8. ORGANIC REGULATION OF PHYTOPLANKTON FERTILITY[1]

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1. Introduction[1]

A. The Problems of "Good" and "Bad" Waters

The search for biologically active substances in sea-water is a recent approach to the age-long problem of "bad" and "good" waters. There is no doubt that water-masses differ in ability or inability to sustain growth of various organisms. Most of the interest in the properties of waters has centered on the words "good" and "bad"[2] for the organisms we harvest from the sea. In this respect, the most fertile areas of the sea are near the coasts, above shallow bottoms (banks), and in zones of water mixing (merging of different bodies of water, upwelling, etc.). Because all life in the sea depends on the primary producers of organic matter, most of our knowledge centers on the algae and we will restrict our presentation of data almost solely to them.

The early work on the physical and chemical conditions governing growth of phytoplankton in the sea was guided by the knowledge of the time that photosynthetic organisms require only mineral nutrients. Of these, nitrogen, and especially phosphorus, assumed primary importance because they are essential and are often present in waters in "limiting" quantities; hence the growth of phytoplankton, it was thought, should correlate with the N and P content of waters. This is often, but not always so: e.g. productivity along the coast of California is far less than around the British Isles, yet the phosphate content of California waters is many times higher. In the meantime, many nutritionists found that algae need, besides N and P, some organic substances present in several natural extracts (soil, peat, seaweeds, etc.) and also in sea-water (Allen, 1914). This led to the recognition that many algae need vitamins (reviewed by Provasoli, 1956; Droop, 1957; Provasoli, 1958).

The present interest in the organic components of waters, and particularly in the biologically active substances, is, however, due to the powerful arguments advanced by Lucas (1947, 1949, 1955, 1958) that some water organisms might affect the growth of other organisms by producing necessary nutrients, by removing inhibitory compounds, and by excreting inhibitory substances. The vitamin requirements of algae, what we already know of the vitamin cycle in sea-water and the production of antibiotics by marine organisms dramatically

[1] Aided in part by contract NR 104–202 with the U.S. Office of Naval Research and by grant G-10783 from the National Science Foundation.

[2] The utilitarian meaning attached to the terms "fertility" and "good and bad waters" is far too general and often misleading. Waters favorable for spring diatoms are not necessarily favorable for dinoflagellates, or tuna, and no water is bad enough not to support some sort of community. While it is obvious that the plants and animals living in the same biocoenosis share common requirements and resistance to a number of environmental factors, we can expect that other factors are peculiarly important for each species or taxonomic groups. Therefore, any discussion about the quality of waters should be in reference to a specific organism or group.

[*MS received October, 1960*]

confirm Lucas's postulates. But all this, perhaps even for the algae, is only a fraction of a bigger, more fascinating picture.

B. Considerations on the Chemical Approach

Extracting and identifying the organic compounds in sea-water is complex because the dissolved organic C averages 2 mg/l. (maxima up to 20 mg/l.). These minimal quantities have to be separated from the 35,000 mg of inorganic salts in a liter of sea-water; the salt content of fresh water varies from 50–800 mg/l. In these few mg of organic matter we can expect to find any known and a few unknown organic compounds; some of them, like vitamins and other biologically active substances, are present in 10^{-9} quantities or less. The main obstacle is the separation of organic components from inorganic salts. This necessary step for any detailed analysis is by no means simple even for fresh waters. The extraction and identification of the microcomponents is further complicated by the need to process very large quantities of water. Jeffrey and Hood (1958) have reviewed and evaluated the various methods for isolation of organic matter.

To determine the effectiveness of various methods, they use a ^{14}C-labeled test solution: a large sample of sea-water fertilized with N, P and labeled bicarbonate was kept in light for 90 days to favor algal growth, then placed in darkness for a long period to allow decay of the organic materials and filtered through an HA millipore filter. The effectiveness of the various methods was also tested on the recovery of biologically important compounds dissolved in natural sea-water.

Column absorption, electrodialysis and co-precipitation permit the isolation of almost all the dissolved organic matter. Co-precipitation with $FeCl_3$ or other metals at alkaline pH values is the most promising method. The recovery is close to 100% and the concentration factor is 10,000. However, the removal of the co-precipitating ions without altering the organic materials offers some problems; further, it is not known whether the metal ions and alkali destroy or change some of the organic compounds. Column absorption, especially with pretreated activated carbon, is very effective, but recovery from the absorbent is difficult and partial. Electrodialysis with cellulose membranes retains 97% of the organic matter and is ideal for the separation of colloidal micellae and large molecules, but inorganic sulfate is also retained and has to be eliminated by electrochromatography, and large volumes of water must be evaporated at low temperature. Electrodialysis with ion-exchange membranes employed for industrial desalting of sea-water permits work with large volumes; the recovery of organics is equal to that with electrodialysis. Electrodialysis perhaps may be improved by the use of several large cells separated by physical barriers of membranes of increasingly fine porosity; large sheets of the existing dialysing membranes of very fine and graded porosity are produced for other uses.

Ion-exchange and solvent extraction permit only a partial recovery of the dissolved organic matter. Ion-exchange resins offer no advantages: impracti-

cally large volumes of resins are necessary, huge amounts of liquid must be evaporated, and the resins give a large organic blank.

Extraction with water-immiscible solvents or with solvents after evaporation of sea-water is a simple and quick way to prepare organic concentrates without altering materially the organic compounds. Phenol, 2,4-lutidine, ethyl acetate and benzoyl alcohol remove substantial quantities of organic material. Even though any one solvent separates only a fraction of the organic matter, this method has been successful in extracting and identifying several organic substances in sea-water.

It might be interesting to try the method of "gel filtration", which acts as a molecular sieve. Like dialysis, it separates large molecules from small, but apparently is far more rapid and versatile. Columns or beds made of small grains of cross-linked dextran [Sephadex G–25 and G–50 produced by Pharmacia Laboratories (American address, Box 1010, Rochester, Minnesota)] form a gel which retards and apparently traps small molecules in the intramolecular spaces of the polymer, while large molecules do not enter the gel phase and flow freely in the interstitial fluid (i.e. between the grains of polymer molecules). This system has been employed to separate into two distinct fractions ammonium chloride from the proteins in serum (Porath and Flodin, 1959), and for the fractionation of polypeptides and proteins (Porath, 1959, 1960). It offers several advantages over dialysis: it is as rapid as filtration and can be used with the same results on a small or a large scale; high recovery is achieved in the presence of electrolytes; the polymer is inert to charged groups and stable in the pH range 2–10; and the gel beds retain their original properties even after daily use for a period of months. Two grades of cross-linkages are available, and perhaps others will be designed to trap or retard smaller molecules. Chlathrates or other systems of molecular sieves may be even better.

The direct chemical approach, hampered as it is by the inherent technical difficulties, has contributed only a few data on some of the organic substances present in relatively large quantities in sea-water. Better methods of extraction could be developed if one could direct the marine chemist to specific compounds.

The problem of the organic substances in waters and their fertility, being fundamentally biological, has also been studied by biologists. The first isolated attempts to solve the problem for algae have developed along several lines which have contributed some valuable information: the nutritional approach through culture of organisms, the biological analysis of sea-water, and the chemical analysis of the organisms and their excretions. These independent developments are all part of a comprehensive logical attack which, though mainly tested on algae, could be applied to all water organisms.

We can look at the problem in two ways: the origin and fate of the organic compounds in waters, and the nutritional needs of the organisms.

C. Origin and Fate of Organic Substances

Obviously, almost the totality of organic substances is derived, in the present state of our planet, from the activities of living organisms.

Most water organisms are bound forever to the water environment and the cycle of organic matter is intimately dependent upon their activities. Lucas's theory of "non-predatory" relationships between water organisms includes an important part of these events because in waters the food chain is simultaneously a food pyramid and a pile of assorted excretions and metabolic products of micro-organisms, plants and animals. These organic substances fully express their biological activity because they are soluble or coupled in various ways to hydrophilic molecules and freely diffusible in the continuum of rivers, lakes and seas.

The other sources of organic matter in the sea are the rivers, which carry in the left-overs of the activities of fresh-water organisms, the elutes of rocks, and leachings of soils rich in trace metals and in the chemical residues of the activity of terrigenous microbes, plants and animals. Fluvial contributions to the open seas are minimal, and if felt at all, must be long-term; but they are significant locally, particularly in locations such as great rivers ending in relatively land-locked parts of the sea. One example is the effect of rivers and the effluent from Lake Okichobee after heavy rains on blooms of *Gymnodinium breve* (Wilson and Collier, 1955). Therefore quantitatively the plankton, especially the phytoplankton which represents the bulk of life, is the main source of organic matter in the sea, either through its secretions or decomposition.

The fate of the organic substances thus produced, and therefore the quantities of organic substances actually found in the waters, depends on the kinetics of production and consumption. Only slowly metabolizable organic substances should tend to accumulate and, being in larger quantities, may be easier to extract. Though these substances are of little or no nutritional value, they may have important functions such as the solubilization and chelation of trace metals (Shapiro, 1957; Fogg and Westlake, 1955); antibiosis, CO_2 and rH buffering; emulsification of fatty substances; and chemical conditioning of sexuality, spawning, moulting, etc. Such substances do accumulate in fresh waters as the humic substances, the yellow acids (Shapiro, 1957) and peptides (Domogalla, Juday and Peterson, 1925). In sea-water the situation seems to be different: the C/N ratio of the organic solutes is low (2–6) while in fresh waters it is high (> 10). This difference may, however, be only apparent, because in the sea the origin of the soluble organics is almost entirely autochthonous (from organisms) while in fresh waters the effect of the surrounding soil and its leachings are very important (see discussion on page 173).

Conversely, organic substances which are in continuous demand as nutrients should be present in small quantities. Direct chemical analysis of the waters can be of little use; studies of turnover of labeled compounds seem more helpful. Granted that most of the inherent difficulties of identifying and following the fate of a variety of organic compounds in sea-water remain, this analysis is inescapable if we want to trace the important chemical events in the environment. The important thing is to know what is worth tracing.

Since we are dealing with knowns and unknowns produced by organisms, we must go to the origin, to the aseptic cultures of the organisms themselves:

chances are better there of finding larger quantities of the products of their metabolism. Besides, one can determine the conditions under which some substances are produced and released into the environment, study the chemical composition of the organisms, employ them as food organisms, or let them die and be broken down by micro-organisms. This experimental simplification does not mean that the task is simple.

In effect, what we are looking for is the chemical relationships between organisms mediated by the water environment; of the hundreds of organic substances present in the waters only a few may be important for a particular receiver species.

D. Nutritional Requirements

The complementary approach to the problem is the study of the nutritional, morphogenetic and physiological requirements of the organisms. Obviously, the nutritional requirements can be identified only in the absence of any other organisms.[1] Axenic cultures are also indispensable in discerning the chemical relationships between the organisms of a given biocenosis. The lack of these exogenous contributions often results in no, or poor, growth, in abnormal morphogenesis, incomplete life cycles, mortality of larvae, difficulties in moulting, lack of copulation and ovideposition, etc. To impute these events to the artificiality of *in vitro* experimentation is to reject the opportunity of finding the cause and to miss potential bioassays.

The methodology and principles of nutritional studies are well established. Briefly, when attempting the isolation in axenic culture of the organism, it is advantageous to reproduce as far as possible the environmental conditions, both physical and chemical. Since often we do not have a precise knowledge of the environment, or we are resorting to culturing as a means to learn more about the biotic factors of the environment, we have to break new ground. We cannot afford to neglect any scrap of information or hint, experimental or observational. For instance, in dealing with an exacting alga, knowledge of the nutrition of other algae may be inadequate or irrelevant; one may have to resort to information on the nutrition of higher plants, plant-tissue culture, and animal nutrition. This applies to inorganic and organic requirements as well as to how to solve particular technical problems such as pH, rH, trace-metal buffering, emulsifiers, etc. In any case it is also advisable to try a variety of natural products and extracts rich in unknowns (soil, algal extracts; powders, infusions or hydrolysates of various organs; blood, coconut milk; etc.). If any of these proves useful, substitution of the natural products with their known major components will follow and may lead to a complete substitution with chemically defined compounds or even to a new growth factor or regulator (Hutner, Cury and Baker, 1958; Hutner, Provasoli and Baker, 1961).

Nutritional studies, in defining the factors needed for growth, indicate to the marine chemist and the ecologist which are the important parameters of the environment. The assumption is, if an organism requires absolutely a

[1] This is a wholly unnatural condition but so is the light-and-dark-bottle method. Somehow we seem to understand more easily the mechanism of a watch when we tear it apart, even if unable to reassemble the parts, let alone reconstitute a functional unit.

substance *in vitro*, this metabolite, or its physiological equivalent, must be in the environment: most of the algal flagellates of the littoral zone need vitamin B_{12} and indeed this vitamin is measurable in sea-water. However, the nutritional studies, to have ecological meaning, should be extensive and not limited to growth factors (which are, after all, only one of many important variables).

As explained recently (Provasoli, 1958), one needs to know—besides the minimal mineral and organic requirements—the idiosyncrasies, tolerances and abilities to utilize a host of N, C, and P compounds. The success of an organism is controlled by the quantity and quality of metabolites; deficiency or excess are both detrimental.

Similarly, emphasis on the limiting factors of the environment implies belief in a simplification that can rarely be true. Just as interactions between organisms are sometimes fundamental, so are interrelations between chemical and physical factors.

Thermophilic bacteria, *Ochromonas malhamensis*, *Euglena gracilis*, etc., can be grown above the normal "lethal" temperature limit of the species if the vitamins and metals are increased manyfold; temperature is a nutritional variable (Baker *et al.*, 1955; Hutner, Baker *et al.*, 1957; Hutner, Aaronson *et al.*, 1958). Braarud (1945) noted that some boreal diatoms (*Chaetoceros* spp.) bloom in the warmer water (18°–20°C) of the Oslo Fjiord, but only in polluted areas. Temperature and salinity interact affecting the growth of marine fungi (Ritchie, 1957, 1959). Gradients of light and temperature markedly affect production and the ratios of the photosynthetic and accessory pigments of algae (Halldal, 1958). Similarly, the folic acid requirement of some organisms can be spared or substituted by *p*-amino-benzoic acid, the B_{12} requirement by other cobalamins, methionine or deoxyribosides; some photosynthetic organisms can grow in darkness on exogenous carbon sources.

Axenic cultures of ecologically important organisms are the most fruitful biological approach to the problem of fertility of waters. In the course of determining nutritional needs of organisms one often detects the indispensability of organic substances which act at concentrations so low that they are not easily assayable chemically. The organism which has this need, or others more technically suitable because of their rapid and abundant growth and because of their specificity, can be employed for the quantitative determination of the active principle (see Chapter 9). Axenic cultures are also necessary, as mentioned, to determine the production of "external metabolites" and excretions which might affect growth and morphogenesis of other organisms. Their chemical composition can be analyzed giving information on the nutritional needs of their predators.

