties to resuspend the dried algal cells without disintegrating them, and the fast settling of the food particles in the bivalve cultures. In this regard, dried *T. suecica* was found to be valuable in the culture of pedal feeders, such as *P. abrupta*, when it was introduced in the substrate.

Dried yeast (source not specified), was reported as being of low value as food for juvenile *C. gigas* at substitution levels

ranging from 25 to 100%. Also, feeding Topal (Artemia Systems N.V.-S.A.) resulted in poor growth and high mortality for larvae of *Dosinia dunkerii* and yielded poor growth in spat of *C. gigas* and *T. philippinarum*. Manipulated yeasts (University of Ghent) gave satisfactory results as an 80% algal substitute for spat of *M. mercenaria*, *T. philippinarum*, and *C. gigas*. Preliminary tests with broodstock of *T. decussata* and *T. philippinarum* indicated an acceptable conditioning index, but a retarded maturation in clams fed a 20/80% mixture of algae and manipulated yeasts.

The replacement of the algal diet fed to spat of *C. gigas* and *T. philippinarum* by microcapsules (Frippak Booster) yielded poor growth. However, the combined feeding of the experimental micro-encapsulated diet (James Cook University) with dissolved yeast extract resulted in a better growth of giant clam larvae (*T. gigas*, *H. hippopus*) than controls fed either *I. galbana* or *Pavlova salina*. Feeding these microcapsules to larvae of *S. commercialis* yielded up to 81% of the shell growth and similar ash free dry weight growth compared to algal-fed controls during a one-week experiment. By contrast, poor growth was reported when the same diet was fed to larvae of pearl oysters (*P. margaritifera*).

Cornflour may serve as a 20% algal supplement for broodstock of the pacific oyster, although increased bacterial growth was observed when fed to spat cultures.

5) TOTAL ALGAL PRODUCTION IN 1990

Most hatcheries cultured between two and five different algal species (Fig. 74). Five of the six hatcheries in which giant clam larvae were reared did not maintain any algal culture.

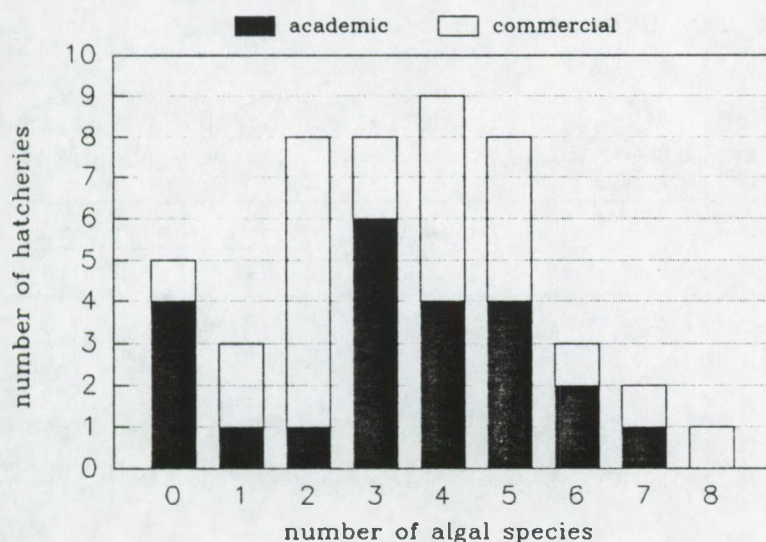


Fig. 74: Number of algal species cultured in 47 bivalve hatcheries.

An inventory of the algal species recorded in this study is presented in Table 64. Eight algal species (*I. galbana*, clone T-Iso; *C. gracilis*; *C. calcitrans*; *T. suecica*; *T. pseudonana*, clone 3H; *P. lutheri*; *I. galbana*; *S. costatum*) were widely used and represented over 90% of the volume of algal culture produced in 23 facilities. The other species were used less frequently and about a third was reported only once.

The total capacity of the algal culture showed a wide range from less than 1 m³ for a few research laboratories up to nearly 500 m³ for one commercial hatchery (Fig. 75). Because the yield of the algal culture greatly varies according to the culture and climatological conditions, the volume available for algal culture is a poor estimate for the productivity. The latter was computed as the daily volume of algae produced and converted to dry weight assuming an average culture density of 100 g/m³ (Gladue, 1991).

Apparently, about 50% and 60% of, respectively, the commercial and academic hatcheries produced daily less than 5 m³ of algal culture, i.e. about 0.5 kg dry biomass (Fig. 76). A quarter of the commercial hatcheries produced between 10 and 50 m³ algal culture per day, and six out of 21 companies reported a daily production between 30 and 110 m³. The total volume of algal culture produced per day by 37 hatcheries amounted to about 500 m³, i.e. 50 kg dry biomass.

Table 64: Frequency of use and total daily production of various algal species. Species are ranked according to decreasing frequency of use.

Algal species	frequency of use [†]	total daily production	
		n [‡]	volume (m ³)
<i>Isochrysis galbana</i> , clone T-Iso	31	18	23.8
<i>Chaetoceros gracilis</i>	23	11	14.1
<i>Chaetoceros calcitrans</i>	16	10	6.0
<i>Tetraselmis suecica</i>	15	10	39.1
<i>Thalassiosira pseudonana</i> , clone 3H	14	9	112.0
<i>Pavlova lutheri</i>	11	7	11.7
<i>Isochrysis galbana</i>	8	5	9.1
<i>Skeletonema costatum</i>	6	3	58.8
<i>Chroomonas salina</i>	5	3	0.76
<i>Dunaliella tertiolecta</i>	4	2	2.2
<i>Chaetoceros simplex</i>	3	3	1.76
<i>Chaetoceros muelleri</i>	3	2	5.0
<i>Nannochloropsis</i> sp.	3	2	0.20
<i>Cyclotella</i> sp.	2	1	0.36
<i>Phaeodactylum tricornutum</i>	2	1	2.0
<i>Tetraselmis chui</i>	2	0	-
<i>Pavlova salina</i>	1	1	3.18
<i>Dicruteria</i> sp.	1	1	4.07
<i>Tetraselmis levis</i>	1	0	-
<i>Dunaliella perva</i>	1	1	0.012
<i>Thalassiosira weissflogii</i>	1	1	0.12
<i>Chlamydomonas</i> sp.	1	1	0.52
<i>Chlorella</i> sp.	1	1	0.36
TOTAL	43	23	295

†: number of hatcheries growing each algal species (from 43 completed forms)

‡: number of hatcheries providing data which allowed to calculate daily production per algal species (from 23 completed forms)

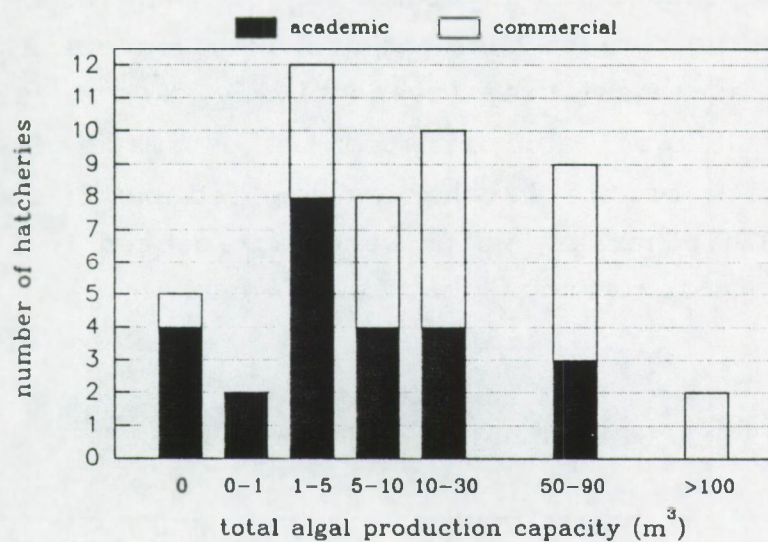


Fig. 75: Total capacity of algal culture in 48 hatcheries.

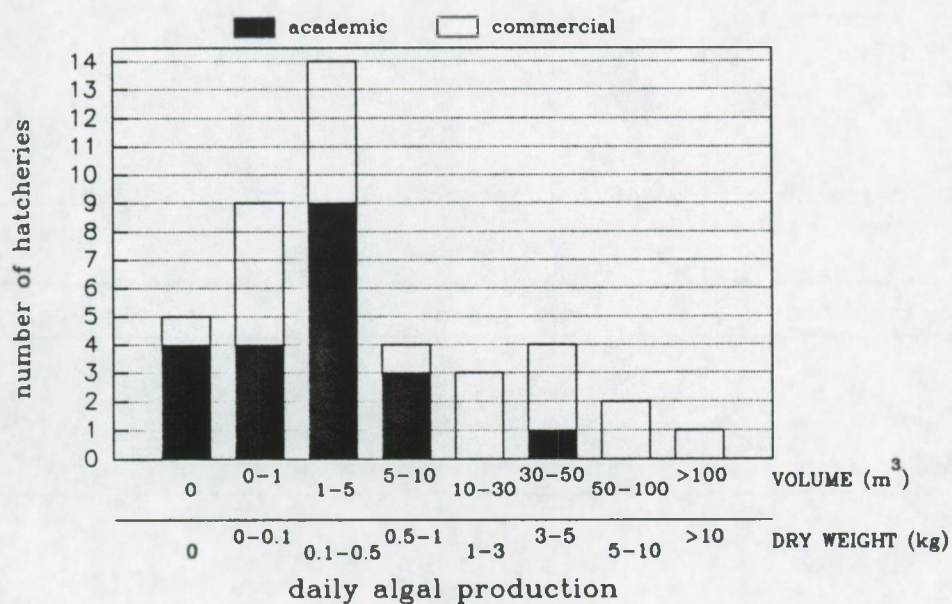


Fig. 76: Daily algal production in 42 hatcheries expressed either as volume of algal culture or as dry algal biomass.

