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The Development of Artificial Media for Marine Algae

By

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The culturing of marine algae has proceeded slowly since MIQUEL (1890—93) succeeded in growing a few diatoms in the laboratory. Until recently most media were composed of sea water or sea water-like artificial solutions which are prone to precipitate because of the presence of several salts in concentration near saturation. In order to avoid precipitates such media must be sterilized either by filtration or by bringing them for a few seconds to 118—120° C (15 lb./in.^{2}). Phosphates, iron and other trace metals, if added to the medium before sterilization, cause precipitates that can be avoided only by tedious aseptic addition to the sterile base. Precipitates *per se* are not objectionable and poorly-soluble salts might be an effective way to provide a non-toxic reservoir of nutrients (PRINGSHEIM, 1946b); however it is difficult to reproduce precisely the same kind of precipitate in a medium as complex as sea water and the difficulty in repeating results is a common complaint from the early days (ALLEN and NELSON, 1910, p. 441). The addition of soil extract to sea water enriched with minerals ("Erd-SCHREIBER", FØYN, 1934a) while still presenting the difficulties mentioned above, provided a marine medium better suited for the growth of a variety of marine organisms (BARKER, 1935; GROSS, 1937).

In the last few years the recognition that many algae needed vitamins (PROVASOLI and PINTNER, 1953) and the inclusion of vitamins or organic extracts containing them in marine media has greatly increased the number of algae which it has been possible to culture axenically. The use of chelating substances (HUTNER, PROVASOLI, SCHATZ and HASKINS, 1950) has permitted the development of marine media which are not dependent on soil extract and natural sea water for their heavy metal control. Such synthetic solutions are more reproducible and appropriately enriched support at least the growth of a wide variety of algae. The goal of analysing ecological problems of the phytoplankton by the use of bacteria-free cultures under controlled laboratory conditions now seems more nearly attainable.

Natural sea water media

The first cultures of marine algae (diatoms) were achieved by MIQUEL in 1890—93. He observed that the waters of lakes, ponds, and the sea could not support in the laboratory continued and luxuriant growth of algae. Natural waters had to be enriched by the addition of some mineral salts that he compounded in the famous solutions A and B (table 1).

These solutions, soon neglected for fresh-water work because new formulae took hold, became the standard method for enriching sea water after the work of ALLEN and NELSON (1910). The original formula and the numerous modifications proposed

Table 1. *Sea water inorganic enrichments of the* MIQUEL*-type*

Solution A

	MIQUEL (1890)	ALLEN and NELSON (1910) "MIQUEL Sea water"	KILIAN (1911)	KETCHUM and REDFIELD (1938)	MATUDAIRA (1942)
$MgSO_4$	10 g.				
NaCl	10 g.				
Na_2SO_4	5 g.				
NH_4NO_3	1 g.		1 g.		1 g.
KNO_3	2 g.	20.2 g.	2 g.	20.2 g.	2 g.
$NaNO_3$	2 g.		2 g.		2 g.
KBr	0.2 g.				0.2 g.
KI	0.2 g.				0.1 g.
H_2O	100 g.	100 g.	100 g.	100 g.	100 ml.
quantity of sol. A added to 1 l. sea water	40 drops (2 ml.?)	2 ml.	2 ml.	0.55 ml.	1 ml.

Solution B

	MIQUEL (1890)	ALLEN and NELSON (1910) "MIQUEL Sea water"	KILIAN (1911)	KETCHUM and REDFIELD (1938)	MATUDAIRA (1942) [1]
$MgSO_4$				4 g.	
$Na_2HPO_4 \cdot 12\ H_2O$	4 g.[2]	4 g.	4 g.[2]	4 g.	4 g.
$CaCl_2 \cdot 6\ H_2O$	4 g.[3]	4 g.	4 g.[3]	4 g.[2]	4 g.
HCl conc.	2 ml.	2 ml.	2 g.	2 ml.	1 ml.
$FeCl_3$ (melted)	2 ml.	2 ml.	2 g.	2 ml.	
H_2O	80 ml.	80 ml.	80 ml.	100 ml.	100 ml.
quantity of sol. B added to 1 l. of sea water	20 drops (1 ml.?)	1 ml.	1 ml.	0.5 ml.	1 ml.