E. Biological Analysis of Sea-Water

Another interesting approach to the fertility of waters is the so called "biological analysis". Selected organisms can serve to differentiate water-masses into "good" or "bad" by their growth responses or other measurable physiological events (hatching, setting of larvae, etc.). Once established, one can try with different treatments to see whether the "bad" waters lack some nutrients

or contain inhibitory substances for the test species. When sufficient nutritional information is available for a group of organisms, we can add to freshly collected samples of waters (with their living population) a mixture of all the nutrients required, the mixture minus one of the nutrients, and each single nutrient. We have thus a means of discerning the lack of some nutrients and of evaluating by the incidence of scarcity, which parameter is more ecologically important. Other profitable uses of this approach can be found: H. Kylin (1941), employing germlings of *Ulva*, found that waters of 70-m depth were low in N, P, Fe, Zn, Co, Mn; A. Kylin (1943) determined the need of them and level of trace metals favorable for *Ulva*.

2. Data from Chemical Analysis

A. Quantities of Organic Matter in the Seas

Organic matter into water is arbitrarily divided into: (*a*) particulate (living organisms + detritus = seston) and (*b*) "dissolved" organic matter (in general particles < 1 μ). Strikingly, the amount of dissolved organics exceeds by many orders of magnitude the amount of particulate: in the euphotic zone, rich in phytoplankton, it is seven to eight times greater and in deep waters, in which the plankton is scarce, it may be up to a thousand or more times greater.

Only recently has it been possible to analyze the dissolved organic carbon in sea-water. The first reliable method was developed by Krogh and Keys (1934); newer methods were developed by Kay (1954), Skopintsev (1959) and Duursma (1960). Duursma, in a comprehensive, stimulating paper, reviews methods for determining the dissolved organic C, N and P in sea-water, the data obtained and their significance. The quantities of total organic C in open seas vary from 0.2–2.7 mg C/l. Higher values are found in more landlocked areas: 3.3 in the Black Sea, 4.6 in the Baltic, 6 in the Sea of Azov, and 8 in the landlocked coastal area of the Dutch Wadden Sea (Table I). Contrary to the early data of Krogh, who found an almost homogeneous distribution from surface to deep water, wide variations were found by the other investigators. Especially significant are the data of Duursma derived from several hundred analyses in the Wadden, North and Norwegian Seas, and in the North Atlantic Ocean. He found that "there are well defined areas where maximum and minimum concentrations occur, both horizontally and along the vertical" (Duursma, *op. cit.*, p. 133). Water-masses can be characterized by their peculiar concentrations in organic nitrogen and carbon, besides the usual variations in salinity and temperature. His distribution charts and data indicate that concentrations of dissolved organics may permit the identification of smaller water-masses, especially if one considers also the C/N ratios, which vary widely and independently. In a year's study of the station L.V. *Texel* in the North Sea, Duursma found clear seasonal variation in dissolved organic matter. Since the highest concentrations of organic C are reached several weeks after phytoplankton blooms, he considers that nearly all the dissolved organic compounds are produced by breakdown of the dead phytoplankton and that the excretion

TABLE I

Dissolved Organic Matter in Sea-Water[a]

Author	Locality	mg C/l.	mg N/l.
Krogh (1934)	Atlantic Ocean (Bermuda)	2.35 ± 0.07 (along vertical)	0.244 ± 0.08 (same)
Dazko (1939)	Black Sea	2.4	
Dazko (1951)	Black Sea	2.83–3.36 (seasonal variations)	
Dazko (1955)	Sea of Azov	4.63–6.02	
Kay (1954)	Baltic	2.0–4.6 (maximum in euphotic zone)	
Plunkett and Rakestraw (1955)	Pacific (3 stations)	0.6–2.7	
Skopintsev (1955)	North Atlantic	1.04–1.97	
Skopintsev (1959) (referring to other Russian investigators)	Atlantic Ocean	2.40–2.48	0.24–0.26
	Pacific	0.98–2.68	0.07–0.11
	Greenland Sea	2.0–2.1	0.03–0.38
Duursma (1960)	North Atlantic	0.2–1.3	0.04–0.4
	Norwegian Sea	0.45–1.38	0.1–0.21
	North Sea (L.V. *Texel*)	0.5–1.8	0.08–0.54
	Wadden Sea	1.0–8.0	0.1–0.6

[a] Derived from Duursma's table II (1960), with added concentration ranges found by Duursma.

of dissolved organic matter by living phytoplankton is not directly demonstrable in the sea. This conclusion is contrary to the finding, under experimental conditions, that several species of algae excrete considerable amounts of organic solutes. This discrepancy deserves further work to find out if the excretions are produced by healthy cells during logarithmic growth or by resting or dying cells, and what are the conditions favoring release of external metabolites. More detailed field studies are also needed to see whether Duursma's findings are general. He determined at the microscope "the relative proportions of the number of several species" (Duursma, *op. cit.*, p. 29), but the plankton populations were not studied in detail; this could give a more precise meaning to the data on particulate matter and chlorophyll determinations.

The practical and precise method for analyzing dissolved organic carbon of Duursma allows finally a detailed and more biologically significant definition

of water-masses, and already poses some new puzzles. It is interesting for instance to compare the C/N ratios found in fresh water and sea-water. Birge and Juday (1934), in grouping the data from several hundred lakes according to total organic carbon, found a C/N ratio of 12.2 for lakes containing 1.0–1.9 mg C/l. Duursma found in North Atlantic deep waters of similar C content a C/N ratio varying from 2.5–6.5 with a mean of 2.7. The surface waters (0–200 m) of the same transect (a meridional section running southward from Cape Farewell in Greenland, 60°N, to about 43°N) show a tendency toward large variations in C/N ratios (up to 20–30) at higher temperatures ($>7°C$) whereas at lower temperatures ($<7°C$), and especially in September, the C/N ratios are closer to the values of deep water. Living phytoplankton and zooplankton have an average C/N ratio of 5.7–6.7 with limits between 4 and 14 (Fleming, 1940), proteins of 2–3, and skeletons of invertebrates 2.9–3.3 (Vinogradov, 1953). Why the striking difference in C/N ratios between fresh waters and sea-water? Are the high values in fresh water only due to the influence of the land on lakes, or to physical conditions such as depth and size of the basins? Hutchinson (1957) has calculated from the data of Birge and Juday the amounts of the two types of dissolved organic matter found in lakes: one (allochthonous) derived from bogs, peat and soil has a brown color and an approximate C/N ratio of 45–50; the other (autochthonous) derived from the decomposition of plankton has an approximate ratio of 12. Skopintsev (1959) found that the end product of complete decomposition of marine organisms is a "water humus", a carbon–protein complex (possibly a pectin or uron) of high biochemical stability. This product has a C/N ratio of 10, remarkably close to the one calculated by Hutchinson. In the open sea we cannot expect to find significant amounts of allochthonous dissolved organic matter but we could expect to find the autochthonous, yet we find lower ratios (2–6) in some surface waters and in deep waters. The warm surface waters of the North Atlantic have variable and high ratios, while the cooler surface waters have ratios similar to the deep waters. This could be due to a slower decomposition in cool waters: the dead organisms will be decomposed only partially before they sink; in deep water the scarcity of micro-organisms and the cool temperature may account for the stability of C/N ratios of the dissolved organic portion. If so, the dissolved organic matter in deep water should still be a good nutrient and not a product resistant to bacterial attack. Many experiments with oxygen bottles in fact show that the deep waters can support a good population of micro-organisms (provided solid surfaces are available). This, however, does not explain the difference, presumably in the early phases of decomposition, between fresh water and sea-water. From the starting point of 6 (ratio in living plankton) decomposition produces in fresh waters higher ratios (i.e. N is preferentially consumed) and in sea-water lower ratios (C is preferentially consumed or high-N compounds are produced). If we assume that, during decomposition in sea-water, high-N products are released in large amounts, then the low C/N ratios found do not exclude the presence, though in low quantities, of stable products like "water humus" which have high C/N ratios. Quite likely decomposition

proceeds differently in sea-water: production of amines is after all quite noticeable in sea-water food at the market; and the bacterial flora of sea-water is quite different from that of fresh waters.

B. The Nature of Dissolved Organic Substances

Vallentyne (1957) has comprehensively reviewed the organic compounds found in lakes, oceans, sewage and soil. Data on the identification of some of the organic solutes in sea-water are summarized in Table II.

TABLE II

Organic Compounds Identified in Sea-Water

Substances	Quantities	Locality	Method	Authors
"Rhamnoside" Dehydroascorbic acid	Up to 0.1 g/l. present	Inshore waters Gulf of Mexico	Activated charcoal absorption, ethanol elution	Wangersky (1952)
"Carbohydrates" (as arabinose equivalents)	0.0–20 mg/l.	Estuary, Gulf of Mexico	*N*-Ethyl carbazole	Collier *et al.* (1953)
"Carbohydrates" (as sucrose equivalents)	0.14–0.45 mg/l.	Pacific Coast, U.S.A.	Anthrone and *N*-ethyl carbazole	Lewis and Rakestraw (1955)
"Carbohydrates" (as arabinose equivalents)	0.0–2.6 mg/l. (max. of 12 mg/l. at surface,29°N, 80°, 31'W)	South Atlantic (30°N–25°N)	*N*-Ethyl carbazole	Anderson and Gehringer (1958)
	0.0–3.0 mg/l. (23% = 0.0; 50% = 0.2–1 mg/l.)	Continental Shelf, Gulf of Mexico (50 mg/l. in red tides of *G. breve*)	*N*-Ethyl carbazole	Collier (1958)
Citric acid	0.025–0.145 mg/l.	Littoral Atlantic (French Coast)		Creac'h (1955)
Malic acid	0.028–0.277 mg/l.	Northeast Pacific (surface and inshore)	Chloroform or ether extraction at pH 3; partition chromat. on silica gel column	Koyama and Thompson (1959)
Acetic and formic acids[a]	< 0.1 mg/l.			
Fatty acids (up to 20 carbons)	0.4–0.5 mg/l. (weight of methyl esters)	Gulf of Mexico	Ethyl acetate extraction at pH 2. Gas–liquid chromatography	Slowey *et al.* (1962)

TABLE II—(*cont.*)

Substances	Quantities	Locality	Method	Authors
Amino acids (from hydrol. proteins)	Traces to 13 mg/ m^3 [b]	Gulf of Mexico, Yucatan Strait, Reef (British Honduras), Caribbean	Co-precipitation of organic material with $FeCl_3 + NaOH$; acid hydrolysis; paper and ion-exchange chromat.	Tatsumoto *et al.* (1961)
Vitamin B_{12}	Present	See Table XII		
Plant hormones	Present	North Sea	Chloroform extraction at pH 5; ether extract of residue. Measured biologically	Bentley (1959)

[a] Acetic, formic, lactic, and glycolic (up to 1.4 mg/l.) acids are liberated from breakdown of larger organic molecules during the long extraction procedure (4–5 weeks).

[b] 18 amino acids were found in the hydrolysates. The amounts and kind of amino acids vary widely in samples.

The data are few and limited. Aside from the "carbohydrates" estimation, based on diverse localities and depths, the other data represent successful extractions and identifications of the components of the dissolved organic matter and are limited to a very few samples of sea-water. The *N*-ethyl carbazole or anthrone methods are not strictly specific for carbohydrate. Nevertheless, this colorimetric reaction can be quantitated and serves to measure a biologically active organic fraction which affects quantitatively the pumping rate of oysters (Collier *et al.*, 1950, 1953). This organic fraction may be of algal origin; the filtrate of cultures of several marine flagellates and diatoms contains organic substances reacting with *N*-ethyl carbazole (Collier, 1958; Guillard and Wangersky, 1958). The oyster activity of such filtrates has not been reported. Perhaps it may be possible to separate, and later analyze, the *N*-ethyl carbazole reactive fraction from the organic solutes of sea-water extracted by co-precipitation with Fe in alkali.

3. Organic Products of Algae and Bacteria

A. Excretion of Carbohydrates

Several fresh-water green algae, mostly *Chlamydomonas* species, liberate soluble organic products in the culture medium. Six *Chlamydomonas* release as

solutes from 10 to 45% of the total organic matter produced (Allen, 1956; measured as organic material oxidizable by dichromate). The products formed include polysaccharides, glycolic, oxalic and pyruvic acids and only traces of organic nitrogen. Fifteen species of fresh-water *Chlamydomonas*, two *Chlorosarcina* and one *Gloeocytis* liberate in the medium 10–115 mg/l. of soluble polysaccharides. This excretion constitutes 2 to 25% (*Chlamydomonas mexicana*) of the total organic matter produced (Lewin, 1956). The main components of the polysaccharides (precipitated by ethanol and hydrolyzed with H_2SO_4) are galactose and arabinose for all species except *C. ulvaensis* (glucose and xylose). The associated sugar moieties are fucose, rhamnose, mannose, uronic acids and several unidentified components (identification by chromatography).

Allen found that the production of extracellular compounds parallels growth, i.e. the substances are released by living and dividing cells; the same applies to *C. parvula* (Lewin, 1956) and *Anabaena cylindrica* (Fogg, 1952); however, production of mucilage around the cells continues in old cultures, reaching 40–60% of the total organic matter produced (*Chlamydomonas parvula* and *C. peterfii*) (Lewin, 1956).

Also unicellular marine algae, in bacteria-free culture, excrete large amounts of "carbohydrates" (Table III). These results were obtained with the *N*-ethyl carbazole hydrolyzing method, which gives a purple-red coloration with carbohydrates; optical densities were converted to glucose equivalents, except for *Prorocentrum* sp. (arabinose equivalent). Guillard and Wangersky (1958) found that excretion of carbohydrates does not parallel growth: it is very low and does not exceed 3 mg/l. during exponential growth. Carbohydrates accumulate in the medium at or right after maximum growth (10^6–10^7 cells/ml). This accumulation may come from lysis of dead cells or a modified metabolism when division is hampered by nutritional deficiencies but photosynthesis is active; *Navicula pelliculosa* only under these conditions makes huge amounts of capsular polysaccharides (J. C. Lewin, 1955).