Detailed data, obtained from 21 hatcheries, showed that most of the algae (*i.e.* 88% and 72% in terms of volume and dry weight, respectively) are produced in systems larger than 1 m³ (Table 65). It is interesting to note that the average production yields of large and small scale cultures (respectively, 89 and 248 g/m³; weighed average: 108 g/m³) approximated the estimate of Gladue (1991). The majority of the hatcheries applied batch cultures (Table 66). Only 3 hatcheries, which were all located in Europe, grew algae solely in continuous cultures.

Table 65: Total production capacity and daily production of small and large scale algal culture systems in 21 hatcheries.

scale algal culture	average yield (g/m ³) [†]	daily algal production			
		m ³	z	kg DW [§]	z
< 1 m ³	248	31	12	7.7	28
≥ 1 m ³	89	228	88	20.3	77
TOTAL		259	100	28.0	100

†: derived from data on algal culture densities from 19 hatcheries and conversion to dry weight based on data from Brown (1991)

§: estimated from average yield x daily production (m³)

Table 66: Frequency of use of batch, semicontinuous, and continuous algal culture systems in 42 hatcheries.

culture method	applied	solely applied
batch	38	27
semicontinuous	5	1
continuous	10	3

6) ADDITIONAL QUESTIONS

6A) Total algal production cost in 1990

6B) Algal cost as percentage of total seed production cost

6C) Algal cost per kg dry weight of algae

The total cost of algal production in 1990 reported by 20 hatcheries amounted to nearly US \$ 700,000 (Table 67). The cost of live food production per hatchery greatly varied as could be expected from the large differences in production capacity, and averaged about US \$ 40,000 and 24,000 for commercial and academic hatcheries, respectively (Fig. 77). The algal production cost averaged about 30% of the total seed production cost (Table 67). Although few data were collected concerning the cost of live algae per unit dry weight, six out of nine estimates were in the range of US \$ 300-400.

Table 67: Algal production cost in bivalve hatcheries. Replies to the questions 6A, 6B, 6C (from n hatcheries).

	ALGAL PRODUCTION COST (C= commercial, A= academic hatchery)					
	total per hatchery (US \$)		fraction of total seed cost (%)		cost per unit weight (US \$ (kg DW) ⁻¹)	
	C	A	C	A	C	A
total	442,000	212,000				
n	11	9	8	15	4	5
average	40,000	24,000	33	27	400/338/150/ 50-100	365/322/318/ 300/75
min	5,000	4,000	5	6		
max	160,000	74,000	60	60		

6D) Do you consider algal production as a limiting factor for your production ?

6E) Will algal production be expanded in the near future ?

6F) Would you consider using an artificial diet if one would be available ?

The replies on the questions 6D, E, and F appeared contradictory. Although algal production was felt as a limiting factor by only a third of the interviewees, over 50% planned an enlargement of the algal culture units and nearly everybody considered the use of an artificial diet (Table 68).

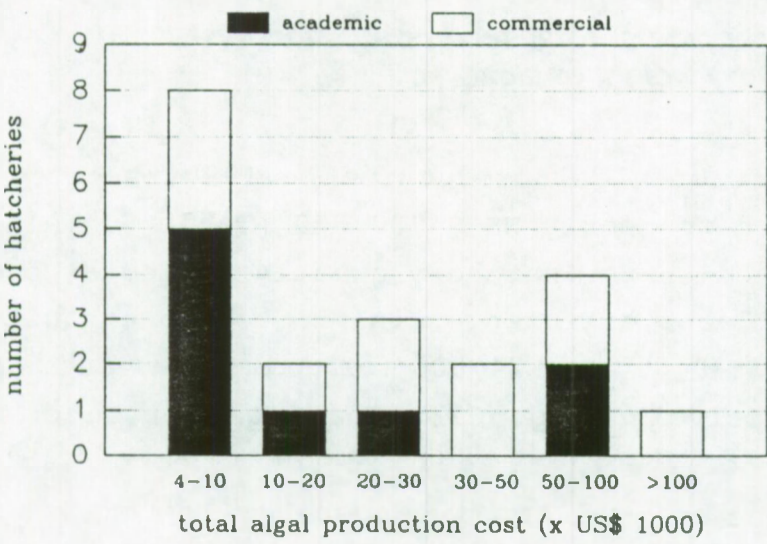


Fig. 77: Total algal production cost in 20 bivalve hatcheries.

Table 68: Response to questions 6D, 6E, 6F.

question	positive replies/total number completed	
	ratio	%
6D	14/43	33
6E	24/43	56
6F	37/40	93

6G) Rank, in order of importance, the features of an artificial diet

The relative importance of the various characteristics of an artificial diet was estimated by summation of the quotations (between 1 and 5, 5= most important) which were given to the features listed in each questionnaire. Food value, price, ease of use, and shelf-life were recognized as the most significant parameters that determine the value of an artificial diet (Fig. 78).

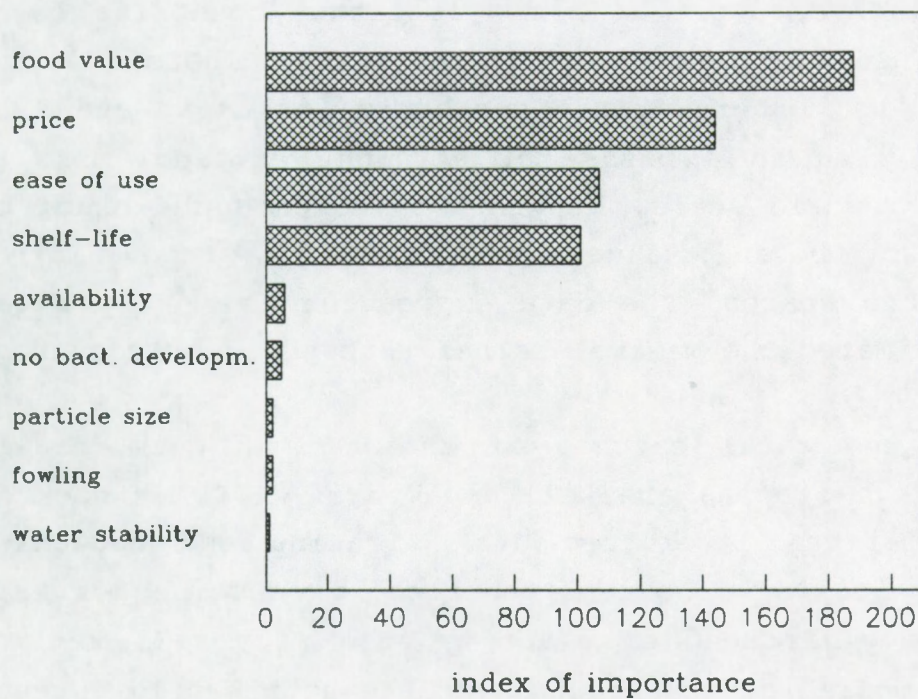


Fig. 78: Index of importance of various parameters of an artificial diet for bivalve rearing, based on 50 completed questionnaires (maximal index = 250).

X.4. DISCUSSION

The success of the questionnaire allowed the compilation of data from a significant number of companies, and research and demonstration centra involved in bivalve seed production. However, it is difficult to estimate the fraction which is represented by the latter in comparison with the total existing number of hatcheries. Also, the contributing hatcheries may not be a representative sample as a few important countries (e.g. France, China) did not participate in the survey. Therefore, the quantitative data concerning the production of algae and seed should not be used as a basis for straight extrapolation to production on a world scale, but rather indicate the order of magnitude. Also, because algal production is often varying according to the season, the data expressing the daily algal production estimated the maximal rather than the yearly average production.

Although the total catches of the various commercially important bivalve species are well documented (FAO Yearbook of Fisheries Statistics), literature data estimating seed production are completely lacking. The reported total production for 1990 of *Crassostrea gigas* seed of planting size (i.e. 183 million oysters) is comparable with about 3% of the total world catches for this species in 1987 (i.e. 620,000 metric ton, FAO Yearbook of Fisheries Statistics; assuming a market size of 100 g). The pacific oyster harvest on the west coast of North America amounted to 25,000 mt in 1989 (Chew, 1990), i.e. approximately 250 million oysters. The production of over 25 billion eyed larvae for remote setting, reported by the hatcheries along the Pacific coast of the United States, confirms that this technique is providing the main part of the seed to the farmers in this region (Chew, 1990).

Walne (cited in Persoone & Claus, 1980) composed in 1978 a preference list of algal species suitable for hatchery rearing of bivalves on the basis of a survey held among ten institutes active in intensive bivalve culture in Europe and North America (Table 69). It is clear that the relative importance of several

algal species has significantly changed over the last decade, in particular with the recognition of *I. galbana*, clone T-Iso (Helm & Laing, 1987) and *C. gracilis* (Enright et al., 1986a) as valuable species.

Table 69: Relative importance of various algal species in bivalve hatcheries as reported in the survey of Walne (1978, in Persoone & Claus, 1980) and in the present study.

algal species	Frequency of use	
	Walne (out of 10)	Present study (out of 43)
<i>Isochrysis galbana</i>	8	8
<i>Pavlova lutheri</i>	7	11
<i>Tetraselmis suecica</i>	6	15
<i>Phaeodactylum tricornutum</i>	5	2
<i>Pseudoisochrysis paradoxa</i>	5	-
<i>Thalassiosira pseudonana</i>	4	14
<i>Chaetoceros calcitrans</i>	4	16
<i>Skeletonema costatum</i>	2	6
<i>Isochrysis galbana</i> , clone T-Iso	2	31
<i>Chlamydomonas</i> sp.	1	1
<i>Pyramimonas obovata</i>	1	-
<i>Tetraselmis chui</i>	1	2
<i>Rhodomonas</i> sp.	1	-

The algal production capacity recorded for the various hatcheries, varying from 1 to over 100 m³ day⁻¹, can be related to the estimates of Helm (1990a) for the quantities of algae required in a hatchery operation. The latter author calculated that one million juvenile clams or oysters of 3 mm shell length will consume about 1.4 m³ of dense algal culture each day at the optimum rearing temperature of 24°C, while one million of larvae require only 15 liter of algal culture.