[1] Besides sol. A and B, MATUDAIRA adds: 1 ml. of sol. C (ferric chloride 1 g./100 ml.) 1 ml. of sol. D (3/20 n $NaHCO_3$), 0.5 ml. of sol. E (SiO_2, as Na silicate, 1 g./liter) and 1 ml. of sol. F ($MnCl_2$ 0.4 mg., H_3BO_3 0.4 mg., $CuSO_4$ 0.02 mg., H_2O 100 ml.) to 80 ml. of sterile sea water to which sol. A and B have been added. Sterilize at 90° C. for 30 minutes, decant off the precipitate before adding sol. C, D, E, and F.

[2] Anhydrous. [3] "dry" $CaCl_2$.

allow the growth of bacteria-free cultures of a few unexacting photoautotrophic organisms and bacterized cultures of many algae.

Inspection of the components of solution A reveals that MIQUEL designed it primarily for enriching fresh-water because some of its components (Mg, Na, K, Br, I, Cl, and SO_4) are found in low concentrations in fresh-water and are quite abundant in sea water. While MIQUEL employed it as such, even for enriching sea water, ALLEN and NELSON simplified solution A by eliminating the salts present in sea water as obviously unnecessary. They found that the nitrates were the active components and that KNO_3 could replace completely the whole of solution A. KILIAN (1911), KETCHUM and REDFIELD (1938), MATUDAIRA (1942), and others (table 1) suggested several modifications of solution A.

Solution B on the contrary has been seldom modified, though it has been employed at different concentrations. Its addition to sea water or fresh-water causes a precipitate upon sterilization even at 70° C. According to MIQUEL, this precipitate is necessary to the growth of freshwater algae but the enriched sea water should be freed of it by decanting the supernatant aseptically.

ALLEN and NELSON have made a detailed analysis of the components of MIQUEL's solutions and conclude that: (a) both solutions A and B are needed; (b) the nitrates are the only active ingredients of solution A for enriching sea water; (c) the beneficial action of solution B is only partially due to the addition of nutrients, because the analysis of the precipitate shows that almost all the phosphate and calcium, and all the iron added were precipitated; (d) the precipitate itself or single additions of the components of solution B are ineffective. ALLEN and NELSON concluded (p. 456) that the action of this solution B might be a "protective" one, i. e. "any removal or neutralization of harmful substances", since the combined addition of calcium and phosphate or of animal-charcoal could replace it. It might also be observed that solution B lowers the p_H of sea water and that the formation of a precipitate seems to be the essential feature because precipitates are formed by all effective combinations, solution B, calcium and phosphate, and animal charcoal [which according to THORPE (in ALLEN and NELSON, p.431) contains large quantities of Ca and Mg phosphates]. The protective action might be due to a co-precipitation of substances present in sea water in concentrations nearing toxicity at a p_H lower than that of sea water.

ALLEN and NELSON grew, in persistent but bacterized cultures, many of the ecologically important diatoms (*Asterionella, Biddulphia, Chaetoceros, Coscinodiscus, Nitzschia, Rhizosolenia, Sceletonema, Thalassiosira*) and reported that "MIQUEL sea water" supports growth also of several species of unidentified Rhodophyceae, Myxophyceae, filamentous Chlorophyceae (*Enteromorpha, Vaucheria*, etc.) and even young plants of *Laminaria*. This later observation led DREW (1910) to cultivate this alga artificially and so to discover its early life history. However, according to ALLEN and NELSON, "throughout the work we have had very great difficulty, in spite of much care and many precautions, in obtaining consistent results. It may even happen that, in two flasks containing the same culture medium, inoculated with the same culture of diatom and standing side by side, under exactly identical conditions, so far as can be recognized, quite different degrees of growth will be observed" (p. 436—37). This type of inconsistency is quite familiar all through the literature (e. g. ALLEN and NELSON, 1910; WILSON, 1951).