Not all marine organisms behave similarly: multiplying *Katodinium dorsalisulcum* produces a polysaccharide mucilage (McLaughlin *et al.*, 1960), so much so that mucoid masses form; in stationary cultures they float to the top because of entrapped bubbles of photosynthetic oxygen. When growth has reached an optimal cell concentration (10^4 cells/ml), the cells keep producing polysaccharide until the medium gels (1.4–2.6 g/l. of polysaccharide are produced in a month). Hydrolysis of the polysaccharide yielded glucose, galactose and fructose. The supernatant of *Katodinium* was the only one to give a carbazole reaction typical of sugar (purple); the supernatants of *Monochrysis lutheri* and *Prymnesium parvum* gave different colors, respectively straw-green and light green. Guillard and Wangersky (1958) report for the same strains of these organisms, and with the same reagent, a good production of "carbohydrates" (Table III). They do not mention any discrepancy of the color reaction, yet they (Wangersky and Guillard, 1960) noted a different color reaction (blue) obtained with the *N*-ethyl carbazole method in *Amphidinium carteri* supernatants. This filtrate gave the usual purple carbohydrate

TABLE III

Production of "Carbohydrates" in Marine Algae[a]

Species	Carbohydrate produced (mg/l.)	
	Before maximal growth	Highest value (stationary phase)
CHLOROPHYTES		
Dunaliella euchlora	3.1	9.0
Chlorella sp. (No. 580 Indiana Univ. Cult. Coll.)		9.0
Chlamydomonas sp. ("Y" R. Lewin)	2.1	10.6
Chlorococcum sp.		27.0
Pyramimonas inconstans	2.8	5.4
DIATOMS		
Cyclotella sp.		1.5
Nitzschia brevirostris		25.6
Melosira sp.		60.0
CHRYSOMONADS and CRYPTOMONADS		
Isochrysis galbana		25.0
Monochrysis lutheri	1.7	15.7
Prymnesium parvum	5.8–15.9	123.0
Rhodomonas sp.	1.9	8.8
DINOFLAGELLATES		
Amphidinium carteri (interference, see text)		> 5.0
Prorocentrum sp.		~ 20.0
Katodinium dorsalisulcum[b]		0.6–2.4 g/l.

[a] Data from Guillard and Wangersky (1958); *N*-ethyl carbamate as glucose equivalents, except for *Prorocentrum* (data from Collier, 1958; as arabinose equiv.).

[b] From McLaughlin *et al.*, 1960; for extraction of the polysaccharide, see text.

reaction after the blue-reacting material was eliminated by dialysis. The substance responsible for the blue coloration and fishy odor is apparently the end-product of the hydrolysis of an analog of acetylcholine.

Brown and red seaweeds produce large quantities of mucilaginous polysaccharides; several of them, e.g. alginic acid, agar, etc., are economically important [see papers in: Braarud and Sorensen (1956), and Lewin (1955) for polysaccharides of other marine algae]. These polysaccharides, being utilized by several bacteria as C sources, may be ecologically important; the specialized microflora which they support probably produces vitamins and other growth factors.

B. Excretion of Organic Nitrogen

Extra-cellular nitrogenous products have been noted frequently in cultures of bacteria or blue-green algae fixing nitrogen, but no data were available on the nature of these substances before the work of Fogg (1952) on *Anabaena cylindrica*: liberation of extra-cellular organic nitrogen accompanies its growth. The relative amounts of the excreted nitrogen vary during growth: as much as 50% of the total nitrogen taken up is excreted during the early logarithmic growth; it decreases to a minimum (10–20%) at half growth; increases again in older but still healthy and growing cultures; and moribund material liberates large quantities of soluble organic nitrogen (but cultures in these conditions were not used by Fogg in his work). The amount excreted is not affected appreciably by cultural conditions except in the later stages of growth: it may be increased by nutritional deficiencies such as Fe, or slightly decreased by Mo deficiencies (Mo deficiencies affect nitrogen fixation and nitrite assimilation in *A. cylindrica*; Wolfe, 1954).

The nitrogenous excretion consists principally of polypeptides with lesser amounts of amide N (amides are high in young cultures and decrease as they become older). As much as 4–8 mg/l. of extra-cellular N are produced in culture. The polypeptides after acid hydrolysis yield amino acids. Chromatographic analysis suggests that the polypeptide fraction varies in composition: the polypeptide of a 28-day culture in a medium without added N contained in decreasing quantities serine, threonine, glutamic acid, glycine and tyrosine, and traces of alanine, valine, leucine; a 12-day culture in a medium with ammonium phosphate gave in decreasing quantities, glutamic acid, alanine, valine, leucine, glutamine, glycine, tyrosine, phenylalanine, aspartic acid.

Production of polypeptides is not restricted to nitrogen-fixing organisms: algae representing four different classes liberate considerable amounts of extra-cellular N and polypeptides (Fogg and Westlake, 1955). However, since far greater amounts are released in old cultures, autolysis rather than excretion is responsible for the increased amount of soluble organic N (Table IV). The production of polypeptides may be widespread amongst micro-organisms: it is common in non-nitrogen-fixing bacteria (Proom and Woiwood, 1949); several bacilli produce polypeptides with antibiotic properties like polymixin, bacitracin, valinomycin, tyrocidin and gramicidin.

The extra-cellular nitrogenous substances may or may not serve as nutrients to other organisms. The polypeptide of *Anabaena* does not give a perceptible reaction with ninhydrin, suggesting a ring structure which may not be susceptible to attack by the usual proteolytic enzymes. It has no antibiotic properties and is not utilizable as a N-source by *A. cylindrica*, *Chlorella* sp. and *Oscillatoria* sp.; it is not known whether bacteria can utilize them (Fogg, 1952).

Peptide nitrogen occurs dissolved in lakes in amounts ranging from 0.057 to 0.436 mg N/l. (Domogalla, Juday and Peterson, 1925). Fogg and Westlake (1955) found smaller quantities of peptide N in English lakes: the concentration

TABLE IV

Extra-cellular Organic Nitrogen Produced by Fresh-Water Algae
(from Fogg and Westlake, 1955)

Species	Age of culture (days)	Cells per ml $\times 10^6$	Extra-cellular N (mg/l.)		
			Total organic	Free α-amino N	Peptide α-amino N
Chlamydomonas mocwusii (1)	30	3.8	2.8	0.17	1.50
(2)	24	—	—	0.027	0.2
Chlorella pyrenoidosa (1)	15	—	—	0.053	0.019
(2)	31	7.5	—	0.043	0.000
Tribonema æquale	60	—	—	0.041	0.065
Navicula pelliculosa	27	—	—	0.089	0.093
Anabaena cylindrica (1)	30	—	0.51	0.013	0.073
(2)	100	—	5.6	0.41	3.0

of peptide N shows no apparent correlation with the trophic state of the lake and the occurrence of peptide does not depend on any particular algae.

We have no data on production of extra-cellular organic nitrogen in cultures of marine algae. The high C/N ratios in the organic solutes found in sea-water indicate, as mentioned, that in the sea the situation is quite different from that in fresh waters; however, we know nothing about their nature. Furthermore, Duursma's results indicate that the release of organics in the sea occurs after the algal blooms.

C. Growth Factors

Vitamin B_{12} (cyanocobalamin), thiamine and biotin are indispensable requirements for many marine algae (Table VIII). Hence these growth factors should be present in the environment: cyanocobalamin and thiamine have been measured in sea-water. The search for the producers is going on, stimulated by the pioneer work of Lochhead and collaborators (1951, 1957) on the growth-factor relationships among the micro-organisms of the soil. Most of the work in the sea centers around vitamin B_{12} and the family of cobalamins[1]; cyanoco-

[1] A series of cobalamins is produced by micro-organisms; these cobalamins differ one from another largely in respect to the type of nucleotide portion of the molecule or its absence (factor B). For cyanocobalamin the nucleotide is 5,6-dimethylbenzimidazole; for pseudo-B_{12}, adenine; for factor A, 2-methyladenine; for factor H, 2-methylhypoxanthine; for factor III (factor I), 5-hydroxybenzimidazole—other cobalamins have been obtained by biosynthesis with *Escherichia coli* (Bernhauer, 1956). The vitamin B_{12}-requiring organisms have characteristic patterns of response toward the cobalamins (see Table X, and chapter on bioassays). It suffices here to mention that most algae respond only to

balamin being the growth-factor needed by the majority of the auxotrophic algae.

The data indicate that bacteria are the major producers of vitamin B_{12} in the sea. Ericson and Lewis (1953) isolated 34 bacteria from the Baltic Sea and epiphytic on seaweeds; 70% of them (mainly *Pseudomonas* and *Achromobacter* spp.) are cobalamin producers (*Escherichia coli* bioassay). An equal number of marine bacteria was isolated by Starr *et al.* (1957) from mud and waters from the southeast and west coast of Texas, a lagoon, laboratory tanks, and mullet intestine: 70% of them had activity for *Escherichia coli* and 30% for *Euglena gracilis*. Twenty per cent of 60 bacteria isolated from muds of the Bahia Fosforescente (Puerto Rico) had *Escherichia coli* activity (Burkholder and Burkholder, 1958). Burkholder (1959) isolated from muds and water of Long Island Sound 344 bacteria; 24% of them produced cobalamins. Marine bacteria collected near shore produce more non-B_{12} cobalamins than true vitamin B_{12}: this is indicated by the following data. Chromatographic analysis of the filtrates show more non-B_{12} cobalamins than true B_{12} (Ericson and Lewis, 1953); more bacterial strains produce cobalamins active for *Escherichia coli* than for *Euglena gracilis* (Starr *et al.*, 1957). Most marine bacteria live epiphytically on particulate matter. Suspended solids and muds of Sapelo Island, Georgia coast, the Bahia Fosforescente and Long Island Sound are relatively rich in *Escherichia coli*-active cobalamins (Starr, 1956; Burkholder and Burkholder, 1956, 1958; and *in litt.*). A differential assay with *Ochromonas malhamensis* and *Escherichia coli* on the same bottom deposits of the Bahia Fosforescente shows that the quantity of true B_{12} is 7–23% of the *Escherichia coli*-active cobalamins (Burkholder and Burkholder, 1958). A similar situation should exist in sea-water but data are lacking. Most determinations of "B_{12}" in sea-water have been done with the *Euglena gracilis*, which, as noted, is not specific for true B_{12}. Cowey (1956) did a few differential assays: in sea-water from Aberdeen Bay almost half the total cobalamins was B_{12}; in oceanic waters the non-B_{12} cobalamins were present in far lower relative quantities. More work is needed to find out whether this holds generally; if it is so then we should explain the discrepancy between what is actually found in the waters, what has been produced by marine bacteria, and what is present in muds. In any case, differential assays for sea-water are indispensable because the B_{12}-requiring algae themselves have different specificity patterns; those of low specificity (*Escherichia coli* pattern) utilize all cobalamins, but the others able to utilize only vitamin B_{12} may be discriminated against, depending on the ratio of non-B_{12} cobalamins to vitamin B_{12}.

Many marine bacteria require vitamins for growth and they compete for

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cyanocobalamin (*Ochromonas malhamensis* and "vertebrate" pattern); *Euglena gracilis* responds also to pseudo-B_{12} and factor A, and *Escherichia coli* to all cobalamins including factor B (no nucleotide), and methionine. The *Escherichia coli* bioassay hence is equivalent to "total cobalamins", the *Ochromonas* assay only to cyanocobalamin, and the *Euglena gracilis* assay is intermediate between the *Ochromonas* and *Escherichia coli* assays.

vitamins with all the other organisms. Since their metabolism and rate of division are higher than other consumers, they may be quantitatively the most important consumers. Table V points to vitamin exchanges between some

TABLE V

Marine Bacteria (Burkholder, in press)

No. of cultures	Patterns of requirements				No. of vitamin producers			
	Biotin	Thiamine	Nicotinic acid	B_{12}	Biotin	Thiamine	Nicotinic acid	B_{12}
29	+					3	29	1
7		+			6		7	
2			+		1			
3				+	3	1	3	
3	+			+			3	
5		+		+	3		5	
1		+	+		1			
1	+	+	+					
1	+	+		+			1	

marine bacteria (Burkholder, in press). Here nutritional interdependency is made dramatically manifest by vitamin producers being themselves dependent on other vitamins. This chemical symbiosis supports Lucas's postulates on the importance of external metabolites in the sea.

Apparently bacteria are not the only producers of vitamins. Several unicellular algae contain appreciable amounts of vitamins (Table VI); these data refer to the content in vitamins of the cells and not of the supernatants. Therefore, we do not know whether these algae excrete the vitamins; if they do not the vitamins could become available for the consumers only after death of the cells. The vitamins are synthesized by the algae since several analyses were done on algae grown in bacteria-free culture and in media lacking the vitamin tested (see Brown *et al.*, 1955; Robbins *et al.*, 1951). Furthermore, the B_{12} content of the various *Chlorella* species is of the same order for species grown under impure or aseptic conditions. However, the species of Table VI are freshwater algae and belong to the green and blue-green algae. Species belonging to these algal groups are not very abundant in the sea, except for some blue-green algae, like *Trichodesmium erythraeum*, which form blooms in warm seas.

The content in cobalamins of many seaweeds has been analyzed by Robbins *et al.* (1951), Ericson and Lewis (1953), Southcott and Tarr (1953) and Hashimoto (1954). These data and their ecological significance are discussed by Provasoli (1958a). In brief, the red seaweeds are richer in cobalamins than the brown seaweeds, and more species of reds than browns contain cobalamins. Since the algae were collected from nature, we do not know whether the

TABLE VI

Vitamin Content of Algae

Species	Vitamin	Quantities per 100 g dry matter[a]	Author
Chlorella pyrenoidosa	thiamine	1–4.1 mg	Combs (1952)
	riboflavin	3.6–8 mg	
	nicotinic ac.	12–24 mg	
	pyridoxine	2.3 mg	
	pantothenic ac.	0.8–2.0 mg	
	biotin	14.8 μg	
	choline	300 mg	
	B_{12} (*E. g.*)[b]	2.2–10 μg	
Chlorella vulgaris[d]	B_{12} (*E. c*)[b]	6.3 μg	Brown, Cuthbertson and Fogg (1955)
Chlorella ellipsoidea	B_{12} (*E. g.*)[b]	4.2–8.9 μg	Hashimoto (1954)
Anabaena cylindrica[d]	B_{12} (*E. c*)[c]	63–110 μg	Brown, Cuthbertson and Fogg (1955)
Microcystis sp. (bloom)	B_{12} (*E. g.*)[b]	12.3 μg	Hashimoto (1954)
Plectonema nostocorum[d]	B_{12} (*E. g.*)[b]	6–7 μg	Robbins *et al.* (1951)
Calothrix parietina[d]	B_{12} (*E. g.*)[b]	64 μg	,,
Aphanizonemum flos-aquae[d]	B_{12} (*E. g.*)[b]	28 μg	,,
Diplocystis aeruginosa[d]	B_{12} (*E. g.*)[b]	24 μg	,,

[a] Dry weight of alga.

[b] B_{12} as assayed with micro-organism: *E. g.* = *Euglena gracilis*, *E. c.* = *E. coli*.

[c] 65–70% of this is true B_{12}; the remainder is pseudo-B_{12} and factor A.

[d] Bacteria-free.