The algal production cost per unit dry weight appeared to be known (or released) by few hatchery operators. The few values, reported by hatcheries producing less than 5 m³ algal culture per day mostly exceeded US \$ 300 (kg DW)⁻¹, which is higher than most data in the literature (see III.4.1.). Lower estimates, ranging

between US \$ 50 and 100, were reported by hatcheries producing relatively large quantities of algae (Fig. 79). Obviously, large scale culture systems, which provide the bulk of the algal biomass grown in bivalve hatcheries, yield lower production costs per unit of dry weight.

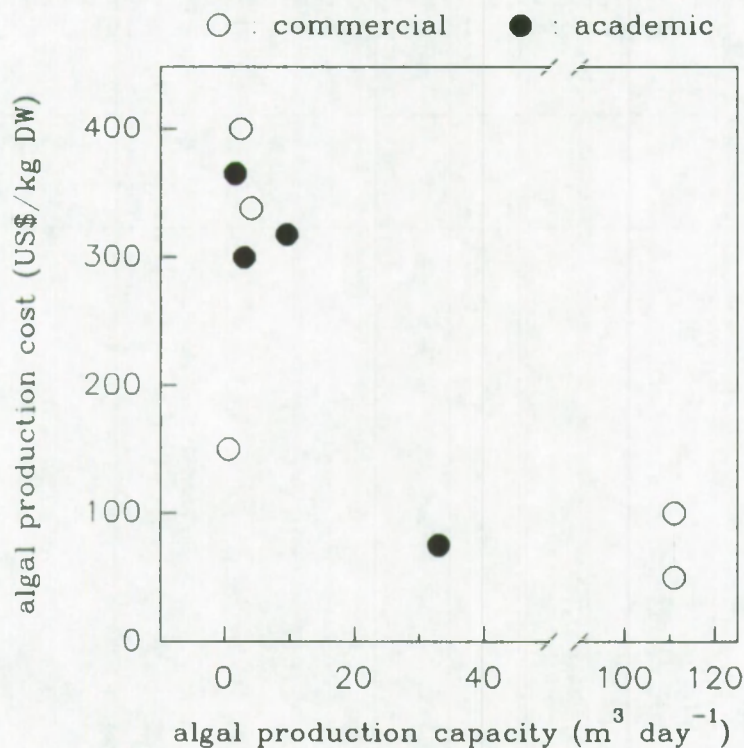


Fig. 79: Algal production cost as a function of the production capacity for 8 bivalve hatcheries. Dotted line connects estimates from one company.

The survey revealed that more than half of the questioned operators had experimented with algal substitutes in their hatchery. Despite these efforts, artificial diets are included in the routine production process of only a few hatcheries and more often considered as a useful backup diet. In either case, the live algae could only be partially replaced by dried *Tetraselmis suecica* (up to 25-50%) or a preserved algal paste (up to 75%). The hatcheries rearing giant clams appeared to be an exceptional case as they mostly lack algal culture facilities, and the feeding regime of the larvae and early postset consists solely of artificial diets. This may be ascribed to the lower food requirements of giant clam larvae compared to other

bivalves, and to the relatively high costs associated with maintaining algal cultures on the often remote sites where giant clam farms are located.

It is interesting to note that the results obtained with the same artificial diet greatly vary between experimenters and are often inferior to those reported in scientific papers. In this way, the unsatisfactory results, reported for the micro-encapsulated Frippak diet fed to spat of the Manila clam and the Pacific oyster, conflict with the successful experiments performed by Laing (1987). The latter author obtained for the same bivalve species a similar growth as the algal-fed controls when substituting up to 60-85% of the algal diet by microcapsules. Also, the limited replacement of live algae by dried *T. suecica* is in contrast with reports of successful substitution of up to 75% and 90% of the live algae in the spat rearing of, respectively, *Crassostrea virginica* (Helm & Hancock, 1990) and *Tapes philippinarum* (Laing, 1991). Although various authors have demonstrated through laboratory experiments that live algae could be substituted for up to 50% by dried yeast (*Candida utilis*) in the juvenile rearing of several bivalve species (Epifanio, 1979a; Alatalo, 1980; Urban & Langdon, 1984), no confirmation of this was revealed in the survey. The inconsistent performance of artificial diets may have several explanations. Certain products, such as the dried algae, appeared to be difficult to use and may not always have been presented in the optimal form to the animals. Alternatively, the experimental conditions, including quality and quantity of the algal control diet, stocking density, water quality, and scale of the experiment, may affect the performance of the artificial diet. In this regard, the specific conditions of laboratory experiments can be expected to differ from those encountered in a hatchery.

Chapter XI

SUBSTITUTION DIETS FOR LIVE MICRO-ALGAE IN THE HATCHERY AND NURSERY REARING OF BIVALVE MOLLUSCS: GENERAL CONCLUSIONS

I. THE USE OF MANIPULATED YEAST DIETS AS AN ALGAL SUBSTITUTE IN THE CULTURE OF BIVALVE SEED

Growth experiments were performed with juveniles of different bivalve species (*Tapes philippinarum*, *Mercenaria mercenaria*, *Crassostrea gigas*) at various locations to evaluate the use of manipulated yeast diets as a substitute for live algae. The yeast diets were manipulated with regard to their digestibility and nutritional composition by, respectively, chemical treatment and lipid-enrichment. Furthermore, prior to the evaluation of the artificial diets, standardization experiments allowed to identify the quantitative algal requirements under the experimental conditions for juveniles of the hard clam *M. mercenaria* and the Manila clam *T. philippinarum*. In this way, the present study contributed to the knowledge of the following two aspects of bivalve feeding:

1. THE EFFECT OF ALGAL RATION ON GROWTH IN JUVENILE CLAMS (*M. MERCENARIA*, *T. PHILIPPINARUM*)

The optimal daily ration for maximal growth in *T. philippinarum* (1-40 mg unit live weight) fed *Chaetoceros gracilis* ranged between 1 and 1.5% dry weight algae per wet weight of clams (DW WW⁻¹). This was supported by the following observations:

-Growth of clams showed a linear response to increasing rations up to 1% day⁻¹, was not significantly affected by rations ranging

from 1 to 1.5%, and eventually decreased with a further increase of ration.

-Preliminary tests suggested that a daily ration of 1.3% day⁻¹ resulted in maximal growth in clams fed either *Isochrysis galbana* (clone T-iso), *C. gracilis* or a mixture of both algal species.

-The maximal amount of *C. gracilis* that was removed from suspension during a growth test averaged 1.16% DW WW⁻¹ day⁻¹, which was equivalent to 67% of the offered ration.

The nutritional value of *C. gracilis* for *T. philippinarum* was lower after concentration and storage at 4 °C in the dark compared to that of algae fed directly from the algal culture without separation of the cells from the culture medium.

Growth of *M. mercenaria* (0.4-6 mg unit live weight) fed a mixture (50/50 on dry weight basis) of *I. galbana* (clone T-iso) and *C. gracilis* was maximized at a daily ration of 2% DW WW⁻¹.

2. THE USE OF MANIPULATED YEASTS AS AN ALGAL SUBSTITUTE IN JUVENILE REARING OF CLAMS (*M. MERCENARIA*, *T. PHILIPPINARUM*)) AND OYSTERS (*C. GIGAS*)

The results obtained by substituting the control diet of live micro-algae by manipulated yeast diets in the rearing of bivalve juveniles largely depended on the experimental location.

-A preliminary experiment, which was performed with *T. philippinarum* at a Spanish commercial hatchery, revealed that an 80% replacement of the algal control diet (consisting of a mixture of five algal species) by the yeast product yielded a daily growth rate of 82-93% of that obtained in the algae-fed controls over a 4-week culture period. A 50/50% algae/yeast diet supported similar growth as the 100% algal diet. However, growth of clams fed a sole diet of yeast was strongly reduced.

-The performance of the yeast diets was very limited in a series of experiments performed at the Laboratory of Aquaculture with *T. philippinarum* fed *C. gracilis* as a control diet. The best daily growth rate which was obtained with a 50% and 80% replacement of the control diet amounted to, respectively, 88% and 64% of the growth rate observed in the algae-fed controls over a period of three weeks. The supplementary effect of the yeast diets could not be improved by increasing the nutritional value of the control diet through the use of a mixture of algal species.

-During 2-weeks experiments performed at the South Carolina Wildlife and Marine Resources Department (SC, USA), it was shown that replacing 50% of the algal ration by the yeast diet did not affect growth rate of *M. mercenaria* fed a mixture of *C. gracilis* and *I. galbana* (clone T-iso). Clams fed a 20/80% algae/yeast diet exhibited a daily growth rate of 75-94% of that observed in algae-fed controls.

-A growth trial with *C. gigas* and *T. philippinarum* at a British commercial hatchery demonstrated that replacing 80% of the algal diet yielded an average daily growth rate of 70-80% of that measured in the algal control treatments during a 3-week experimental period.

The effect of the addition of various compounds on the nutritional value of the yeast diet was not consistent between experiments. In some clam experiments, a significant growth improvement was observed by the incorporation of the clay kaolinite. A positive effect due to the addition of an extract of seaweeds, which was revealed by the preliminary test with *T. philippinarum*, could not be confirmed in subsequent experiments. The supplementation of rice starch or fat-soluble vitamins did not significantly improve the nutritional value of the yeast diet for the two clam species examined.

Experiments performed with *T. philippinarum* at the Laboratory of Aquaculture and the British hatchery could not detect a significant difference between the nutritional value of the yeast diet and that of dried *Tetraselmis suecica* as an 80% replacement diet for live algae. The use of dried *Cyclotella cryptica* as an 80% algal substitute for *C. gigas* resulted in slower growth compared to that obtained with the yeast diet under the same conditions.

The best results reported in the present study confirmed the literature data with regard to the successful use of yeasts as a 50% algal substitute for rearing bivalve seed, and demonstrated considerably better performance of the manipulated yeasts at higher levels of partial substitution. This may be ascribed to the improved digestibility and nutritional value of the yeast diet through, respectively, chemical treatment and lipid-enrichment. However, this study identified the following problems associated with the replacement of live algae by manipulated yeast diets and artificial diets in general:

-Growth rate of juveniles fed a mixed diet of algae and artificial diets often decreased after 1-2 weeks of culture compared to that of the algae-fed controls. This demonstrated that the nutritional value of the mixture is inferior to that of a full ration of live algae.

-The discrepancy between the results obtained with the artificial diets at the Laboratory of Aquaculture and those recorded at other locations, indicated that the culture conditions may influence the success of the algal replacement.

Although this study demonstrated that manipulated yeasts offer interesting possibilities as a cheap, partial substitute for live algae in bivalve seed rearing, further research is required to improve the nutritional value of the yeast diets and to unravel the impact of culture conditions on the performance of the artificial diets.

II. THE USE OF ALGAL SUBSTITUTES AND THE REQUIREMENT FOR LIVE ALGAE IN THE HATCHERY AND NURSERY REARING OF BIVALVE MOLLUSCS: AN INTERNATIONAL SURVEY

By means of an international survey among operators of bivalve hatcheries, information was collected concerning the quality and quantity of the produced algae and bivalve seed, the algal production costs, and the experience with artificial diets. On the basis of the replies of 50 operators of commercial and experimental hatcheries, the following conclusions could be drawn:

-Commercial hatcheries focus on the production of a few species of oysters (*Crassostrea gigas*, *Crassostrea virginica*, *Saccostrea commercialis*, *Ostrea edulis*), clams (*Tapes philippinarum*, *Mercenaria mercenaria*, *Tapes decussata*), and scallops (*Argopecten purpuratus*, *Patinopecten yessoensis*, *Argopecten irradians*), representing over 98% of the total reported seed production.

-The capacity of the algal production facilities ranged between 1 m³ for a few research laboratories to nearly 500 m³ for one commercial hatchery. The total algal production capacity reported by 37 hatcheries amounted to about 500 m³ algal culture day⁻¹, which is equivalent to about 50 kg of dry biomass. The total cost of algal production in 1990 reported by 20 hatcheries approximated US \$ 700,000 and averaged about 30% of the total seed production cost. The estimates for the algal production cost per unit dry weight ranged from \$ US 50 to 400 kg⁻¹.

-About a third of the questioned operators considered the algal production as a limiting factor in the rearing of bivalve seed, whereas over 50% planned an expansion of the algal cultures and more than 90% was interested in the use of a suitable artificial diet. The most important characteristics of the latter were ranked as, in descending order, food value, price, ease of use, and shelf-life.

-The large interest for alternatives for on-site algal production from people that are involved in the practice of bivalve seed production, was demonstrated by the fact that more than 50% of the operators claimed to have experimented with artificial diets. Despite the extensive research efforts, artificial diets are rarely applied in the routine process of bivalve seed production and are mostly considered as a useful backup diet. A few operators reported the successful partial substitution of live algae by dried *Tetraselmis* (up to 25-50%) and preserved algal pastes (up to 75%). To date, the need for algal cultures could only be eliminated in the hatcheries rearing seed of giant clams.

-The results obtained with the same artificial diet greatly varied between experimenters and are often inferior to those reported in scientific papers. This supports the hypothesis that the performance of an artificial diet may depend on the culture conditions. Furthermore, this demonstrates the necessity to verify the nutritional value of artificial diets under the conditions which are encountered in a commercial hatchery.

SUMMARY

The culture of many commercially important molluscs, fishes, and crustaceans still relies on the production and use of micro-algae as a live food during at least part of their life cycle. The high costs and unpredictability associated with the mass-production of selected species of unicellular algae has prompted a search for alternative diets. The present work attempted to document the application of baker's yeast *Saccharomyces cerevisiae* as a substitute for live algae in filter-feeding organisms using the brine shrimp *Artemia* as a test-organism and juveniles of bivalve molluscs as commercially important species.

EXPERIMENTAL PART 1: STUDY OF FEEDING AND GROWTH IN *ARTEMIA* USING YEAST AS A FOOD SOURCE

I. BAKER'S YEAST AS A FOOD SOURCE FOR THE BRINE SHRIMP *ARTEMIA*

Preliminary experiments revealed poor growth and survival in *Artemia* fed solely on baker's yeast. Standardized growth tests evaluating various yeast preparations and the determination of assimilation efficiency using ^{14}C -labelled yeast, demonstrated that the nutritional value of baker's yeast for the brine shrimp is primarily limited by the low digestibility of the yeast's rigid cell wall. Furthermore, it was hypothesized that the latter was due to the external mannoprotein layer of the yeast cell wall, which forms a permeability barrier for the digestive enzymes of *Artemia* and thus prevents the efficient digestion of the internal skeletal glucan layer. A simple chemical treatment was conceived, which improved the digestibility of baker's yeast without affecting the integrity of the cell wall. The application of the chemically-treated baker's yeast, preserved either in a fresh form by freezing or as a dried, lipid-enriched product, seriously reduced the requirement for micro-algae to cultivate brine shrimp at a laboratory scale. A total substitution of the alga *Dunaliella tertiolecta* by the fresh yeast was achieved in

small scale growth tests resulting after one week of culture in an average survival and body length of 70% and 4 mm, respectively. A 75% substitution of *D. tertiolecta* by the dried yeast diet yielded similar survival and significantly faster growth and sexual differentiation compared to *Artemia* fed solely on algae.

II. STUDY OF FEEDING, ASSIMILATION, AND GROWTH IN ARTEMIA USING BAKER'S YEAST

The present study contributed to the knowledge of the following three aspects of the feeding biology of *Artemia*:

- THE EFFECT OF FOOD CONCENTRATION ON FEEDING AND GROWTH

The functional response curves, determined for various stages of *Artemia* fed chemically-treated yeast by means of the cell count method, revealed an increase of maximal feeding and clearance rate, and a decrease of the incipient limiting concentration in the course of development. The functional response curve of adult *Artemia* was influenced by the experimental environment. *Artemia*, grown at various continuous concentrations of the treated yeast in a recirculating system, maximized its growth rate during the first week after hatching at a concentration between 800 and 1200 cells μl^{-1} .

- THE EFFECT OF CULTURE CONDITIONS ON FEEDING

The ingestion rate of adult *Artemia* at food saturating concentrations of treated yeast was measured by means of the cell count method at various conditions of animal density, water quality, mechanical disturbance, and light intensity in short-term grazing tests. Crowding did not affect feeding of *Artemia* up to densities of 6.7 animals ml^{-1} in a recirculating system, which allowed to exclude side-effects from changes in water quality. By contrast, feeding rate was depressed at animal

densities of 3 adults ml⁻¹ in closed, rotating tubes. Feeding rate was significantly reduced by short-term exposures to concentrations of ionized ammonia and nitrite as high as, respectively, 1,000 and 100 ppm, whereas nitrate did not influence feeding in the range of 0-1,000 ppm. In aerated culture systems, maximal feeding rates were observed at an intermediate aeration intensity. Light intensity did not alter feeding rate in a measurable manner.

- THE EFFECT OF FOOD DIGESTIBILITY ON FEEDING AND ASSIMILATION

Strongly reduced ingestion rates were observed by means of the cell count method in *Artemia* fed untreated yeast compared to those feeding treated yeast. By contrast, radiotracer experiments revealed 2 to 5 times lower ingestion rates in *Artemia* fed the treated yeast compared to animals fed untreated yeast. The discrepancy between the indirect (cell counting) and the direct (¹⁴C) estimates of ingestion evidenced an important recycling of defecated yeast cells in brine shrimp fed untreated yeast. Elevated feeding rates in *Artemia* fed untreated yeast were associated with a minimal gut passage time of about 30 min, whereas lower and more variable feeding rates in animals fed the treated yeast was coupled to a minimal gut passage time of 60-100 min. A study of the ¹⁴C-budget revealed assimilation efficiencies of 24-31% and 72-76% in *Artemia* fed at non-limiting concentrations of untreated and treated yeast, respectively. The effect of food concentration on the assimilation efficiency depended on the yeast type and the acclimation conditions prior to the experiment. After acclimating to the experimental food concentration, assimilation efficiency decreased with increasing concentrations of untreated yeast, whereas a high assimilation efficiency, irrespective of food concentration, was observed in *Artemia* fed treated yeast. As a result, assimilation rate in *Artemia* showed a saturation response towards increasing concentrations of treated yeast, whereas this parameter decreased with increasing levels of the untreated yeast.

The above observations allowed to relate the concept of

superfluous feeding in zooplankton to the digestibility of the food and to postulate a compensatory mechanism adjusting ingestion rate as a function of assimilation in the brine shrimp.

EXPERIMENTAL PART 2: SUBSTITUTION DIETS FOR LIVE MICRO-ALGAE IN THE JUVENILE REARING OF BIVALVE MOLLUSCS

I. THE USE OF MANIPULATED YEAST DIETS AS AN ALGAL SUBSTITUTE IN THE CULTURE OF BIVALVE SEED

The present study contributed to the knowledge of the following two aspects of bivalve feeding:

- THE EFFECT OF ALGAL RATION ON GROWTH IN JUVENILE CLAMS (*M. MERCENARIA*, *T. PHILIPPINARUM*)

The optimal daily ration for maximal growth in *T. philippinarum* (1-40 mg unit live weight) fed *Chaetoceros gracilis* ranged between 1 and 1.5% dry weight algae per wet weight of clams (DW WW⁻¹). The nutritional value of *C. gracilis* for *T. philippinarum* was lower after concentration and storage at 4 °C compared to that of algae fed directly from the algal culture without separation of the cells from the culture medium.

Growth of *M. mercenaria* (0.4-6 mg unit live weight) fed a mixture (50/50 on dry weight basis) of *I. galbana* (clone T-iso) and *C. gracilis* was maximized at a daily ration of 2% DW WW⁻¹.

- THE USE OF MANIPULATED YEASTS AS AN ALGAL SUBSTITUTE IN JUVENILE REARING OF CLAMS (*M. MERCENARIA*, *T. PHILIPPINARUM*) AND OYSTERS (*C. GIGAS*)

Growth experiments, performed with juveniles of different bivalve species (*Tapes philippinarum*, *Mercenaria mercenaria*, *Crassostrea gigas*) at various locations, revealed that an 80% replacement of the algal control diet by manipulated yeast yielded a daily growth rate of 64-93% of that obtained in the algae-fed controls over the total experimental period (2-4

weeks). In most experiments, a 50/50% algae/yeast diet supported similar growth as the 100% algal diet. The considerably lower performance of the artificial diets in experiments performed at the Laboratory of Aquaculture and those recorded at other locations, indicated that the culture conditions may influence the success of the algal replacement. Furthermore, growth rate of juveniles fed a mixed diet of algae and artificial diets often decreased after 1-2 weeks of culture compared to that of the algae-fed controls. This demonstrated that the nutritional value of the mixture is inferior to that of a full ration of live algae.