See also Kanazawa, A., 1962. Studies on the vitamin B-complex in marine algae. I On vitamin content. *Mem. Fac. Fish. Kagoshima Univ.*, **10**, 38–69.

vitamins were synthesized by the seaweeds or absorbed from the environment (it is well known that seaweeds accumulate inorganic ions 100–1000-fold). Ericson (1952) found that *Pelvetia caniculata* has a very poor ability to concentrate radioactive B_{12}, but when collected in nature it contains at least 0.5 μg B_{12}/g; *Polysiphonia fastigiata* concentrates radioactive B_{12} 90-fold, but when collected from nature is devoid of B_{12}. Since the quantity of cobalamins in sea-water is very low, these data favored the hypothesis that B_{12} is synthesized by seaweeds. Later, Ericson and Lewis (1953) found that epiphytic bacteria of seaweeds produce B_{12} and non-B_{12} cobalamins: from this and other considerations they conclude that the B_{12}-compounds in seaweeds are probably of bacterial origin, even though true B_{12} is preponderant in seaweeds and non-B_{12} cobalamins are predominantly excreted by the epiphytic bacteria. The question can only be answered by the use of aseptic cultures of seaweeds; so far the red algae which have been cultured aseptically, *Goniotrichum elegans*

(Fries, 1959), *Bangia fusco-purpurea* and *Antithamnion* sp. (Provasoli and Iwasaki, unpublished), need B_{12} for growth.

Folic, folinic, pantothenic and nicotinic acids were also measured in seaweeds. The content of vitamins in seaweeds varied seasonally, and in the younger and older parts of the thallus (Ericson, 1953; Ericson and Carlson, 1953). Larsen and Haug (1959) found a correlation between salinity and the nicotinic acid and biotin content of *Ascophyllum nodosum* and *Fucus vesciculosus*; vitamin maxima occur in lower salinity and do not seem to depend on pollution carried in by fresh waters.

Whatever the origin of vitamins in seaweeds, the vitamins are bound to be released in coastal waters either by excretion or decomposition of the seaweeds —indirectly, more vitamins probably are produced by the bacteria decomposing the immense quantities of dead seaweeds in the tidal zone.

Phytohormones ("X" and "Z") biologically active on *Avena* coleoptiles were found in phytoplankton samples (predominantly diatom); zooplankton samples showed high activity, on chromatography, in the indoleacetic zone (Bentley, 1958, 1959).

D. Antibiotics

The reciprocal exchange of vitamins is one aspect of the interaction amongst organisms. Another is the production of antibiotics. Co-operation and conflict amongst organisms at the macroscopic level, especially for land plants, is an important branch of ecology. The same phenomenon, but at the chemical levels of antibiosis and growth promotion, is neglected. The possible biological and ecological importance of these phenomena is discussed boldly by Burkholder (1952) and Brian (1957). The high solubility of many biologically active substances peculiar to the water environment permits rapid exchange of metabolites but it also limits their effectiveness through dilution; therefore, only substances active at extreme dilutions (10^{-15}), such as vitamins, are eligible for ecological importance. The antibiotics, at least those now known, are effective at much higher concentrations (10^{-6}–10^{-9}); one wonders if they can play an important part in the *free* water environment. Their action may, however, be quite significant when the dilution factor is minimized, as in symbiosis, cohabitation and parasitism. One example is the relationship *Phaeocystis*–euphausids–Antarctic birds described by Sieburth (1958, 1959); a similar condition may be responsible for the antibiotic effect of corals (Burkholder and Burkholder, 1958a); the production of antibiotics by seaweeds (Chesters and Scott, 1956) may affect the kind of epiphytic flora normally present in seaweeds.

The sea may become a grab bag of medicinals, but the data obtained for these practical motives, e.g. measurements of pathogen-active antibiotic production by marine organisms, obviously lack ecological meaning. Screening procedures are based generally on sensitive terrigenous bacteria followed by a screening on human pathogens. To have any ecological significance, the initial screening should be based on an assay with sensitive marine bacteria, and later on the ecologically important bacterial species. But we cannot say now

Table VII

Vitamin B_{12} Content of Marine Seaweeds

		Species	B_{12} activity as measured by		
			E. coli (Ericson and Lewis), range μg/g dry wt.	*E. gracilis* (Robbins *et al.*), range μg/g dry wt.	*E. gracilis* (Hashimoto), range μg/100 g[a]
Red seaweeds	High content	*Laurentia pinnatifida, Rhodomela subfusca, Polysiphonia brodiaei, Ceramium rubrum,*[b] *C. tenuicorne*	0.20–0.63		
		Ceramium rubrum,[b] *Champia parvula, Chondria tenuissima, Lomentaria baileyana*		0.05–0.1	
		Ceramium rubrum,[b] *C. tenerrimum, Martensia elegans, Acanthopeltis japonica, Gelidium amansii, Chondrococcus japonicus*			0.8–4.0
	Low content	*Rhodymenia palmata, Chondrus crispus,*[b] *Gigartina stellata, Porphyra* sp.	0.4–0.13		
		Agardhiella tenera, Chondrus crispus,[b] *Polysiphonia variegata*		0.01–0.045	
		Batrachospermum moniliforme, Carpopeltis angusta, Meristotheca papulosa			0.1–0.2

Brown Seaweeds	*Himanthalia elongata, Alaria esculenta, Laminaria hyperborea, Sphacelaria arctica, Laminaria digitata*	0.1–0.26		
	Ascophyllum nodosum,[b] Chorda filum, Chordaria flagelliformis, Fucus spiralis, F. vesiculosus,[b] Laminaria agardhii, Mesogloia divaricata, Sargassum filipendula		< 0.01	
	Ectocarpus sp., *Dictyota dichotoma, Padina arborescens, Ishige okamurai, Colpomenia sinuosa, Hydroclathrus cancellatus, Laminaria angustata, Endarachne binghamiae, Scytosiphon lomentaria, Eisenia bicyclis, Ecklonia cava, Undaria pinnatifida, Hijikia fusiformis, Sargassum enerve, S. ringgoldianum, S. horneri, S. serratifolium, S. pilubiferum, S. thunbergii*			0.1–1.2
	Pelvetia canaliculata, Fucus vesiculosus,[b] F. serratus, Ascophyllum nodosum,[b] Laminaria saccharina	0.04–0.075		
Green Seaweeds	*Chara tomentosa, Valonia ventricosa, Ulva lactuca,[b] Enteromorpha intestinalis[b]*	0.2–0.6		
	Ulva lactuca[b] *Enteromorpha intestinalis[b]*		0.01–0.045 0.05–0.1	
	Enteromorpha linza, Chaetomorpha crassa, Monostroma nitidum, Letterstedtia japonica, Ulva pertusa, Caulerpa okamurai, Codium divaricatum			1.4–3.9 0.1–0.8

[a] It is not clear whether the author refers to 100 g dry or wet weight; higher values were found in algae predigested with trypsin.
[b] Species analyzed by more than one author.

which are the ecologically important species; type culture collections are still grossly inadequate, and the species ill-identified at best.

a. Antibiotics of bacterial origin

Two of the most important groups which produce antibiotics in the soil, the actinomycetes and the fungi imperfecti, seem scarce in sea-water. We do not know whether any truly marine representatives of them exist. As a consequence the antibiotic picture of the ocean should be quite different from the soil. The predominant flora of the sea is composed of Gram-negative and pleomorphic Gram-positive bacteria; the commonest genera are *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Achromobacter*, *Bacterium*, and corynebacteria (Zobell, 1946; Wood, 1950). However, our knowledge of the bacterial flora of the seas may be entirely biased by the enrichment media employed. If some marine bacteria shared the nutritional characteristics of the marine algae, they could never be isolated with the extremely rich media in current use: the marine algae, many of which are quite euryhaline, are particularly sensitive to concentrations of organic substances (10–30 mg % of "peptones" drastically inhibit their growth). The only criterion to ensure the qualitative and quantitive appreciation of the marine flora is the matching of microscope counts with colony counts.

A few actinomycetes (*Nocardia*, *Micromonospora*, *Streptomyces*) and mycobacteria have been isolated from marine coastal sediments, nets, cordage and rotting seaweeds. Grein and Meyers (1958), after a survey, consider that the species isolated are probably of terrestrial origin: they are not different morphologically from terrestrial species; the halophilic tolerance of terrestrial actinomycetes is as good as the one of the "marine" species; both types grow better at sea-water concentrations of less than 50%. Several isolates of Grein and Meyers exhibited antibiotic production in culture media. A few marine *Bacillus* and *Micrococcus* (9 out of 58 marine species tested) had antibiotic activity against non-marine forms (Rosenfeld and Zobell, 1947). A conclusion is that marine bacteria may account for some of the observed antibacterial action of sea-water on enteric and fresh-water forms (Greenberg, 1956). The antagonism between marine micro-organisms is probable but unproven.

b. Antibiotics of algal origin

Extracts of several marine seaweeds are antibacterial (Pratt, Mautner *et al.*, 1951; Mautner, Gardner and Pratt, 1953; Vacca and Walsh, 1954; Chesters and Scott, 1956; Allen and Dawson, 1959). This seems a widespread ability in seaweeds: antibiotics were found in green, brown and red seaweeds, but it is not known whether they are excreted in the sea or released after death. Antibiotic activity and antibacterial spectrum vary with species, and activity within each species of seaweed varies in the different months and may even be lacking (Chesters and Scott, 1956). The seaweeds tested by Allen and Dawson inhibit only Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus*

aureus and *Mycobacterium smegmatis*) and not *Escherichia coli*[1] while the species tested by Chesters and Scott inhibited also Gram-negative fresh-water bacteria (*Escherichia coli* and *Pseudomonas aeroginosa*, etc.); marine bacteria are mostly Gram-negative.

Unicellular fresh-water algae also produce antibiotics: antibacterial substances are present in old cultures of *Protosiphon* and *Stichococcus* (Harder and Oppermann, 1953) and in *Chlorella* extracts (Spoehr *et al.*, 1949). Photooxidized unsaturated fatty acids are responsible for the antibacterial action of *Chlorella* and, apparently, also for *Protosiphon* and *Stichococcus* (the evidence is scanty). Unsaturated fatty acids produced and liberated upon death by *Chlamydomonas reinhardii* are apparently responsible for the antibiosis of *C. reinhardii* on *Haematococcus pluvialis* (Proctor, 1957). Since unsaturated fatty acids are produced by algae, including the seaweeds (Collyer and Fogg, 1955, for green algae, and table in Fogg, 1953), one wonders whether some antibacterial effects of seaweeds are not due to fatty-acid toxicity, especially in the alkaline range. This possibility was not considered in the work on seaweeds.

Several laboratories are now testing a variety of marine unicellular algae for antibacterial activity. The only published report is the very instructive story of *Phaeocystis* sp. Arctic birds were known to have a reduced gastro-intestinal microflora; this was confirmed for Antarctic birds by Sieburth (1959), who found that blood serum of penguins was active against Gram-positive bacteria. The antibiotic activity was traced through the food chain: from the crustacean *Euphausia superba*, which is the staple diet of penguins, to *Phaeocystis*-like algae which bloom in the Antarctic and are a food for *Euphausia* (Sieburth, 1959a). The antibacterial substance of *Phaeocystis* inhibits Gram-positives more than Gram-negatives; it is water- and ethanol-soluble and is soluble in non-polar solvents, stable to heat and alkali, but inactivated by heat-drying and mineral acids (Sieburth and Burkholder, 1959). The substance was finally identified as acrylic acid (Sieburth, 1960).

c. Antibiotics of invertebrate origin

Water homogenates of living sponges from the temperate and subtropical zones contain substances which inhibit a variety of marine bacteria (isolated from sponges and sea-water) and the usual forms employed for antimicrobial assays (Jakowska and Nigrelli, 1960). This activity was found in species of *Microciona*, *Halichondria*, *Cliona*, *Tedania*, *Haliclona*, *Dysidea* and *Oligocera*. The active substances are heat-stable and can be selectively extracted with solvents, also from frozen and heat-dried sponges. The activity ranges from inhibition of marine bacteria to "broad-spectrum antibiosis" ("ectyonin" extracted from *Microciona prolifera*) and specific action against *Pseudomonas pyocyanea* and *Candida albicans*.

Extracts of several gorgonian (horny) corals had a strong antibiotic action against both the usual fresh-water assay species and marine bacteria. On the

[1] Gram-positive bacteria are notoriously more sensitive to antibiotics, on the whole, than are Gram-negative.

contrary, extracts of the few stony corals tested had little or no antibacterial action (Burkholder and Burkholder, 1958a). Antibacterial action was also found in extracts of unidentified "hydroids" (Allen and Dawson, 1959). Since most corals have endozoic zooxanthellae and harbor epizoic algae, it is not known whether the living tissues of the hydrozoan or the associated flora produce the antibacterial substances.

d. Antialgal substances

Algae produce, besides antibacterial antibiotics, auto-inhibitors and substances inhibiting the growth of other algae (heteroinhibitors). The phenomenon is widespread among fresh-water algae; the literature is critically discussed by Proctor (1957); Hartman (1960) has tabulated the data on the interacting organisms, including all the species studied by Léfèvre *et al.* (1952). These substances are found in filtrates of old cultures and it is likely that they were mostly released by living mature cells or during autolysis, but excretion by healthy cells is not excluded. The rate of production of these inhibitory substances has not been followed experimentally and their nature is unknown; those found by Léfèvre *et al.* are, in general, heat-labile.[1] Presumably marine algae behave similarly.

e. Crustacean inhibitors

Unicellular algae apparently release inhibitors for Crustacea: old cultures of *Chlorella vulgaris* inhibit filtering rate and growth of *Daphnia magna* (Ryther, 1954). *Tigriopus japonicus* and the brine shrimp *Artemia salina* utilize as food only a limited assortment of marine flagellates; others are unsuitable (Provasoli, Shiraishi and Lance, 1959). The unsuitability of these organisms as food may reflect nutritional incompleteness, but the possibility that nutritionally adequate food organisms are unsuitable because they produce inhibitory substances is not excluded. In the sea two relationships between phytoplankton and zooplankton have been observed: an inverse relationship, i.e. heavy grazing by zooplankton (Harvey, 1934) or avoidance of phytoplankton bloom by predators (Hardy, 1936). Avoidance may indicate that some species of phytoplankton produce inhibitors, or, more simply, obnoxious substances (perhaps tastes and odors, or other substances repressing the feeding reaction).