The effect of the addition of various compounds on the nutritional value of the yeast diet was not consistent between experiments. In some clam experiments, a positive effect was observed by the incorporation of the clay kaolinite or an extract of seaweeds. The supplementation of rice starch or fat-soluble vitamins did not significantly improve the nutritional value of the yeast diet.

The present experiments could not detect a significant difference between the nutritional value of the yeast diet and that of dried *Tetraselmis suecica* as an 80% replacement diet for live algae in the culture of *T. philippinarum*. The use of dried *Cyclotella cryptica* as an 80% algal substitute for *C. gigas* resulted in lower growth compared to that obtained with the yeast diet under the same conditions.

Although further research is required to improve their nutritional value and to unravel the impact of culture conditions on their performance, manipulated yeasts offer interesting possibilities as a cheap, partial substitute for live algae in bivalve seed rearing.

II. THE USE OF ALGAL SUBSTITUTES AND THE REQUIREMENT FOR LIVE ALGAE IN THE HATCHERY AND NURSERY REARING OF BIVALVE MOLLUSCS: AN INTERNATIONAL SURVEY

By means of an international survey among operators of bivalve hatcheries, information was collected concerning the

quality and quantity of the produced algae and bivalve seed, the algal production costs, and the experience with artificial diets. On the basis of the replies of 50 operators of commercial and experimental hatcheries, the following conclusions could be drawn:

- Commercial hatcheries focus on the production of a few species of oysters, clams and scallops
- The capacity of the algal production facilities ranged between 1 m³ for a few research laboratories to nearly 500 m³ for one commercial hatchery. The total algal production capacity reported by 37 hatcheries amounted to about 500 m³ algal culture day⁻¹, which is equivalent to about 50 kg of dry biomass. The total cost of algal production in 1990 reported by 20 hatcheries approximated US \$ 700,000 and averaged about 30% of the total seed production cost. The estimates for the algal production cost per unit dry weight ranged from \$ US 50 to 400 kg⁻¹.
- About a third of the questioned operators considered the algal production as a limiting factor in the rearing of bivalve seed, whereas over 50% planned an expansion of the algal cultures and over 90% was interested in the use of a suitable artificial diet.
- The large interest for alternatives for on site algal production from people that are involved in the practice of bivalve seed production, was demonstrated by the fact that more than 50% of the operators claimed to have experimented with artificial diets. Despite the extensive research efforts, artificial diets are rarely applied in the routine process of bivalve seed production.

SAMENVATTING

Gedurende het voorbije decennium is de aquacultuur, in het bijzonder de gecontroleerde vis- en garnaalkweek, geëvolueerd van een artisanale activiteit naar een volwaardige industrie. Deze ontwikkeling is vooral te danken aan het op punt stellen van geschikte technieken voor de gecontroleerde produktie van jongbroed dat, na één tot meerdere maanden, als pootvis kan uitgezet worden in vijvers en kooien voor het vetmesten tot een marktklaar produkt. De kweek op industriële schaal van de larvale en juveniele stadia van vis, schaal- en schelpdieren, is gebaseerd op het gebruik van drie types van levende prooiorganismen: bepaalde soorten van ééncellige wieren, raderdiertjes *Brachionus plicatilis* en het pekelkreeftje *Artemia*. Ééncellige wieren vormen een essentiële voedselbron voor de kweek van alle stadia van schelpdieren en de eerste larvale stadia van vele garnaal- en sommige vissoorten. Bovendien fungeren wieren als voedsel voor de filtreer-organismen *B. plicatilis* en *Artemia*, welke op hun beurt worden gevoederd aan laat-larvale en juvenile stadia van garnaal en vis. De complexiteit en de hoge kosten, die gepaard gaan met de gecontroleerde produktie van wieren in grootschalige monocultures, hebben het onderzoek naar alternatieve voedsels sterk aangemoedigd. Dit heeft de substitutie van wieren, hetzij gedeeltelijk (vb. voor sommige garnaallarven) hetzij volledig (vb. kweek van rotiferen met bakkersgist, van *Artemia* met vermalen landbouwprodukten), voor de kweek van sommige filtreer-organismen mogelijk gemaakt. Ondanks grote onderzoeksinspanningen, blijven ééncellige wieren evenwel een essentieel voedsel voor de kweek van vele commercieel belangrijke organismen (sommige garnalen, alle schelpdieren).

In deze thesis werd het gebruik van bakkersgist als wiersubstituut voor filtreer-organismen bestudeerd aan de hand van het pekelkreeftje *Artemia* en juvenielen van diverse soorten bivalven. De keuze van *Artemia* als proefdier liet toe om diverse aspecten van de substitutie van ééncellige wieren door gisten te onderzoeken in kleinschalige laboratoriumexperimenten. Juveniele

schelpdieren werden gebruikt om de toepasbaarheid van gisten als voedsel voor commercieel belangrijke filtreer-organismen te evalueren. In de literatuurstudie werd een overzicht geschetst van het gebruik van gisten in de aquacultuur en van de huidige kennis van de voedingsbiologie van het pekelkreeftje en bivalven. Het experimenteel werk viel uiteen in een eerste deel dat handelde over de proeven met het pekelkreeftje, en een tweede deel dat de resultaten met schelpdieren omvatte. De resultaten worden hier samengevat weergegeven.

EXPERIMENTEEL DEEL 1

STUDIE VAN DE VOEDING EN GROEI BIJ *ARTEMIA* AAN DE HAND VAN BAKKERSGIST

I. BAKKERSGIST ALS VOEDSELBRON VOOR HET PEKELKREEFTJE *ARTEMIA*

1. DEMONSTRATIE VAN DE BEPERKTE VERTEERBAARHEID VAN BAKKERSGIST

Wij hebben op diverse wijzen aangetoond dat de voedingswaarde van bakkersgist voor *Artemia* in de eerste plaats beperkt wordt door de geringe verteerbaarheid van de gistcelwand:

-Pogingen om het pekelkreeftje te kweken op een monodieet van bakkersgist faalden als gevolg van de zeer geringe groei.

-Door middel van gestandaardiseerde kweektesten werd aangetoond dat de voedingswaarde van bakkersgist significant kon verbeterd worden door de celwand volledig enzymatisch te verwijderen. Bovendien bleek een behandeling met 2-mercaptoethanol, welke volgens literatuurgegevens de gevoeligheid van gistcellen verhoogt voor *in vitro* vertering door diverse enzympreparaten, tevens de *in vivo* verteerbaarheid van gist te verbeteren voor *Artemia*.

-Onbehandelde gistcellen behielden hun celinhoud (kleurbaar met methyleenblauw) en een fractie vertoonde zelfs metabolische activiteit (onkleurbaar met methyleenblauw) na opname en uitscheiding door het pekelkreeftje. De faeces van *Artemia* gevoed met de chemisch behandelde gist, vertoonden zeer weinig intacte cellen en bestonden voor het grootste deel uit restanten van verteerde celwanden.

-Tijdens een studie van de koolstofbalans in *Artemia* aan de hand van radioactief gemerkte bakkersgist werd bij hoge gistconcentraties een assimilatieëfficiëntie van 24-31% en 72-76% aangetoond voor, respectievelijk, onbehandelde en behandelde gist.

2. IDENTIFICATIE VAN DE MANNOPROTEINELAAG VAN DE GISTCELWAND ALS DE POTENTIELE HINDERPAAL VOOR DE SPIJSVERTERINGSENZYMES VAN *ARTEMIA*

De gistcelwand wordt in de literatuur beschreven als bestaande uit een buitenste laag van mannoproteïnen en een binnenste laag welke voornamelijk opgebouwd is uit glucaan. De glucaancomponent vormt de eigenlijke ruggegraat van de celwand en wordt beschermd tegen extracellulaire enzymen door de lage permeabiliteit van de mannoproteïnelaag, waarvan de proteïnen onderling gebonden zijn via disulfide bruggen. De huidige studie leverde diverse argumenten voor de hypothese dat het spijsverteringsstelsel van *Artemia* niet beschikt over de enzymen nodig voor het aantasten van de externe mannoproteïnelaag van de gistcelwand. Deze laatste vormt aldus de eigenlijke hindernis voor een efficiënte vertering van de gistcel door het pekelkreeftje.

-Uit de literatuur betreffende de *in vitro* vertering van intacte gistcellen bleek dat een efficiënt mycolytisch enzymcomplex bestaat uit twee componenten: een mannanase of een specifieke protease voor het doorbreken van de mannoproteïnelaag, en een glucanase voor het aantasten van de glucaanmicrofibrillen. Bovendien kan de eerste component vervangen worden door een behandeling met een sulfhydrylverbinding, welke de permeabiliteit van de externe laag zou verhogen door een reductie van de disulfidebruggen. Het verteerbaar maken van bakkersgist voor *Artemia* louter door een behandeling met 2-mercaptoethanol, toonde aan dat het spijsverteringsstelsel alleen niet beschikt over de enzymen nodig voor de penetratie van de mannoproteïnelaag. De afwezigheid van mannanase en de aanwezigheid van glucanase in *Artemia* wordt inderdaad bevestigd in de literatuur.

-De aard van de effectieve chemische produkten bevestigde de hypothese dat de verteerbaarheid van de behandelde gist te wijten is aan de reductie van bepaalde bindingen in de celwand. De chemische behandelingen die de voedingswaarde van gist voor *Artemia* verbeterden bevatten een sulfhydrylverbinding (2-

mercaptoethanol, cysteïne), terwijl behandelingen met andere zwavelhoudende verbindingen (methionine), met zuur of met alkali veel minder of niet effectief waren.