E. Poisons

It is well known that from time to time spectacular algal blooms discolor the sea; some of them lead to mass mortality of marine invertebrates and fishes. Though commonly known as "red tides" because many of these blooms

[1] The blue-green algae are notorious for suppressing other algae. The fresh-water *Nostoc muscorum* produces in bacteria-free culture a "dihydroxy-anthraquinone" which inhibits growth of several algae (*Cosmarium*, *Phormidium*, *Euglena*), baker's yeast, and bacteria (Jakob, 1961).

are due to red-pigmented dinoflagellates, other discolorations (brown, yellow, green or cloudy) are also responsible for producing mass mortality; conversely some red blooms like the ones of *Trichodesmium erythreum* (a red-pigmented blue-green alga) in the Red Sea, Indian Ocean and the Sea of California are generally harmless. The poisonous blooms, though not annual, recur in certain coastal regions: in general, where fertilization occurs (upwelling, pollution, heavy soil runoffs). Maps of bloom localities are given by Hayes and Austin (1951) and Ballantine and Abbott (1957); Brongersma-Sanders (1957) gives a detailed review of the outbreaks, the causative agents, and an impressive bibliography.

Most of the mortalities are due to dinoflagellates, specifically to *Gonyaulax polyedra*, *G. monilata*, *Gymnodinium splendens*, *G. breve*, *G. mikimotoi*, *G. galatheanum*, *Cochlodinium catenatum*, *Exuviaella baltica* and *Pyrodinium phoneus*. Two other dinoflagellates, *Gonyaulax catenella* and *G. tamarensis*, do not cause mortality of marine organisms, including shellfish, but are ingested by shellfish, which concentrate the poison in the siphon or digestive glands and so become poisonous to man. Two other algae, the chloromonadine *Horniella marina* (Subrahmanyan, 1954) and the diatom *Thalassiosira decipiens* (Takano, 1956), caused, respectively, extensive mortalities off the Malabar Coast and in Tokyo Bay. Mortalities of fishes in brackish waters in Denmark (Otterstrom and Nielsen, 1939) and in fish ponds in Israel (Shilo and Aschner, 1953) are caused by the chrysomonad *Prymnesium parvum*.

Mass mortalities occur at the peak of the bloom. In bacteria-free cultures of *Gonyaulax catenella*, the toxin is localized in the cells during the logarithmic phase of growth and accumulates in the medium, when death and disintegration affect the population (Provasoli and McLaughlin, unpublished). This does not exclude some toxin excretion by actively dividing cells, i.e. concentrations below the sensitivity limit of the mouse bioassay. The ecological situation may be similar; toxic levels may be reached only when large numbers of dinoflagellates are senescent or dying.

A wealth of information on the poison of *Gonyaulax catenella* and *Prymnesium parvum* (Schantz, 1960; Burke *et al.*, 1960; Shilo and Rosenberger, 1960) and other products of marine organisms appeared in vol. 90, pp. 615–950, of the *Ann. N.Y. Acad. Sci.* devoted to "The biochemistry and pharmacology of compounds derived from marine organisms".

If the poisons of other dinoflagellates are as potent as the one of *Gonyaulax catenella*, we have another example of extremely active biological substances released in sea-water. The poison of *Gonyaulax catenella* is one of the most potent known, i.e. 1 mouse unit = 0.18 μg (1 unit is the dose killing all 20-g mice in 7–15 min); the lethal dose for man = 3–4 mg. This neurotoxin, unlike botulinum toxin, is a strongly basic nitrogeneous compound of low molecular weight (372), yielding under various oxidations and hydrolytic procedures guanidopropionic acid, guanidine, urea, ammonia and carbon dioxide (Schantz, 1960). A survey on the production of poisons by many dinoflagellates reared in bacteria-free culture is under way at the Haskins Laboratories.

4. Nutritional Requirements of Marine Algae

The nutritional requirements of algae have been reviewed recently (fresh-water algae, Krauss, 1958; nutrition and ecology, including marine algae, Provasoli, 1958). Several marine algae have been grown bacteria-free in artificial media of known composition (Provasoli, McLaughlin and Droop, 1957). These media seem adequate for most of the algae growing in the littoral zone, including the red seaweeds (Fries, 1959; Provasoli and Iwasaki, unpublished). The photosynthetic marine algae are fundamentally photoautotrophic, i.e. synthesizing their organic carbon almost exclusively from CO_2. Many species, however, have acquired needs for growth factors while retaining their basic photoautotrophic abilities.

A. Inorganic Requirements

The tolerance to salinity variations are indeed quite great; most neritic marine flagellates can grow from 12–40‰ salinity (optima between 20–24‰). Only species obtained from the Caribbean and Gulf of Mexico seem to prefer a salinity of 33‰. Tolerance to variation in ratios and concentrations of Na, K, Ca and Mg, is, in general, high; this versatility has been exploited in compounding non-precipitating artificial media low in Ca and Mg (Ca 10–20 mg %; Mg 50–70 mg %). Iron, Mn, Mo, Cu, Co and V have been demonstrated to be essential for fresh-water algae; B, Zn and S are also needed but their essentiality has not been proven. We may assume that this holds for marine algae also, even though no available data meet the rigorous requirements for purity of chemicals for these studies. In artificial media the addition of metal-buffered (chelated) trace-metal solutions is favorable for sustained growth despite the trace metals introduced as impurities of the "chemically pure" major salts.[1] Studies on single-trace-metal requirements may be misleading when metal-chelators are present in the media, because addition or removal of single heavy metals affects simultaneously the hold of the chelator on all other chelatable ions in the medium. Removal of chelators results in precipitates if the trace metals are added before sterilization; separate aseptic additions do not guarantee absence of precipitates and are cumbersome for extensive nutritional studies. However, a precise study of the trace-metal requirements is unavoidable in view of the results of Johnston (see pp. 200–201). Almost all photosynthetic marine algae utilize nitrates. Ammonia is utilized, and usually is not toxic at the average ecological levels of N, but at higher levels it becomes rapidly toxic in the alkaline range (Shilo and Shilo, 1953; McLaughlin, 1958). Ammonia toxicity is unlikely to be ecologically important and may be significant only in highly polluted zones. The species which invaded and replaced

[1] Chelators improve growth of higher plants and plant tissues (Wallace, 1960; Klein and Manos, 1960). The finding of Goldberg (1952) that the diatom *Asterionella japonica* utilizes only particulate iron has not been confirmed yet; the other species of algae so far tested grow poorly or do not grow in artificial media when precipitates occurred during sterilization. Oceanic forms may behave differently as postulated by Provasoli (1956).

the normal marine flora of Great South Bay when pollution from duck farms accumulated because of poor flushing of the bay are resistant to ammonia (1 mg atom N/l.) and utilize urea and other organic sources of N (Ryther, 1954a). Amino acids and other organic N are in general poorly utilized, if at all, by photosynthetic marine algae. An exception is *Hemiselmis virescens* which requires glycine; colorless species might be expected to utilize and even to require amino acids as do *Gyrodinium cohnii* (Provasoli and Gold, 1962) and *Oxyrrhis marina* (Droop, 1959). Inorganic phosphate and also certain forms of organic P are good P sources.

This summary of the inorganic requirements shows that only the trace metals and their status in sea-water (as particles or solutes) can be a cause of "good" or of "bad" waters, when inorganic N and P are not deficient.

B. Organic Requirements

As mentioned, photosynthetic marine algae have slight, if any, ability to utilize organic nitrogen.

Glycerophosphoric, adenylic, cytidylic and guanylic acids and perhaps other nucleotides serve as P sources for all the marine species tested (extending the data of Chu, 1946, and Harvey, 1953). The widespread ability to utilize these organic compounds of P suggest that the P fraction of the organic solutes in sea-water should be closely measured and that P need not be mineralized to be available to phytoplankton organisms (see review in Provasoli, 1958, p. 294). This may explain why turnover of P is more rapid than expected and why in the early spring one diatom species after another can bloom so rapidly. In preliminary experiments, we noted that during the logarithmic phase of growth of *Navicula pelliculosa*, P becomes at first undetectable in the medium, but later significant amounts of P are released in the medium, and this before any portion of the population has become senescent or dying.

Organic acids and sugars are in general not utilized as carbon sources by photosynthetic marine algae. At times small concentrations of organic C are stimulatory, but the increase in growth is too little to suspect utilization as a C source. Similarly, very low concentrations (0.1–1 mg %) of amino acids and purines or pyrimidines are stimulatory; this may be due to chelation of trace metals and also to sparing of some substance along the pathways of biosynthesis.

The above remarks on the ability to utilize organic substances are derived from data on algae living in the photic littoral zone and may not apply at all to algae living in other zones of the sea, especially the shallow and deep bottoms and deep waters. Many pennate diatoms live close to, and, in the coastal muds: 11 of 26 species of diatoms isolated from such habitats not only utilize exogenous carbon sources but grow in darkness if supplied with glucose, acetate or lactate (Lewin and Lewin, 1960). A colorless marine diatom, *Nitzschia putrida*, has been found and is also heterotrophic (Pringsheim, 1951); it is probable that more colorless species exist in muds. This heterotrophic ability

fits the need imposed by the environment: several species of diatoms were found to migrate and reproduce in the mud where no light can reach them (Moul and Mason, 1957). But the existence of diatoms in mud is not, apparently, restricted to shallow waters: large populations of *Ethmodiscus rex* were found in material collected in the Marianna Trench (Wiseman and Hendey, 1953); 14 species of diatoms were present in good number in mud samples collected during the *Galathea* Expedition of 1951 from depths of 7000–10,000 m (Ferguson Wood, 1956). This preserved diatoms material is not only composed of empty frustules, but of many cells containing protoplasm which can be stained with the usual cytological dyes. This, along with the observation that no planktonic species were found in the samples, excludes the "rain" theory (how could the protoplasm of the cells remain intact during the slow descent to the depths?), and favors the existence of autochthonous populations of living diatoms in the deep (Ferguson Wood, 1956).

Flagellates below 20 μ (= nanoplankton = μ flagellates), endowed with photosynthetic pigments, had escaped attention until recently because they are not retained by the finest plankton nets, even though Lohmann had demonstrated their presence and abundance in the sea by centrifuging water samples. New methods for the quantitative estimation of the nanoplankton (Knight-Jones, 1951; Ballantine, 1953) reveal its great importance in the productivity of the seas. The nanoplankton in the North Sea is mainly composed of chrysomonads (*Chrysochromulina*) and other small flagellates which, like *Chromulina pusilla*, present very difficult taxonomic problems (Manton, 1959; Manton and Parke, 1960). The pioneering team of Parke and Manton is describing systematically the various species (Parke *et al.*, 1955–1959) by culturing hundreds of isolates, following the life cycles, and studying the morphology with the electron microscope. We can expect many exciting surprises, like the recent finding that *Crystalolithus hyalinus* is the motile phase of the non-motile *Coccolithus pelagicus* (Parke and Adams, 1960). In warm seas the unicellular flagellates and blue-green algae are a conspicuous part of the phytoplankton (500–4000 cells/ml, Bernard, 1958, 1959). The coccolithophorid chrysomonads, especially *Coccolithus fragilis*, are the predominant flagellates in the southern Mediterranean and constitute 75–96% of the phytoplankton volume, while dinoflagellates represent from 3–15% and naked flagellates and *Nostoc* constitute less than 1% of the volume; in the Southern Mediterranean, diatoms are rare (Bernard, 1958a). Remarkably, the richest layers for *Coccolithus* are not only in the euphotic zone (0–50 m) but also in the 250–800 m zone; furthermore, the density of cells in the layer 1000–2500 m is still very high, about two-thirds of the density found at 250–800 m (Bernard, 1958a). Growth of pigmented coccolithophorids in deep waters is not limited to the Mediterranean, but is also found in the Indian Ocean and the tropical Atlantic Ocean (off the Senegal Coast). In the Indian Ocean their maximum is found at 200 m and in the tropical Atlantic the population of the 200, 300, 400 m are between half and three-quarters of the maximum found (50 m). In fact, the growth in the column 0–200 m is several times less than below 200 m, where no photosynthesis can

take place (Bernard, 1958, 1959). Daily collections in the Mediterranean show that *Coccolithus* doubles every five days (Bernard, 1958a); their growth in the non-photic layers should then depend upon organic matter. Several *Chrysochromulina* actively ingest particles (even graphite!) and cells up to 5 μ (Parke *et al.*, 1955–1959) but none of the nanoplankton organisms has been grown in bacteria-free culture and it is not known whether they can utilize dissolved organic matter.[1] The fresh-water chrysomonad, *Ochromonas malhamensis*, grows luxuriously *in vitro* on organic solutes; it can also grow almost as well phagotrophically or photosynthetically. Even supposing that the chrysomonads living in non-euphotic zones of the sea are as versatile as *Ochromonas* nutritionally, where do they find food to enable them to divide at least once in five days? The content of organic solutes in sea-water is apparently quite low (2–20 mg/l.) and bacteria are supposed to be very scarce in deep waters!

C. Growth Factors

The sampling of marine species in bacteria-free culture is indeed small but it is clear that many marine photosynthetic algae need vitamins (Table VIII); fresh-water algae behave similarly. A complete list of the vitamin requirements of marine and fresh-water species is given by Provasoli (1958). Since then J. C. and R. A. Lewin (1960) have studied the requirements of 26 species of marine diatoms, Droop (1959) of *Oxyrrhis marina*, McLaughlin and Zahl (1959) of two symbiotic dinoflagellates, and Fries (1959) of a red alga[2] (all included in Table IX, with new results since ms. preparation). Table IX summarizes the old and new data, including 23 species of *Volvocales* (Pringsheim and Pringsheim, 1959). The tentative conclusions reached several years ago on only two dozen species are still valid (Provasoli and Pintner, 1953). Briefly: (*a*) photosynthetic algae, like autotrophic bacteria, have species that do not and some that do need vitamins (=auxotrophs); (*b*) only three vitamins are required, alone or in combination; in order of incidence, vitamin B_{12}, thiamine and biotin (Table IX); (*c*) auxotrophy does not correlate with any particular environment or with the lack or presence of heterotrophic abilities; (*d*) the algae have an unexpectedly narrow and stereotyped need for only three vitamins even though the algae live in environments rich in all vitamins and many accompanying micro-organisms (bacteria and fungi) have widely different vitamin requirements.

[1] Organic carbon sources improve growth in light but cannot sustain growth of *Hymenomonas* sp., *Pavlova gyrans*, and *Syracosphaera* sp. in darkness. *Coccolithus huxleyi* has very poor heterotrophic abilities (Pintner and Provasoli, in press). Some species of *Chrysochromulina* are now bacteria-free.

[2] Since the writing of the MS the following species have been investigated: the diatom *Cyclotella nana* requires B_{12} and *Detonula confervacea* has no vitamin requirement (Guillard and Ryther, 1962); the chrysomonads *Hymenomonas* sp., *Coccolithus huxleyi*, *Syracosphaera* sp., and *Ochrosphaera neapolitana* require only thiamine; *Pavolva gyrans* requires B_{12} and thiamine (Pintner and Provasoli, in press); the blue-green *Synechocystis* sp. requires only B_{12} (Van Baalen, 1961); the red seaweed *Nemalion multifidum* requires B_{12} and perhaps pyridoxamine (Fries, 1961).

TABLE VIII

Vitamin Requirements of Marine Algae[a]

Species	B_{12}	Thiamine	Biotin
CHLOROPHYCEAE			
Dunaliella salina, D. primolecta, D. euchlora, D. viridis, Nannochloris atomus, N. oculata, Pilinia sp., *Platymonas* sp., *Prasiola stipitata, Stephanoptera gracilis, Stichococcus cylindricus*[b]	O	O	O
Stichococcus cylindricus[b], *Platymonas tetrathele*	R	O	O
Brachiomonas submarina, Pyramimonas inconstans	R	R	O
BACILLARIOPHYCEAE			
Amphora coffaeiformis,[b] *Navicula* sp., *N. incerta, N. menisculus, Nitzschia putrida, N. angularis* var. *affinis, N. filiformis, N. frustulum,*[b] *N. hybridaeformis, N. laevis, N. curvilineata, N. marginata, N. obtusa* var. *scalpelliformis, N.* aff. *ovalis, Phaeodactylum tricornutum, Stauroneis amphoroides.*	O	O	O
Achnanthes brevipes, Amphora perpusilla (*coffaeiformis?*) *A. coffaeiformis,*[b] *A. lineolata, Cyclotella* sp., *Nitzschia frustulum,*[b] *N. ovalis, N. punctata, Synedra affinis, Skeletonema costatum, Stephanopyxis turris*	R	O	O
Amphipleura rutilans, Amphora coffaeiformis,[b] *Nitzschia closterium*[b]	R	R	O
Amphiprora paludosa, var. *duplex, Amphora coffaeiformis,*[b] *Nitzschia closterium*[b]	O	R	O
CHRYSOPHYCEAE			
Stichochrysis immobilis	O	O	O
Hymenomonas carterae	R	O	O
Pleurochrysis scherffelii	O	R	O
Hymenomonas elongata, Isochrysis galbana, Microglena arenicola, Monochrysis lutheri, Prymnesium parvum	R	R	O
CRYPTOPHYCEAE			
Hemiselmis virescens, Rhodomonas sp. (10 strains)	R	R	O
Rhodomonas lens	S	R	O
DINOPHYCEAE			
Exuviaella cassubica, Glenodinium foliaceum, Gonyaulax polyhedra, Gymnodinium splendens, Gyrodinium californicum, G. resplendens, G. uncatenum, Peridinium balticum, P. chattoni, P. trochoideum	R	O	O
Amphidinium klebsii, A. rhynchocephalum, Gymnodinium breve, Oxyrrhis marina	R	R	R
Gyrodinium cohnii	O	S-R[c]	R

TABLE VIII—(*cont.*)

Species	B_{12}	Thiamine	Biotin
CYANOPHYCEAE			
Phormidium persicinum	R	O	O
RHODOPHYCEAE			
Goniotrichum elegans, Bangia fusco-purpurea	R	O	O

[a] R = required; O = not required. Most of the data were contributed by Droop; J. C., and R. A. Lewin; Provasoli; Pintner; McLaughlin; and Gold; other contributors are A. Gibor, Ryther, and B. Sweeney (the author's references, up to 1958, are given in Provasoli, 1958).

[b] Species represented by strains with different vitamin requirements.

[c] Grows slightly but indefinitely without the thiamine; the addition of the thiamine results in a 100 or more-fold increase in growth.

The only perceptible trend is that algal groups differ in the incidence of species requiring vitamins: the Cyanophyceae, Chlorophyceae and Bacillariophyceae are algal groups in which about half or less of the species require vitamins (perhaps the non-requirers predominate); in the other algal groups the vitamin requirers predominate. The latter algal groups are the richest in animal tendencies (i.e. many species have lost the photosynthetic pigments and phagotrophy is widespread even in species with photosynthetic pigments, i.e. many chrysomonads). It has been postulated that incidence of auxotrophy may correlate with developed animal tendencies (Provasoli, 1956). For practical purposes the vitamin B_{12}-like cobalamins and thiamine can be considered the two most important vitamins for the phytoplankton and they may be relevant ecological factors. We have mentioned that assessing the ecological importance of B_{12} is difficult because several cobalamins are present in waters and the vitamin B_{12}-requiring organisms have themselves different patterns of specificity. Tables X and XI summarize the situation for algae. Table XI lumps freshwater and marine algae because, as mentioned, they apparently behave similarly; unpublished data of our laboratory as well as data in collaboration with Droop are included (Droop *et al.*, 1959). Of the three algal groups most important ecologically in the sea, the diatoms (Bacillariophyceae) seem to have the widest specificity. This may be fortuitous; still, they are apparently both the most abundant producers of organic matter in the temperate and cold seas and utilize all the known cobalamins in the environment. Therefore, although dependent on B_{12}-like compounds, they hold the advantage over the other B_{12}-requirers of narrower specificity. However, some cryptomonads—a group not abundant in the sea—have wide specificity. Because of this complex situation, assays on sea-water to be meaningful should be done with at least two bioassay organisms in order to measure "total cobalamins" (*E. coli* assay) and "true B_{12}" [*Ochromonas* or *Thraustochytrium* assay (see Adair and Vishniac, 1958)]. Furthermore, the data of J. C. and R. A. Lewin (1960) indicate that

TABLE IX

Summary of Vitamin Requirements of Fresh-Water and Marine Algae

Algal group	Number of species	No vitamins	Require vitamins	B_{12}	Thiamine	Biotin	B_{12} + thiamine	Biotin + thiamine	B_{12} + biotin + thiamine
Chlorophyceae	68	24	44	10	8		26		
Euglenineae	9	0	9	2	1		6		
Cryptophyceae	11	0	11	2	1		7		
Dinophyceae	17	1	16	11			0	1	4
Chrysophyceae[a]	22	1	21	2	5		9	1	2
Bacillariophyceae	39	21	18	11	3		4		
Cyanophyceae	10	9	1	1					
Rhodophyceae	4	0	4	4					
Totals	180	56	124	43	18		52	2	6
Totals for single vitamins				103	78	10			

[a] Two chrysomonads require B_{12} + biotin.

TABLE X

Patterns of Specificity toward Vitamin B_{12}-Like Compounds

Organism	*Type of nucleotide*		
	Benzimidazole (B_{12}, factor III)	Adenine (pseudo B_{12}, factor A)	No nucleotide (factor B)
Mammals and *Ochromonas malhamensis*	+	0	0
Lactobacillus leichmannii and *Euglena gracilis*	+	+	0
Escherichia coli	+	+	+

TABLE XI

B_{12} Specificity in Fresh-Water and Marine Algae

Algal group	Number of species studied	Specificity pattern		
		mammalian	lactobacillus	coli
Chlorophyceae	10	10		
Chrysophyceae	8	7	1	
Dinophyceae	8	6	2	
Euglenineae	5	1	4	
Cryptophyceae	7	2		5
Bacillariophyceae	10	2		8
Cyanophyceae	1			1
Rhodophyceae				

strains of diatoms of the *same* species may require different vitamins or not require them at all. This physiological variation among strains, which may also occur in other algal groups, obliges us to be extremely careful in extrapolating data pertaining to a strain of any species to other strains or localities. To avoid gross errors one must correlate the data on vitamin content of waters, especially B_{12}, with laboratory nutritional findings obtained exclusively on species isolated from the *same water samples*. Since this entails a great deal of work, such precise analysis should be limited to the history of blooms in localities where, as in Long Island Sound and off the English coast around Plymouth, the succession of forms and other ecological factors have been thoroughly studied.

It is suspected that seaweeds or other highly differentiated algae may need plant hormones for normal morphogenesis. *Ulva lactuca* produced the normal flattening and a short leafy thallus only in nutrient sea-water (+N, P, trace metals, vitamins) enriched with adenine and kinetin (Provasoli, 1958b).

However, this result could not be repeated with other samples of sea-water; the artificial sea-water media are inadequate for growth. *U. lactuca* responds also to indoleacetic acid and gibberellins.

5. Crustacea and Organic Solutes

Recent work on two Crustacea, *Tigriopus japonicus* and *Artemia salina* grown aseptically, exhumes again the hypothesis of Putter on the nutritional role of dissolved organic substances for marine animals. In feeding *Tigriopus* with a variety of aseptic algal flagellates, it was observed that *Chroomonas* sp. and *Isochrysis galbana*, fed singly, cause larval mortality and adult infertility in *Tigriopus* after supporting several normal generations: respectively, after four and eight generations. The number of generations before mortality sets in indicated that biologically potent micro-nutrients could be responsible. The addition of a vitamin mixture or glutathione to the medium restored normal growth for several additional generations (Shiraishi and Provasoli, 1959). This experiment obviously does not tell us whether vitamins added as solutes to the two-membered culture alga *Tigriopus* (*a*) modify the metabolism of the prey, or (*b*) become concentrated in the algae, or (*c*) are ingested directly from the medium by *Tigriopus*. It does show that vitamins in sea-waters affect, directly or indirectly, growth and fertility of the herbivores. Other effects of vitamins on Crustacea have been described. The addition of 200 mg/l. of pantothenic acid to septic cultures of *Daphnia* fed on *Chlamydomonas* tripled the life span and increased egg production tenfold (Fritsch, 1953). All the barnacles (*Balanus* sp.) in a tank when exposed to a maximal concentration of 14 μg/l. of ascorbic acid, immediately initiated copulating activities (Collier, Ray and Wilson, 1956).

The axenic culture of *Artemia* on artificial media shows that organic solutes can be utilized by Crustacea (Provasoli and Shiraishi, 1959). The main nutrients (blood serum, peptone, liver infusion, vitamins, nucleic acids, etc.) are added as solutes. *Artemia* grows to adulthood in this medium if particles (starch or cellulose) are present in abundance, but it dies, soon after the second meta-nauplius has consumed its yolk, if the particles are omitted. The nutrient solutes support growth only if enough drinking takes place; drinking in turn depends upon the feeding reaction caused by ingestion of particles; without particles they do not drink enough. The ecological significance is obvious: the soluble organic matter in sea-water is utilized, but since the quantities of nutrients dissolved are very small, the nutritional dependence of Crustacea on nucleic acids, proteins or amino acids eventually present in sea-water is minimal; only substances like vitamins and hormones, which act at extreme dilutions, can be ecologically significant.

6. Data from Biological Analysis of Sea-Water

The problem of "good" and "bad" waters—the fisherman's preoccupation—parallels frustrations of biologists trying to rear marine animals in the laboratory.

The change of water-masses around Plymouth caused a change in fauna and

distress to the scientists of this laboratory which pioneered so much work in marine biology. During 1930 Wilson experienced great difficulties in rearing larvae of polychaetes in the local sea-water. Later it became clear through the work of Russell, Cooper, Armstrong and Harvey, at the Marine Biological Association at Plymouth, that the local planktonic and hydrographic conditions had changed. This prompted Wilson to analyze the biological properties of the new water-masses (typified by the indicator species *Sagitta setosa*) and compare it with the *S. elegans* water which previously surrounded Plymouth. Employing *Echinus esculentus*, *Ophelia bicornis* and *Sabellaria alveolata* as bioassay organisms, he found the two types of water remarkably different: in *setosa* water, eggs and larvae of the sea urchin and worms developed abnormally; in *elegans* water, they grew normally (Wilson, 1951; Wilson and Armstrong, 1952). In nature *elegans* water supports good growth of phytoplankton and zooplankton. Addition of antibiotics, filter-sterilization, variations in pH, addition of B_{12}, ascorbic acid, or a metal chelating agent (EDTA), and of supernatants of thick cultures of diatoms and flagellates all did little to improve bad waters for the above organisms (Wilson and Armstrong, 1954, 1958). Experiments in which eggs were allowed to develop in a mixture of the two types of water suggested that it is the presence in good water of something beneficial rather than the presence in bad water of something harmful which makes the difference. Extracts with activated carbon and acetone of the bad and good waters gave confusing results: neither supported normal growth. Wilson attributed this to the properties of the samples of waters employed for extraction: both water samples, including the good water, supported abnormal growth.

The original observations of DeValera (1940) that superficial waters of the tidal *Fucus–Ascophyllum* zone permit the normal development from zygotes of *Enteromorpha*, while the water of 30-m depth allows only stunted and slow growth, resulted in a luckier chase. H. Kylin (1941, 1943, 1946) employed germinating zygotes of the seaweeds *Enteromorpha* and *Ulva* as bioassay organisms, and counted the number of cells produced by the germinating filament at a fixed time in various enrichments of the infertile deep water: he found that these waters are poor in NO_3, PO_4, Fe and Mn. A. Kylin (1943, 1945), with the same technique, obtained normal growth with super-added Zn, Mn, Fe and Co, while Ni, Al and Cd were inert. H. Kylin (1946) concluded that fertility of the inshore waters for seaweeds is due to their relative richness in N, P, and trace metals, and that these important elements for plant growth diminish with depth and distance from the shore: waters of 70-m depth are poorer in trace metals than the 30-m depth waters. The success of the two Kylins and the lack of results of Wilson reflect the status of the knowledge of the two fields of nutrition; much was known of algal and plant nutrition and Kylin could make a more educated guess as to what might be lacking in poor waters. Wilson faced the utter unknown; very little, if anything is known about the physiology of sea urchins and marine worms; any of a thousand known substances could be responsible.

At present, with the knowledge acquired on the nutrition of marine algae, it is possible to bioassay the biological properties for the phytoplankton of different water-masses. This possibility, though not fully exploited, has given some extremely interesting results.

Sea-water samples with their natural flora of living micro-organisms and phytoplankton were enriched with nutrients and incubated in continuous light for various periods. The effect of the various enrichments was gauged either by the amount of growth elicited during a fortnight (the samples being observed periodically during this period, Thomas, 1959; Johnston, in press) or by measuring the uptake of $^{14}CO_3$ after 24 h incubation in a light of 1500 ft candles (Ryther and Guillard, 1959). The enrichments were: (*a*) single additions of N, P, Si, trace elements, soil extract (Thomas); (*b*) a complete enrichment: N + P + Si + trace elements + soil extract (Thomas); N + P + Si + thiosulphate + under-chelated trace-metal mixture + vitamins (Ryther and Guillard); N + P + Si + over-chelated trace-metal mixture (Johnston); (*c*) the complete enrichment minus one of the components (Thomas; Ryther and Guillard); complete enrichment minus only the chelated trace metals (Johnston); (*d*) an equal mixture of surface sea-water and deep water (100 or 1000 m) from the same water column (Ryther and Guillard). Though the methods differ somewhat and the samples are from very different areas, all the sea-waters supported good growth only when enriched with trace metals; the other enrichments were indifferent; Si was stimulatory. Thomas treated only two samples: one from an oligotrophic area west of Baja California, and one from a eutrophic area off Central America. The oligotrophic sample had a natural population of 500 cells/l. of diatoms and dinoflagellates; none of these organisms grew in any enrichment. This might be due to the scant natural inoculum more than to an adverse (bad) type of sea-water. The eutrophic sample had 500,000 cells/l. of diatoms and dinoflagellates. The only effective single additions were soil extract and, in a minor way, the trace metals; early growth of diatoms followed by late growth of a *Gymnodinium* was favored by the complete enrichment and the complete enrichment minus P or N; the diatoms did not grow when Si or trace metals were lacking from the complete enrichment but the *Gymnodinium* grew. A similar contrast between the northern spring diatoms (*Skeletonema*, *Chaetoceros*, *Nitzschia*, *Thalassiosira*) and dinoflagellates (mainly *Ceratium*) was found also by Johnston: the type of enrichment he employed favored growth of the spring diatoms in all samples of sea-water (the amount of growth depending upon the sample) while the dinoflagellates present in the natural inoculum failed to grow. Omission of the chelated trace-metal mixture from the enrichment resulted in no or poor growth, including the dinoflagellates, in all types of waters (Johnston employed hundreds of samples collected in different seasons and depths in the North Sea, North Atlantic, Faroe and Icelandic waters).