-Licht- en electronenmicroscopisch onderzoek heeft aangetoond dat gistcellen na een behandeling met 2-mercaptoethanol hun leefbaarheid en integriteit van de cel behouden, wat betekende dat de glucaanstructuren van de celwand nog functioneel zijn. De verhoogde kleurbaarheid van de celwand in electronenmicroscopische preparaten na behandeling van de gist met 2-mercaptoethanol, welke tevens werd waargenomen voor gist na behandeling met fosfomananase, bevestigde de hypothese dat sulfhydrylverbindingen de mannoproteïnelaag aantasten.

-Celwandmutanten van *S. cerevisiae*, waarvoor werd aangetoond dat ze gevoeliger zijn voor vertering door *Artemia* dan hun parentale stam, vertonen een afwijkende mannoproteïnestructuur (osmotisch afhankelijke fragiele mutanten) of een verhoogde glucaan/mannaan verhouding (helicase gevoelige mutanten) in hun celwand.

3. VERBETERING VAN DE VERTEERBAARHEID VAN BAKKERSGIST

De traditionele methoden die gebruikt worden om de verteerbaarheid van microörganismen te verbeteren voor hun toepassing als proteïnebron in de dierlijke en menselijke voeding, vernietigen de integriteit van de cellen en zijn dus ongeschikt voor de bereiding van gist als voedsel voor filtreerorganismen. Deze studie identificeerde drie alternatieven voor het verkrijgen van een verteerbare gist zonder verlies van de cytoplasmatische inhoud van de gistcellen.

-Chemische behandeling met sulfhydrylverbindingen (2-mercaptoethanol, cysteïne)

De meest effectieve thiolbehandelingen werden geïdentificeerd via de evaluatie van de voedingswaarde van de behandelde gistpreparaten in gestandaardiseerde kweektesten met *Artemia*. De

efficiëntie van de sulfhydrylverbindingen was hoger in behandelingsmedia met meer alkalische pH en een complementair effect van ethyleendiaminetetraacetaat werd waargenomen voor de behandeling met 2-mercaptoethanol. Een eenvoudige behandeling met cysteïne en bewaring van de behandelde gist door diepvriezen werden voorgesteld voor het bereiden van bakkersgist als voedsel bij de studie van de voedingsbiologie van *Artemia*.

-Hittebehandeling

Autoclaveren verbeterde de voedingswaarde van bakkersgist voor *Artemia*, doch vernietigde tevens de integriteit van de cel. Dit had op korte termijn een lysis van de gistcellen voor gevolg, welke op zijn beurt resulteerde in een vermindering van de waterkwaliteit tijdens de kweek.

-Celwandmutanten van *S. cerevisiae*

Twee types van mutanten van bakkersgist met een afwijkende celwandstructuur (osmotisch afhankelijke fragiele mutanten en helicase gevoelige mutanten) bleken een hogere voedingswaarde te hebben voor *Artemia* dan hun parentale "wild type" stammen. Hoewel het effect van de mutaties op de verteerbaarheid minder uitgesproken was dan dat van de thiolbehandeling, werd aangetoond dat de verteerbaarheid van gist genetisch kan verbeterd worden. Verder onderzoek is evenwel vereist voor de identificatie van de mutaties die verantwoordelijk zijn voor de verhoogde gevoeligheid en de constructie van mutante stammen die op industriële schaal kunnen geproduceerd worden zonder verlies van hun gunstige karakteristieken.

4. GEBRUIK VAN BEHANDELDE BAKKERSGIST VOOR DE Kweek VAN ARTEMIA OP LABORATORIUM SCHAAL

Tot op heden is de gecontroleerde kweek van *Artemia* voor onderzoeksdoeleinden afhankelijk van de kweek van ééncellige wieren. Het gebruik van de thiolbehandelde gist, die kan bewaard worden als een vers produkt door diepvriezen of als een gedroogd produkt waaraan vetten werden toegevoegd, liet toe om de vereiste aan ééncellige wieren voor de kweek van *Artemia* in het laboratorium sterk te verminderen.

-Een totale substitutie van het wier *Dunaliella tertiolecta* door de verse gist was mogelijk voor de kweek van *Artemia* in een kleinschalige kweektest (40 dieren per 90 ml) gedurende de eerste week na ontluiking (gemiddelde overleving en lichaamslengte bedroegen, respectievelijk, 70% en 4 mm). Een monodieet van deze gist leverde op langere termijn een verhoogde mortaliteit op, wat wees op nutritionele deficiënties.

-Een substitutie tot 95% van het wierdieet door de verse gist resulteerde in een gelijkaardige groei en overleving na 14 dagen in vergelijking met de controles die op een 100% wierdieet werden gekweekt. Het 5% wiersupplement volstond evenwel niet voor een normale sexuele differentiatie. De kweek van *Artemia* op een gemengd dieet, bestaande voor 25% uit *D. tertiolecta* en voor 75% uit het gedroogde gistprodukt, resulteerde in een gelijkaardige overleving en een snellere groei en sexuele differentiatie vergeleken met dieren die alleen met wieren gevoed werden.

II. STUDIE VAN DE VOEDSELOPNAME, ASSIMILATIE, EN GROEI BIJ ARTEMIA AAN DE HAND VAN BAKKERSGIST

Niettegenstaande de uitgebreide literatuur betreffende de voedingsbiologie van zoöplankton, hebben tot op heden weinig onderzoekers de voedingskinetiek bestudeerd van *Artemia* onder condities die deze van de intensieve kweek benaderen en die gebruik maken van wiersubstituten. In dit verband had de thiolbehandelde bakkersgist diverse voordelen ten opzichte van wieren voor de studie van de kinetiek van de voedselopname bij *Artemia*:

- De mogelijkheid om de behandelde gist te bewaren garandeert een constante voedselkwaliteit tijdens een reeks experimenten.
- De hoge stabiliteit van de gistcellen onder de experimentele omstandigheden maakt controleflessen zonder dieren, welke gebruikt worden voor de correctie van spontane veranderingen in de celconcentratie ten gevolge van groei of sedimentatie, overbodig.
- Behandelde en onbehandelde gistcellen zijn voedselpartikels van gelijkaardige grootte en samenstelling die verschillen wat betreft hun verteerbaarheid voor *Artemia* en vormen dus een ideaal testsysteem voor het evalueren van het effect van de verteerbaarheid van het voedsel op de voedingskinetiek van een filtreer-organisme.

Deze studie heeft, gebruik makend van de traditionele celtellingsmethode en radioactieve merkingsproeven, bijgedragen tot de volgende aspecten van de voedingsbiologie van het pekelkreeftje:

1. EFFECT VAN DE VOEDSELCONCENTRATIE OP DE VOEDSELOPNAME EN GROEI

- De voedingssnelheid, welke bepaald werd voor diverse ontwikkelingsstadia van *Artemia* aan de hand van de celtellingsmethode, veranderde in functie van de celconcentratie

volgens een rechte lijnige functionele responscurve. De kritische celconcentratie voor het bereiken van de maximale voedingsnelheid daalde tijdens de eerste week van de ontwikkeling van meer dan 500 tot 80 cellen μl^{-1} voor *Artemia* van, respectievelijk, 0.9 en 5.7 mm grootte. De maximale graas- en voedingssnelheid nam toe met de lichaamslengte van het pekelkreeftje. De combinatie van onze waarnemingen en gegevens uit de literatuur liet toe een allometrische relatie te bepalen van de vorm $\text{CR}_{\text{max}} = a\text{DW}^b$ ($a=0.047$, $b=0.918$) tussen de maximale graassnelheid (CR_{max} , $\text{ml ind}^{-1} \text{h}^{-1}$) en het droog lichaamsgewicht (DW , μg) van *Artemia*. De maximale voedingssnelheid, relatief uitgedrukt ten opzichte van het lichaamsgewicht, vertoonde een maximum van 630% dag^{-1} voor *Artemia* met een lengte van 2.4 mm (5 dagen na onluiking) en daalde in functie van de ontwikkeling tot 170% dag^{-1} voor 14 dagen oude dieren.

-Proeven met radioactief gemerkte gist bevestigden de kritische voedselconcentratie en de maximale voedingssnelheid die werd bepaald aan de hand van de celtellingsmethode voor juveniele *Artemia* (3-4 mm).

-De functionele respons van *Artemia* werd beïnvloed door de experimentele omstandigheden. *Artemia* vertoonde een hogere kritische voedselconcentratie en een lagere maximale graassnelheid in een recirculatiesysteem ten opzichte van dieren die werden geïncubeerd in roterende proefbuizen. Deze waarnemingen wijzen op een lagere filtreerefficiëntie in het recirculatiesysteem, mogelijk als gevolg van de sterkere waterstromingen.

-Een experiment waarbij *Artemia* opgekweekt werd bij verschillende constante concentraties van de thiolbehandelde gist wees uit dat het pekelkreeftje een maximale groei bereikt bij een concentratie tussen 800 en 1200 cellen μl^{-1} .

2. EFFECT VAN KWECKCONDITIES OP DE VOEDSELOPNAMEN

De voedingssnelheid van *Artemia* werd bepaald aan de hand van de celtellingsmethode bij een saturerende voedselconcentratie en verschillende condities van dierdensiteit, waterkwaliteit, mechanische verstoring en lichtintensiteit tijdens korststondige (4-6 h) graasproeven.

-De dierdensiteit had geen invloed op de voedingssnelheid van *Artemia* tot densiteiten van 6.7 adulten ml^{-1} in een recirculatiesysteem dat interferentie van neveneffecten (verhoogde concentraties aan metaboliëten, verlaagde zuurstofconcentraties) uitsloot. Dit laatste was niet het geval tijdens proeven die werden uitgevoerd in roterende proefbuizen, waar de voedingssnelheid reeds geïnhibeerd werd bij densiteiten van 3 adulten ml^{-1} .

-*Artemia* bleek relatief resistent tegen blootstelling aan hoge concentraties van anorganische stikstofverbindingen gedurende korte perioden. De voedingssnelheid werd pas afgeremd bij concentraties van 1000 ppm ammonium en 100 ppm nitriet, terwijl geen significant effect werd waargenomen bij nitraat concentraties begrepen tussen 0 en 1000 ppm.