Ryther and Guillard treated seven samples of surface waters collected from the continental shelf to the Gulf Stream and six samples in the Sargasso Sea; omission of the trace-metal mix from the complete enrichment had the most

pronounced effect, reducing photosynthesis almost to the level of the unenriched controls; all other omissions, except Si, gave the same high ^{14}C uptake as the complete enrichment; omission of Si resulted in about half the amount of ^{14}C uptake of the complete enrichment. Ryther and Guillard do not mention the species composition of the phytoplankton present in the various samples. Two interesting additional observations were made. The mixing of deep waters (1000 m) with the surface waters of the Sargasso results in a photosynthesis as high as the surface water with complete enrichment, defining experimentally the elements contributed by the deep waters which impart renewed fertility after mixing. Even more interesting is that the surface waters of the Sargasso are extremely poor in nitrate and phosphate yet the addition of these two elements had no effect on photosynthesis (growth in complete enrichment minus N or P = growth in complete enrichment). The authors comment ". . . that the phytoplankton may be dependent upon the rate of regeneration of these elements [N and P] and/or their presence as dissolved organic compounds and more-or-less independent of their instantaneous concentrations as inorganic salts". It would seem necessary to measure organic P and N of these deep waters.[1]

Johnston went a step further; he found why the addition of trace metals is necessary. The trace-metal mixture employed by him (PI Metals, see Provasoli *et al.*, 1957) is over-chelated; the ratio chelator/trace metals in milliequivalents is about 2:1, raising the possibility that either the chelated (1:1) trace metals or the excess free chelator of the mixture could be responsible for improving "bad" waters for phytoplankton. In fact, the addition of chelators alone, as EDTA and DTPA, and in a lesser degree of NTA and EDDHA,[2] is as effective as the addition of the chelated trace-metal mixture. These results fit perfectly with the ecological situation: at the normal pH of sea-water, iron, manganese and probably the other trace elements are extremely insoluble. The total Fe in sea-water varies between 1–60 μg atoms/l. (see results of various authors in Harvey, 1955, and Goldberg, 1957); in the offshore waters off the Norwegian coast the total Fe is 3–21 μg atoms/l. (Braarud and Klem, 1931). However, Cooper (1937) calculated that no more than 10^{-8} μg atoms/l. of Fe can remain in solution at pH 8.0–8.5. Therefore the deficiency of trace metals depends quantitatively far more on the physical status governing their availability to the cells than on the total amount. Growth promotion by metal chelators is due to their solubilizing power at the pH of sea-water, making available the trace metals which are present but largely unavailable. Indeed, Johnston found that most of the "bad" waters for phytoplankton became fertile upon addition of chelators, and some "good" waters became poor. The latter happening may be due to very high total trace metals in the samples. The ability to form soluble metal complexes is not at all restricted to the artificial chelates,

[1] See Addendum, page 210.

[2] EDTA = ethylenediamine tetraacetic acid; EDDHA = ethylenediamine-di-(*o*-hydroxyphenylacetic acid); DTPA = diethylenetriamine pentaacetic acid; NTA = nitrilotriacetic acid.

but is shared by many organic compounds, e.g. amino acids, nucleotides and hydroxy acids, which have been found or could occur in sea-water (see Section 2 of this chapter and Chapter 9). Some components, then, of the organic matter in the sea perform the dual role of nutrients and of solubilizers of the indispensable trace metals. The experiments of Johnston show that the quality of waters for phytoplankton may depend largely on the presence or absence of trace-metal solubilizers.[1] The search for these substances is, then, of paramount importance.

Johnston, besides enriching samples of sea-water containing their living phytoplankton, did two other types of assay in his attempts to assess the biological properties of the waters. In one assay (No. 1), filtered sea-water samples were enriched with one-fifth volume of medium S36, then autoclaved, and inoculated with a bacteria-free culture of *Skeletonema costatum* (Droop's strain); medium S36 was developed for the same strain by Droop (1955a). In the second assay (No. 2), filtered sea-water samples were enriched with N, P, Si, and a chelated trace-metal mixture, autoclaved, and inoculated with unialgal cultures (i.e. with unknown bacterial flora) of *S. costatum* and *Peridinium trochoideum*. These two assays show that the quality of sea-water varies from place to place, with depth and season. However, the unialgal cultures of *Peridinium* and *Skeletonema* gave a different assessment of sea-water quality indicating that the quality of sea-water acts differently on different classes of organisms (confirming the previously noted contrast between diatoms and dinoflagellates). Hence we should always specify for which organisms waters are good or bad. The assay response obtained with bacteria-free *S. costatum* (No. 1) does not correlate with temperature, salinity, oxygen, blue fluorescence, phosphate, phytoplankton and zooplankton abundance or dominance; it hints that waters of superior quality for *S. costatum* are recently mixed oceanic and neritic waters, and that waters collected after the bloom of spring diatoms are poor. The assay response with unialgal cultures (No. 2) correlates with plankton classification.

Earlier experiments had detected a very important difference between the assay with bacteria-free and with unialgal *Skeletonema*. Bacteria-free *Skeletonema* grew very poorly or not at all in 215 samples of sea-water collected in different seasons and localities of the northern seas when vitamins were omitted from the enrichment (N, P, Si, with and without chelated trace-metal mixture). Samples of sea-water similarly enriched but inoculated with unialgal (i.e. bacterized) cultures of *Skeletonema* supported poor to good growth depending upon the sea-water samples. Evidently the bacteria of the unialgal cultures

[1] In fresh waters some terminal products of microbial metabolism, like the "humic substances", perform this action. The yellow organic acids extracted by Shapiro (1957) are good trace-metal chelators and so are the polypeptides produced extracellularly by blue-green algae (Fogg and Westlake, 1955). Their ecological importance is discussed in these papers and by Fogg (1958). Similar substances have not yet been extracted from sea-water, though the "Gelbstoff" of Kalle (1949), which may be similar to the yellow acids of Shapiro and the "water humus" of Skopintsev (1959), may perform the same function.

almost always produce enough vitamin B_{12} to satisfy the B_{12} requirement of the diatom. The addition of vitamins improved only 38% of the sea-water assayed with unialgal *Skeletonema* (Johnston, in press). This explains why Ryther and Guillard did not report any decrease in growth when the vitamins were omitted from the complete enrichment. These authors enriched sea-waters which contained their natural microflora. It is well known that when sea-water is put in glass containers the added surface of the containers promotes heavy bacterial growth (Zobell and Anderson, 1936; Jones, Thomas and Haxo, 1958) and that many marine bacteria produce vitamins.

These observations prompted Johnston to enrich sea-water samples (to be assayed with bacteria-free *Skeletonema*) with a one-fifth volume of medium S36, which contains vitamins along with N, P, Si and trace metals. With this enrichment, the growth of bacteria-free *Skeletonema* in the numerous samples of sea-water varied from substantially inferior to superior to the controls (i.e. growth obtained in undiluted medium S36). The clear need for a vitamin supplement demonstrates that *Skeletonema* requires higher levels of vitamins than are present in the water tested. This experimental evidence contradicts the assertion of Droop (1957a) that the lowest amount of B_{12} found by Cowey (1956) in the North Sea—0.1 mμg/l.—should support a crop of twenty-five million cells of *Skeletonema* per liter. Johnston employed Droop's bacteria-free culture of *Skeletonema* for his assay of waters from the North Sea. The assertion of Droop was based on a calculation of the molecules of B_{12} required to produce 1 μ^3 of living protoplasm of B_{12}-requiring algae.[1] Daisley (1957) had already contested the validity of applying such data to a dynamic ecological situation. The results of Johnston are even more remarkable because *Skeletonema* can utilize, besides true B_{12}, the widest range of B_{12}-like cobalamins; in nature this species should have an advantage over other vitamin B_{12} requirers with narrow specificity.

Another important result obtained with the two *Skeletonema* assays is that the enriched sea-water samples elicit "poor" to "very good growth", indicating that unknown substances, which are none of the nutrients added with the enrichment (vitamins, trace metals, N, P and Si), affect *Skeletonema* growth, else all waters should have responded alike to the enrichments. For instance, the following behavior, observed in many samples, supports the postulate of unknown beneficial factors:

Sea-water + one-fifth medium S36 = often far more growth than 100% S36 (for bacteria-free and unialgal *Skeletonema* assay).

Sea-water + vitamins + trace metals = often far more growth than 100% S36 (unialgal *Skeletonema* assay).

[1] This value was obtained by dividing the volume of a cell of *Monochrysis lutheri* by the number of cells obtained with given amounts of B_{12}. The value obtained is three molecules of B_{12} for 1 μ^3 protoplasm. The values obtained, with similar calculations, for the existing growth data for *Euglena* and *Stichococcus*, being of the same order, made him confident of the validity of this coefficient. Applying this coefficient to the volume of a *Skeletonema* cell, Droop derived the number of cells of *Skeletonema* that could be supported by 0.1 mμg of B_{12}. See also Droop (1961).

It is evident that comprehensive biological assays of sea-water with unialgal and bacteria-free cultures of varied organisms will be extremely useful.

7. Prospects

It is evident that the biological approach to the problem of "bad" and "good" waters for phytoplankton has been quite successful; two variables, vitamins and trace metals, have emerged and seem to be, with N and P, the important parameters of the environment for phytoplankton. In the absence of chemical methods, the biological approach (i.e. nutritional requirements, organics produced and excreted, and biological analysis of water) may also be the way to attack similar problems for the marine herbivores and carnivores. Preliminary work on the nutrition of Crustacea in bacteria-free culture seems promising.

The nutritional studies reveal new important cycles. One of them, the B_{12} cycle, can now be roughly sketched: the main producers are the micro-organisms (mostly bacteria) though it is not excluded that photoautotrophic algae may be as important either as direct producers of vitamins or, after their death, as food for vitamin-producing micro-organisms. Micro-organisms (bacteria and unicellular algae, as far as we know) are the main consumers; possibly animals are also important consumers. Filter-feeding organisms—if the scant knowledge on *Tigriopus* can be extrapolated—may absorb vitamins directly as solutes. Perhaps animals with extensive gill systems absorb vitamins or other organic micro-nutrients in these highly permeable organs. The non-living particles (clay, organic and inorganic micelles, detritus) absorb large quantities of vitamin B_{12} and on ingestion may supply additional vitamins. How much the removal of vitamins from the particles affects vitamin cycles is unknown; we do not know whether these particles fix the vitamins in a stable way or only transiently; does partial elution maintain a certain level of vitamins as solutes during high consumption of vitamins by phytoplankton? Elution in deep muds might fertilize upwelling waters.

Consumption seems rarely to bring to zero the soluble B_{12}-like compounds in sea-water (Table XII). Though data are scarce, clearly coastal and bay waters, because of the influence of soil, are richer in cobalamins than open waters (Kashiwada *et al.*, 1957a; Droop, 1955; Lewin, 1954; Cowey, 1956). Surface waters show clearly a seasonal variation (Cowey, 1956) and growth of the spring diatoms (the dominant species is *Skeletonema costatum*, a B_{12} requirer) in Long Island Sound is responsible for a sharp drop in B_{12} level (Vishniac and Riley, 1959, 1961). There is more B_{12} in deep waters (Kashiwada *et al.*, 1957; Daisley and Fisher, 1958).[1]

Vitamin B_{12}-like growth factors, therefore, behave like the other ecologically significant nutritional variables—but are the cobalamins limiting, and where? Ecological judgment is uncertain because most of the measurements have been done with bioassay organisms which are not specific for true B_{12} (*E. gracilis* and

[1] See Addendum, page 210.

L. leichmannii) and many unicellular algae utilize only true B_{12} (Table XI). But the assays for Long Island Sound (Vishniac and Riley, 1959) were done with a true B_{12}-specific organism. The quantities found in Long Island Sound do not differ substantially from the data of Droop and Lewin for other coastal waters; all of them show ample B_{12}. It is probable, then, that in inshore waters B_{12} is rarely limiting and not a constraint on fertility. But when the quantities of B_{12}-like cobalamins are far lower, as in the open sea, the specificity of the various algae toward the different cobalamins may be decisive. For this reason much work has been done to determine the specificity of the algal species (Droop *et al.*, 1959). Since specificities vary widely (Table XI) both among the species and the ecologically important algal groups, it becomes necessary to measure the B_{12}-like cobalamins with several bioassay organisms and to determine the ratio true B_{12}/total cobalamins (as recommended by Cowey, 1956; Droop, 1957, and Provasoli, 1958a). Specificity alone is not enough!

Sensitivity of the various algal species toward true B_{12} (Provasoli, 1958a) and probably to the other cobalamins varies also (Ford, 1953). While there are no reasons to doubt that the *specificity* data based on *in vitro* studies can be transferred to the ecological situations, *sensitivity* data are biased. Sensitivity, i.e. the dose–growth response, does not depend solely on the variable to be measured but is obviously influenced by all other cultural conditions, especially the composition of the medium and temperature. After all, the improvement of media and of cultural conditions and the standardization of the inoculum are the routine procedure employed to develop a bioassay, speed of growth and sensitivity being the desiderata of a workable assay (Hutner, Cury and Baker, 1959; Hutner, Provasoli and Baker, 1961). Laboratory assay basal media are, from an ecological standpoint, grotesque in the way *all* constraints on growth have been removed save for the vitamin being assayed: this permits maximum sensitivity *in the laboratory*. The routine artificial marine media, even when not tailored for a specific organism, also lack many constraints: they generally allow a growth equal or superior to the one recorded in natural blooms, even though light conditions are in general not optimal (200–400 ft-candles of fluorescent light).