-In beluchte kweeksystemen werd een maximale voedingssnelheid gemeten bij een intermediaire beluchtingsintensiteit, welke het voedsel in suspensie hield en resulteerde in een optimale verdeling van de dieren over het kweekvolume zonder deze mechanisch te verstoren.

-De lichtintensiteit had geen significant effect op de voedingssnelheid van het pekelkreeftje.

3. EFFECT VAN DE VERTEERBAARHEID VAN BAKKERSGIST OP DE VOEDSELOPNAMEN EN ASSIMILATIE

-Aan de hand van de celtellingsmethode werd aangetoond dat *Artemia* de onbehandelde gist aan een significant lagere snelheid opneemt uit suspensie dan de behandelde gist. Het tegendeel werd geobserveerd in proeven met radioactief gemerkte gist, waar 2 tot 5 maal hogere voedingssnelheden werden vastgesteld bij *Artemia* die zich voedt met behandelde gist. De schijnbaar contradictorische resultaten tussen de indirecte (celtelling) en de directe (^{14}C) bepaling van de voedingssnelheid wezen op het recycleren van uitgescheiden gistcellen bij de voeding van *Artemia* met onbehandelde gist. Dit resulteerde in een onderschatting van de voedingssnelheid bepaald met de celtellingsmethode.

-De retentietijd van de diverse gistpreparaten in de darm was omgekeerd evenredig met de voedingssnelheid. De hogere snelheden waarmee de onbehandelde gist werd geconsumeerd, waren geassocieerd met een minimale retentietijd van ongeveer 30 min. De lagere en meer variable voedingssnelheden resulteerde in een retentietijd van 60-100 min voor de behandelde gist.

-Het effect van de voedselconcentratie op de assimilatie-efficiëntie was afhankelijk van het gistpreparaat en de condities van de acclimatisatie voor de aanvang van de proef. Na een acclimatisatie van de dieren aan de experimentele voedselconcentratie daalde de assimilatie-efficiëntie voor de onbehandelde gist met toenemende voedselconcentraties, terwijl een hoge assimilatie-efficiëntie ($> 72\%$), onafhankelijk van de voedselconcentratie, werd waargenomen voor behandelde gist. Een acclimatisatie aan hoge voedselconcentraties resulteerde in een lage assimilatie-efficiëntie ($< 37\%$) voor de onbehandelde gist onafhankelijk van de gistconcentratie. Het effect van de acclimatisatie werd besproken in functie van de respons van de spijsverteringsenzymen bij *Artemia*.

-Het verloop van de assimilatiesnelheid in functie van de voedselconcentratie vertoonde een saturatierespons voor *Artemia* die zich voedt met behandelde gist en daalde met een toenemende concentratie aan onbehandelde gist.

-De bovenstaande waarnemingen lieten toe om het concept van "superfluous feeding" in zoöplankton te koppelen aan de verteerbaarheid van het voedsel. De variatie van de assimilatiesnelheid en assimilatieëfficiëntie in functie van de voedingssnelheid liet bovendien toe om een kritische waarde voor deze laatste parameter te bepalen (ongeveer $10 \mu\text{g C ind}^{-1} \text{ h}^{-1}$). Deze drempelwaarde werd overschreden door *Artemia* die zich voedt op onbehandelde gist, wat een daling van de snelheid en efficiëntie van de assimilatie impliceerde. Een compenserend mechanisme waarbij de voedingssnelheid aangepast wordt in functie van de assimilatie werd voorgesteld om de verhoogde voedingssnelheid te verklaren bij *Artemia* die zich voedt met weinig verteerbare gistcellen.

EXPERIMENTEEL DEEL 2

SUBSTITUTIE DIETEN VOOR LEVENDE WIEREN IN DE Kweek VAN LARVALE EN JUVENIELE BIVALVE MOLLUSKEN

I. HET GEBRUIK VAN GEMANIPULEERDE GISTDIETEN ALS WIERSUBSTITUUT IN DE Kweek VAN JUVENIELE SCHELPDIEREN

Tijdens deze studie werd het gebruik van gemanipuleerde gisten als substituuut voor levende wieren geëvalueerd door middel van kweekproeven die uitgevoerd werden met juveniele stadia van verschillende commercieel belangrijke soorten bivalven (*Tapes philippinarum*, *Mercenaria mercenaria*, *Crassostrea gigas*). De proeven werden uitgevoerd in verschillende locaties (UG, België; South Carolina Wildlife & Marine Resources Department, SC, USA; Tinamenor, S.A., Spanje; Guernsey Sea Farms Ltd., UK). De gistdiëten waren gemanipuleerd wat betreft hun verteerbaarheid en nutritionele samenstelling door, respectievelijk, chemische behandeling en aanrijking met lipiden. De behoeften aan ééncellige wieren werden gekwantificeerd voor juveniele tapijtschelpen ("clams") aan de hand van een aantal voorproeven. Deze studie heeft aldus bijgedragen tot de kennis van de volgende twee aspecten van de voeding van bivalven:

1. HET EFFECT VAN HET VOEDERREGIME OP DE GROEI VAN JUVENIELE TAPIJTSCHELPEN (*M. MERCENARIA*, *T. PHILIPPINARUM*)

Het voederregime voor het wier *Chaetoceros gracilis* dat optimale groei verzekerde in *T. philippinarum* (1-40 mg individueel levend gewicht), correspondeerde met 1 à 1.5% droge stof wieren per nat gewicht schelpdieren per dag ($DW\ WW^{-1}$). Dit werd bevestigd door de volgende waarnemingen:

-Het verloop van de groeisnelheid van *T. philippinarum* in functie van de dosering van *C. gracilis* vertoonde een saturatie tussen 1 en 1.5% $DW\ WW^{-1}\ dag^{-1}$ en daalde bij een verdere toename van de voederdosering.

-In preliminaire testen, waarbij *T. philippinarum* werd gekweekt met *C. gracilis*, *Isochrysis galbana* (kloon T-iso) of een mengsel van beide wiersoorten, werd een optimale groei bekomen wanneer 1.3% DW WW⁻¹ dag⁻¹ werd gevoederd.

-De maximale hoeveelheid *C. gracilis* die door *T. philippinarum* werd verwijderd uit suspensie tijdens een kweektest waarin diverse voederregimes werden uitgetest, bedroeg gemiddeld 1.16% DW WW⁻¹ day⁻¹.

De voedingswaarde van *C. gracilis* voor *T. philippinarum* verminderde door het afscheiden van de wercellen van het kweekmedium door centrifugatie en het bewaren van het wierconcentraat bij 4°C in het duister.

Het voederregime dat resulteerde in maximale groei van *M. mercenaria* (0.4-6 mg individueel levend gewicht), die gekweekt werd met een mengsel van *C. gracilis* en *Isochrysis galbana* (kloon T-iso), was equivalent met 2% DW WW⁻¹ dag⁻¹.

2. HET GEBRUIK VAN GEMANIPULEERDE GISTDIETEN ALS WIERSUBSTITUUT IN DE KWEK VAN JUVENIELE TAPIJTSCHELPEN (*M. MERCENARIA*, *T. PHILIPPINARUM*) EN OESTERS (*C. GIGAS*)

De resultaten die bekomen werden met de substitutie van het controledieet van levende wieren door gemanipuleerde gisten was afhankelijk van de experimentele locatie.

-Een preliminaire test, die werd uitgevoerd met *T. philippinarum* in een commerciële kwekerij in Spanje, toonde aan dat een 80% substitutie van het controledieet (een mengsel van 5 wiersoorten) door gistprodukten resulteerde in een relatieve groeisnelheid van 82-93% ten opzichte van de groei van de schelpdieren in de controlebehandeling. Een 50/50% wieren/gist dieet gaf vergelijkbare resultaten als het 100% wierdieet. Een monodieet van gist had evenwel een zeer lage voedingswaarde voor de tapijtschelp.

-De voedingswaarde van de gistdiëten voor *T. philippinarum* bleek gering tijdens een reeks experimenten die werd uitgevoerd met *C. gracilis* als controledieet in het Laboratorium voor Aquacultuur. De beste groeisnelheid die werd bekomen voor een 50% en 80% substitutie van het controledieet bedroeg, respectievelijk, 88% en 64% van deze die werd waargenomen voor de controlebehandeling. Het supplementair effect van de gistdiëten kon niet verhoogd worden door het verbeteren van de nutritionele waarde van het controledieet als gevolg van het gebruik van een mengsel van twee wiersoorten.

-Tijdens experimenten met *M. mercenaria*, uitgevoerd aan het SC Wildlife and Marine Resources Department (South Carolina, USA), bleek een 50% substitutie van het controledieet (mengsel van *C. gracilis* en *Isochrysis galbana*, kloon T-iso) de groeisnelheid niet significant te beïnvloeden. Het voederen van een 20/80% wier/gist dieet resulteerde in een relatieve groeisnelheid van 75-94% ten opzichte van de controlebehandeling.

-Een verificatietest, uitgevoerd in een Britse commerciële kwekerij met *C. gigas* en *T. philippinarum*, toonde aan dat het vervangen van 80% van het wierdieet resulteerde in een relatieve groeisnelheid van 70-80% in vergelijking met de controlereeks.

Het effect van de toevoeging van diverse componenten op de nutritionele waarde van het gistedieet bleek niet reproduceerbaar. In sommige experimenten met tapijtschelpen werd een verbetering van de groei bekomen door de additie van kleideeltjes (kaolien). Het groeistimulerend effect van een extract van macrowieren, dat werd waargenomen tijdens de preliminaire test met *T. philippinarum*, kon niet worden bevestigd in daaropvolgende proeven. De supplementatie met rijstzetmeel of vetoplosbare vitamines had geen significant effect op de voedingswaarde van het gistedieet.

De voedingswaarde van de gemanipuleerde gisten werd vergeleken met die van gedroogde wierpreparaten tijdens proeven

in het Laboratorium voor Aquacultuur en in de Britse kwekerij. Het vervangen van het wierdieet voor 80% door gistdiëten of gedroogde *Tetraselmis suecica* (commercieel beschikbaar preparaat) resulteerde in een gelijkaardige groeisnelheid bij *T. philippinarum*. Onder dezelfde condities bleek de voedingswaarde van gedroogde *Cyclotella cryptica* (experimenteel preparaat) voor de oester *C. gigas* lager te zijn dan deze van de gistdiëten.

De beste resultaten die behaald werden met de gemanipuleerde gistprodukten bevestigden literatuurgegevens betreffende het succesvol gebruik van gisten als 50% substituut voor levende wieren in de kweek van juveniele schelpdieren. Bovendien werden beduidend betere resultaten geboekt voor een hogere mate van wiersubstitutie ten opzichte van de tot op heden gepubliceerde resultaten. Dit is mogelijks te wijten aan de verbeterde verteerbaarheid en nutritionele samenstelling van de gemanipuleerde gistdiëten. Deze studie identificeerde evenwel de volgende problemen die geassocieerd zijn met het gebruik van gistdiëten, en van artificiële voedsels in het algemeen, als wiersubstituut voor bivalven:

-De groeisnelheid van de juveniele schelpdieren die gekweekt werden op een gemengd dieet van levende wieren en artificiële diëten, nam in veel experimenten af na een periode van 1 tot 2 weken ten opzichte van de groei in de controlebehandelingen. Dit toonde aan dat het gemengd dieet een minderwaardige voedingswaarde heeft ten opzichte van een compleet wierdieet.

-De discrepantie tussen de resultaten behaald met dezelfde artificiële voedsels in het Laboratorium voor Aquacultuur en deze bekomen in andere locaties, toonde aan dat het succes van artificiële diëten beïnvloed wordt door de kweekomstandigheden.

Alhoewel deze studie heeft aangetoond dat gemanipuleerde gistdiëten interessante perspectieven bieden als wiersubstituut voor juveniele schelpdieren, is verder onderzoek vereist voor het verbeteren van de nutritionele samenstelling van de gistprodukten

en het ontrafelen van het effect van kweekcondities op het resultaat van de wiersubstitutie.

II. HET GEBRUIK VAN WIERSUBSTITUTEN EN DE VEREISTE VOOR LEVENDE WIEREN IN DE KWEK VAN LARVALE EN JUVENIELE SCHELPDIEREN: EEN INTERNATIONALE ENQUETE

Niettegenstaande de uitgebreide literatuur betreffende de evaluatie van artificiële diëten voor bivalven op laboratorium-schaal, is er nauwelijks informatie beschikbaar aangaande de nood voor en het effectief gebruik van de diverse alternatieven voor levende wieren in de dagelijkse praktijk van de produktie van bivalvenzaad.

Aan de hand van een internationale enquête, die werd georganiseerd onder de exploitanten van experimentele en commerciële kwekerijen, werd informatie verzameld betreffende de kwaliteit en kwantiteit van de geproduceerde wieren en bivalven, de kosten van de wierproduktie en de ervaring met artificiële diëten. De antwoorden van 50 exploitanten lieten toe om de volgende tendensen vast te stellen:

-De kweek van bivalvenzaad op commerciële schaal is beperkt tot enkele soorten oesters (*Crassostrea gigas*, *Crassostrea virginica*, *Saccostrea commercialis*, *Ostrea edulis*), tapijtschelpen (*Tapes philippinarum*, *Mercenaria mercenaria*, *Tapes decussata*), en sint-jacobsschelpen (*Argopecten purpuratus*, *Patinopecten yessoensis*, *Argopecten irradians*). Deze soorten maakten meer dan 98% uit van de totale produktie die werd gerapporteerd in dit onderzoek.

-De capaciteit van de wierfaciliteiten varieerde van 1 m³ voor enkele experimentele kwekerijen tot meer dan 500 m³ voor een commercieel bedrijf. De totale wierproduktie die gerapporteerd werd door 37 broedhuizen bedroeg bijna 500 m³ wiercultuur per dag, d.i. ongeveer 50 kg droge biomassa. De totale kost, geassocieerd met de kweek van wieren in 20 broedhuizen, benaderde 700 000 US \$ en nam gemiddeld 30% in van de totale produktiekost

van bivalvenzaad. De produktiekost voor levende wieren werd geschat op 50 tot 400 US \$ per kg droge stof.

-Ongeveer één derde van de ondervraagde exploitanten beschouwde de capaciteit van de wierproduktie als een limiterende factor voor het totale kweekproces. De helft van de geïnterviewden planden een uitbreiding van de wierfaciliteiten, terwijl meer dan 90% interesse vertoonde voor een geschikt artificieel dieet. Dit laatste werd geëvalueerd aan de hand van de volgende criteria (in volgorde van afnemend belang): voedingswaarde, kostprijs, gebruiksvriendelijkheid en stabiliteit tijdens bewaring.

-De grote interesse voor wiersubstituten werd verder bevestigd door het feit dat meer dan de helft van de exploitanten in meer of mindere mate hebben geëxperimenteerd met artificiële diëten. Niettegenstaande deze inspanningen, worden alternatieven voor levende wieren zelden toegepast in de dagelijkse routine en meestal beschouwd als "bruikbaar" in geval van een tekort aan levende wieren. Enkele kwekers meldden een succesvolle, gedeeltelijke substitutie van levende wieren door gedroogde *Tetraselmis* (tot 25-50%) en door een geconserveerd wierconcentraat (tot 75%). Tot op heden kon de vereiste voor levende wieren enkel geëlimineerd worden in de kweek van jongbroed van doopvontschelpen (*Tridacna* sp., *Hippopus* sp.).

-De resultaten die werden vermeld voor eenzelfde wiersubstituut waren sterk verschillend naargelang de experimentator en vaak inferieur aan deze die gepubliceerd werden in de wetenschappelijke literatuur. Dit bevestigde de hypothese dat het succes van een artificieel dieet afhangt van de condities waarin de schelpdieren gekweekt worden. Bovendien illustreerde dit de noodzaak om de voedingswaarde van een kunstmatig voedsel uit te testen onder de omstandigheden van een commerciële kwekerij.

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APPENDIX

Questionnaire:

The use of algal substitutes and the requirement for live algae in hatchery and nursery rearing of bivalve molluscs (Chapter X)

QUESTIONNAIRE: THE USE OF ALGAL SUBSTITUTES AND THE REQUIREMENT FOR LIVE ALGAE IN HATCHERY AND NURSERY REARING OF BIVALVE MOLLUSCS

Atn:

	<p><i>Please check if name and address are correct !</i></p>
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Return to: Peter Couteau, Laboratory of Aquaculture & Artemia Reference Center, State University of Ghent, Rozier 44,
B-9000 Ghent, Belgium (label enclosed)

1) SECRECY CLAUSE

The answers on this questionnaire are confidential, and no reference may be made to the above name and address in all further reports.

YES	NO
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(strike out what is not applicable)

2) PROFILE OF THE FARM

a) Production data for 1990 (bivalve species, numbers produced)

Bivalve species	HATCHERY		NURSERY	
	Size (shell length)	Number produced	Size (shell length)	Number produced

b) Number of employees:

Hatchery only:

Hatchery and nursery:

Hatchery, nursery, and grow-out:

3) INVENTORY OF ALGAL SUBSTITUTES

➡ One of the goals of this questionnaire is to inventory all known algal replacement diets for mollusc culture. Therefore we ask you to fill in all the commercial and experimental algal substitutes you have knowledge of.

name of the product	Cost & availability: C = Commercial E = Experimental		contact address for retrieving more information and sample
	C/E	Cost (US\$/kg)	

4) EXPERIENCE WITH ALGAL SUBSTITUTES FOR THE HATCHERY & NURSERY CULTURE OF BIVALVES

Algal substitute:	Culture phase:	Bivalve species:	Frequency of use:	Substitution level:	Feeding level artificial diet:	Remarks:
product name	broodstock, larvae, spat (note size range of spat)		experimental, routinely, as backup,... turnover/year	% of standard algal regime replaced	mg/l (flow-through), or mg/l.day (batch)	with regard to results, cost, possible (dis)advantages, improvements, ...
EXAMPLE: Product X	spat (1-5 mm)	C. gigas	experimental	50% (DW basis)	2 mg/l/day	growth \pm 40 % lower compared to standard algal regime, fast settling, too expensive

5) TOTAL ALGAL PRODUCTION IN 1990:



This question should allow us to calculate the total algal production expressed as cell numbers or dry weight.
Therefore the use of a separate row is recommended when an algal species is produced with different culture techniques or at strongly different cell densities
Winter versus summer production: when production yield is not dependant on the season, fill in only one column and strike out "winter" & "summer"

Algal species: Systematic name of species and strain	Culture technique:			Volumes and cell densities produced in 1990			
	algal growing container - bag, tank, kallwall, ... - greenhouse/outdoor	number of containers x size	continuous/ batch	SUMMER*		WINTER*	
				Volume (m³/day)	Cell density (cells/µl)	Volume (m³/day)	Cell density (cells/µl)
<u>EXAMPLE:</u> Isochrysis galbana, Tahitian strain (Tiso)	bags, greenhouse	30 x 350 l	continuous	20	1000	15	600
	tanks, outdoor	10 x 15 m³	batch	10	800	6	400

6) ADDITIONAL QUESTIONS

6A

TOTAL ALGAL PRODUCTION COST IN 1990: taking into account <u>all costs</u> (labor, maintenance, depreciation of installations, energy, nutrients,...)US \$
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6B

ESTIMATE OF ALGAL COST AS PERCENTAGE OF TOTAL SEED PRODUCTION COST:%
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6C

ESTIMATE OF PRODUCTION COST PER KG DRY WEIGHT OF ALGAE:US \$/kg DW
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6D

DO YOU CONSIDER ALGAL PRODUCTION TO BE A LIMITING FACTOR FOR YOUR PRODUCTION ?	YES
	NO

6E

ALGAL PRODUCTION WILL BE EXPANDED IN THE NEAR FUTURE	YES
	NO

6F

WOULD YOU CONSIDER USING AN ARTIFICIAL DIET IF ONE WOULD BE AVAILABLE?	YES
	NO

6G

IF YES, RANK IN ORDER OF IMPORTANCE THE FEATURES OF SUCH A DIET (1 = most important) -price/kg -storage life -food value (= performance compared to live algae) -ease of use "...."	1	
	2	
	3	
	4	
	5	