Artificial media are extremely useful in detecting the needed and the utilized metabolites; this knowledge is of ecological importance but the data on the sensitivity to metabolites, acquired in artificial media, cannot be extrapolated directly to the natural environment because each sample of sea-water is in effect a different basal medium (Johnston, in press). Samples of sea-water enriched with one-fifth volume of S36 medium (which contains vitamins, N, P, Si and trace metals) inoculated with bacteria-free *Skeletonema costatum* support, depending upon the sample of sea-water, more or less growth than occurs in the undiluted S36 medium taken as a yardstick. Obviously other properties of these different sea-waters affect the growth response of equal levels of nutrients, including vitamins. Conversely, a level of vitamins and nutrients which in artificial media supports a defined number of cells may support more or less

TABLE

Quantity of Vitamin B_{12}-Like

Locality and type of water	Assay organism	Method
Vineyard Sound, Woods Hole (coastal)	*Euglena gracilis*[a]	Dialysis of SW.
Northwest Arm, Halifax, N.S. (coastal, polluted)	*Stichococcus* sp.[b]	Direct measurement
Pier at Millport, Scotland (coastal)	*Monochrysis lutheri*[c]	Direct measurement
Aberdeen Bay, Scotland (coastal)	*Lactobacillus leichmannii*[d] *Ochromonas malhamensis*[e]	Phenol extract. of SW.
Northern North Sea	*L. leichmannii* and *O. malhamensis*[f]	Phenol extract. of SW.
Butt of Lewis	*L. leichmannii* and *O. malhamensis*	Phenol extract. of SW.
Norwegian Deeps	*L. leichmannii* and *O. malhamensis*	Phenol extract. of SW.
Bay of Biscay	*Euglena gracilis*[a]	Direct on diluted SW.
12 stations 0–30°N along 130°E (North Pacific)	*Euglena gracilis*[a]	Dialysis of SW.
Kagoshima Bay (variations in depth and diurnal)	*Euglena gracilis*[a]	Dialysis of SW.
Bahia Fosforescente (Puerto Rico)	*Escherichia coli*[g]	Phenol extract. of SW.
Long Island Sound (coastal)	*Thraustochytrium globosum*[h]	Direct measurement

[a] Responds to true B_{12}, pseudo-B_{12} and factors A, C, C_2.
[b] The specificity of *Stichococcus* toward various cobalamins is unknown.
[c] Responds to true B_{12}, pseudo-B_{12} and factors A, I and H.
[d] Responds to true B_{12}, pseudo-B_{12}, factors A, I and also desoxyribosides.
[e] Responds only to true B_{12} and factor I.

XII

Cobalamins Found in Sea-Water

Quantity or range	Remarks	Authors
30–200 mμg/l.	Variations due to length of aging in laboratory	Provasoli and Pintner (1953a)
10 mμg/l.		Lewin, R. A. (1954)
5–10 mμg/l.		Droop (1955)
6 mμg/l.	Same sample assayed with 2 bioassay organisms	Cowey (1956)
4 mμg/l.		
0.1 mμg/l. (Aug)–1.2 mμg/l. (Oct.)	Pronounced seasonal variations	Cowey (1956)
0.4 mμg/l. (Apr.)–2 mμg/l. (Feb.)		Cowey (1956)
0. 5 mμg/l. (Aug.)–2 mμg/l. (Apr.)		Cowey (1956)
2.26 mμg/l. (mean of 34 samples)	190–2190 m depth	Daisley and Fisher (1958)
0.57 mμg/l. (mean of 7 samples)	< 190 m and > 2110 m depth	
0.3–1.5 mμg/l. (range of 90% samples; min. = 0.0; max. = 20 mμg/l.)	Surface (0–100 m)	Kashiwada *et al.* (1957)
0.5–2.5 mμg/l. (range 80% samples)	Below 200 m	
7–26 mμg/l. (max. 20% samples)		
3.2 mμg/l. (aver. 34 samples; 6 samples 0.0)	Mouth of Bay	Kashiwada *et al.* (1957a)
5.3 mμg/l. (aver. 57 samples; 7 samples 0.0)	Middle of Bay	
6.7 mμg/l. (aver. 44 samples; 2 samples 0.0)	Deep inland part of Bay	
3.0–3.5 mμg/l. (inside Bay)	Bahia Fosforescente, Puerto Rico	Burkholder & Burkholder (1958)
1.3 mμg/l. (outside Bay)		
4.5–11.4 mμg/l. (after spring diatom bloom)	Pronounced seasonal variation	Vishniac and Riley (1959, 1961)
12–14.6 mμg/l. (winter)		

[f] The values obtained with the two assay organisms are similar, i.e. true B_{12} may be the major cobalamin (80–90%) in these waters.

[g] Responds to true B_{12}, pseudo-B_{12}, all factors including factor B and to methionine.

[h] Responds only to true B_{12}.

cells in different waters. Consequently, the bioassay of vitamins in *direct* assays of sea-water should be based on (Droop, 1955) or include (Daisley, 1958) "internal standards". The sample of sea-water is split in two portions equally enriched with N, P, Si and chelated trace metals. Two parallel growth curves are obtained either by diluting one portion 3 × with artificial sea-water and adding to both portions graded amounts of B_{12} (Droop, 1955) or by adding B_{12} to one portion and diluting both portions stepwise with H_2O (Daisley, 1958). One can calculate, by the interval separating the two regression curves, the amount of B_{12} present in sea-water without referring to an "external standard" (i.e. the growth curve obtained with increasing amounts of vitamins in artificial media). Assays based on B_{12} extracted from sea-water or separated by dialysis, do not need internal standards.

Since we cannot transfer laboratory sensitivity data directly to ecological situations, other means must be devised to judge whether vitamins are limiting. One method was found by Johnston (in press). He employed bacteria-free cultures of *Skeletonema*: no, or poor, growth occurred in over 200 samples of sea-water from the northern seas enriched with N, P, Si, with and without trace metals. Since the same types of waters enriched with S36 medium—which has vitamins—give much better growth, the content of vitamin B_{12}-like cobalamins was limiting in these waters. Over sixty of these samples were collected in winter. According to the data of Coway (1956) one would expect that 1–2 mμg/l. of vitamin B_{12}-like cobalamins were present. This quantity, according to laboratory sensitivity data (Provasoli, 1958a), is above the minimal quantity required by several marine algae. Unfortunately we have no laboratory data on the sensitivity of *Skeletonema*; however, a good density was obtained by Droop (1955a) in the second serial transfer in "no B_{12}" from a culture grown in 100 mμg/l. (the dilution factor for each transfer was 100). Similar assays with bacteria-free cultures of the important ecological species of algae of a given environment can tell directly whether the waters are deficient in vitamins. The assay with bacteria-free strains of the local ecological species permits a reliable judgment. The only inconvenience may be the duration of the assay, but this disadvantage is offset by an avoidance of the measurement of the quantity of vitamin (which has little meaning except for a comparison between different waters); this in turn requires differential assays and the determination of the vitamin specificity patterns of each organism of the environment. Bacterized unialgal cultures obviously cannot be used for this purpose—bacteria produce vitamins.

Trace metals are the other new parameter. The bioassays of Kylin, Thomas, Ryther and Guillard, and Johnston, extending the pioneering work of Harvey, prove that waters from the northern seas, as well as the Sargasso and tropical Pacific, benefit greatly from the addition of trace metals. As noted earlier, Johnston demonstrated that, in the northern waters, the deficiency is due not to lack of trace metals but to their unavailability; the addition of a metal chelator, because of its solubilizing power, is as effective as the addition of trace metals. Conceivably other waters, especially the tropical ones, may be

deficient in total trace metals, and availability is less important; a suitable assay could tell.

Since trace-metal utilization and deficiency may depend either on total quantity, on availability of trace metals, or on both, the presence or absence in sea-water of organic solubilizers becomes a primary factor. Many organic substances (amino, hydroxy and nucleic acids) which have been detected or quite likely exist in sea-water can chelate the heavy metals in various degrees. In fresh waters the "humic" and "yellow" acids are probably the more important solubilizers quantitatively; autochthonous substances like the polypeptides produced by blue-green algae may also be important. A similar situation, though quantitatively less significant, may exist in the sea-waters exposed to the influence of soil and large rivers, and may be partially responsible for the fertility of bays, estuaries and, perhaps, banks where the winter mixing more easily enriches the surface waters with the organic substances of the muddy bottoms. Perhaps other substances of autochthonous origin, i.e. products of marine organisms, operate in the sea. Since fertility may depend in large degree on the availability of trace metals, and chemical extraction of organic substances is rather difficult, we have to resort provisionally to other means.

The C/N ratio may give some indications, particularly if substances similar to the "humic" and "yellow" acids (which have a very high C/N ratio) are present in sea-water. If we assume that plankton has a C/N ratio of about 6, higher values would indicate the presence of some substances of the humic type and lower values the presence of proteins, amino acids or amines. There is a possibility that either very low or very high C/N ratios may coincide with the chelating ability of sea-water.

Clearly, chemical or biological methods are needed for measuring the chelating power in sea-water. Perhaps chelating power may be measurable by employing one of the ions which are preferentially bound, like Cu or Hg, if a way is found to detect them when they appear in sea-water in free form.

A biological way of detecting the presence of chelators would be to measure chelation as a function of its ability to remove the toxicity caused by poisonous ions of high stability constant such as Cu and Hg. Fogg and Westlake (1955) have demonstrated the chelating power of the polypeptides produced by blue-green algae by comparing the toxic action of graded amounts of Cu in the presence or absence of polypeptides on the motility of a filamentous blue-green alga. The rate of movement of the filaments was perceptibly reduced at a concentration of Cu of 0.5 mg/l. whereas a similar effect in the presence of the polypeptide was observed at 8–16 mg Cu/l. *Euglena gracilis* grows to high densities in a medium visibly blue by the addition of Cu sulfate if it is overchelated by EDTA.

A more complex type of assay than the one used by Johnston could be useful: samples of sea-water uniformly enriched with N, P, Si and vitamins, filter sterilized, could be enriched with aseptic additions of graded low concentrations of (*a*) a chelator; (*b*) a non-chelated trace-metal mixture of Fe,

Mn, Zn and Co ; (*c*) the same mixture but 1 : 1 chelated with EDTA. It is possible that a differential assay of this type may distinguish between the gradations of the four possible combinations, i.e. waters (1) rich both in chelators and trace metals ; (2) rich in trace metals and deficient in chelators ; (3) deficient in trace metals but rich in chelators ; (4) deficient in both trace metals and chelators. If these different combinations exist in sea-water then it is conceivable that the favorable effect of mixing may depend on mutual compensation of multiple deficiencies as well as a straight enrichment in nutrients by deeper waters.

Johnston was encouraged by his results with the addition of chelating agents to sea-water to reopen the question of artificial fertilization of sea-water, proposing to lower the cost of the operation by employing a chelator and minimal doses of N and P to be determined experimentally. Promising, inexpensive results were obtained by Buljan (1957) : to 70–80 l. of sea-water were added 5 l. of commercial concentrated H_2SO_4, 100 kg of superphosphate, and two spades-full of garden or forest soil. The phosphates were dissolved with stirring and sea-water added up to 200 l., the whole being allowed to drain slowly into the water of the bay from a moving boat. Use of sulphuric acid permitted the solution of such cheap sources of phosphorus, as finely crushed phosphate, degreased bone meal, guano, etc. ; the soil added vitamins, chelators and trace metals. A total of 37 mg of $P\text{-}PO_4$ was added in one year per ton of bay water. Blooms of surface algae and a conspicuous increase of phytobenthos resulted from the fertilization of the sheltered, shallow inlet of Valiko Jezero in the Adriatic Sea. The area became an excellent feeding ground for oysters (*O. edulis*). Growth of oysters was four times larger than it was for individuals of the same age during the two years preceding fertilization ; the weight of oysters showed an average growth of 26 g per individual per year, and the rate of weight increase was five times larger than in the unfertilized part of the bay.

One of the most attractive features of fertilization experiments is that they provide a means of judging whether in laboratory experiments, or in analysis of sea-water for a limited number of constituents, the really important factors may have been missed. Perhaps experimental ecology will gradually reduce the "trial-and-error" procedures that have characterized fertilization experiments in the past.

Addendum

Since the preparation of the MS a few publications worth mentioning have appeared.

Vishniac (1961) perfected a fungus bioassay for thiamine in sea-water (sensitive to 25 mμg/l.).

The occurrence of vitamin B_{12} in the Sargasso Sea (Menzel and Spaeth, 1962) has been measured for one year using the diatom *Cyclotella nana* (for the bioassay method see Ryther and Guillard, 1962). The quantity of B_{12} in waters above 50 m fluctuates from undetectable to 0.03 mμg/l. from May to October and this paucity of B_{12} seems to control the species composition of

the phytoplankton. The dominant organism throughout the year is *Coccolithus huxleyi*, which does not require B_{12} (but requires thiamine), and the bloom of diatoms occurs in April after the level of B_{12} has increased to 0.06–0.1 mμg/l. (a dozen species of diatoms isolated from the Sargasso require B_{12}).

A new series of enrichments of depleted Sargasso waters (at the end of the diatom bloom) containing their natural populations shows clearly that the relative proportions of various nutrients are directly responsible for species composition (Menzel, Hulburt and Ryther, in press). The initial population in the samples was dominated by *C. huxleyi* (90% numerically). One series of enrichments was done in glass carboys, the other in polyethylene bottles.

In glass carboys the enrichment with N + P caused dominancy of diatoms (70% *Skeletonema costatum* + 25% of three species of *Chaetocerus*); N + P + Fe produced a short, rich bloom of *Chaetocerus simplex* (later supplanted by unidentified flagellates). In the polyethylene bottles N + P enrichment caused a small, short-lived *Skeletonema* bloom followed by a large *Coccolithus* bloom; N + P + Fe favored preponderance of flagellates; N + P + Si favored a large *Nitzschia closterium* bloom concomitant with a smaller *Coccolithus* bloom; N + P + Si + Fe caused a dense bloom of *C. simplex*. In an environment which is almost stable for temperature and light, as the Sargasso Sea, the chemical environment seems, more than any other factor, to be responsible for the distribution and seasonal succession of forms.

Menzel and Ryther (1961) confirm and extend the results of Ryther and Guillard (1959). Iron is the most limiting factor for primary productivity of the Sargasso Sea and iron deficiency is not removed by the addition of EDTA. However, the addition of iron alone results in a short burst of growth, and sustained productivity is achieved only by adding Fe + N + P; evidently N + P are also limiting. These data from enrichments fit into the ecology of the Sargasso Sea: N and P are minimal and total Fe averages 10 μg/l. without any seasonal peak; *C. huxleyi*, the yearly dominant species, has an unusually low requirement for Fe [growth for many serial transfers is not affected by lack of Fe in the enrichment (Ryther and Kramer, 1961)].

From these results it is clear that the combining of chemical and bioassay analysis of the waters with enrichment experiments and the determination *in vitro* of the nutritional characteristics of the dominant species permits good insight into the ecological events.

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