Differences in sea water itself may play a role: ALLEN and NELSON found consistently that, when not enriched, "outside sea water" (collected well outside Plymouth breakwater) was less favourable than the "tank" water (water taken from the *closed* circulation of the tanks of Plymouth's aquarium) where excretory products of marine organisms accumulate. This latter water supports, without artificial enrichment, the

growth in the tanks of minute naviculoid diatoms, also species of *Ectocarpus*, *Cladophora*, *Enteromorpha*, and *Vaucheria*, but it is quite unfavourable to planktonic diatoms or large seaweeds such as *Fucus* and *Laminaria* (the latter grows in outside sea water enriched with solutions A and B).

However, even in the presence of bacteria, the mineral enrichments do not support growth of all the marine algae. FØYN (1934) in his search for a good medium for a *Cladophora* formulated the "Erd-SCHREIBER" medium, composed of SCHREIBER's (1927) mineral sea water enrichment to which is added soil extract (table 2). Extracts of humus substances had proved useful for growing many species of freshwater algae which would not otherwise thrive in culture (PRINGSHEIM, 1912, 1913, 1926). FØYN classified the different marine solutions for their ability to grow *Cladophora* in the following order of increasing effectiveness: pure sea water, SCHREIBER's medium, ALLEN and NELSON medium ("MIQUEL sea water"), KILIAN medium, sea water soil extract, Erd-SCHREIBER.

Erd-SCHREIBER is a true step forward. Besides growing the algae which thrive in the ALLEN and NELSON medium it has permitted the unialgal culturing of *Cladophora* and *Ulva* (FØYN, 1934 a and b), of *Acetabularia* (HÄMMERLING, 1934), of *Dunaliella*[1] sp. and *Prorocentrum* (GROSS, 1934, 1937), of *Peridinium trochoideum*, *Exuviaella baltica* and *Syracosphaera carterae* (BRAARUD, 1951). Erd-SCHREIBER has since been used extensively for unialgal cultures of marine diatoms and flagellates at Plymouth (PARKE, 1949, 1955) and Burnham-on-Crouch (BUTCHER, 1952). GROSS (1937) had found that practically any sample of sea water, if enriched with the Erd-SCHREIBER components, gives a thriving culture of several species of small (2—10 μ.) chrysomonads and cryptomonads, thus revealing a new and abundant category of nannoplankton not retained by the finest net. One might tend to discount the importance of these μ-flagellates because of their minute size, but they are ubiquitous and abundant and because of their small size are the food of many early animal larvae. Two of these, *Pyramimonas grossii* and *Isochrysis galbana*, have proved to be the choice experimental food organisms for the larvae of the European oyster (BRUCE, KNIGHT and PARKE, 1940). Media similar to Erd-SCHREIBER were employed by BARKER (1935) and by SWEENEY (1951) for several dinoflagellates. BARKER grew in bacteria-free culture *Prorocentrum micans* and *P. gracile* and two unidentified *Peridinia* and, in bacterized culture, two *Gonyaulax*, *Exuviaella*, and a *Ceratium*. SWEENEY grew bacteria-free *Gymnodinium splendens*.

Prorocentrum had been grown bacterized by GROSS, but in contrast to BARKER, this author had been unable to grow *Ceratium* in Erd-SCHREIBER. BARKER's success might have been due to his having prepared the medium with "aged" sea water.

Although the Erd-SCHREIBER type of media represent a definite improvement over the MIQUEL-ALLEN and NELSON type they do not eliminate the latter's chief defects: precipitates and inconsistent results. Better results are achieved by the precaution of sterilizing the sea water and the enrichment separately or by filtration. The usual, more reliable and less bothersome sterilization at 15 lb./in.^{2} (121°C.) causes precipitates and erratic results unless the exposure at 121°C. is limited to one minute, which is scarcely sufficient to ensure complete sterilization. The success of these media may depend in great part on the type of "aged" sea water and soil employed for the extract and its age: SWEENEY (1951) finds that her soil extract becomes active one month after preparation and loses its activity 2—3 months later. This, however, is not our experience with our extracts.

The "aged" sea water that BARKER and SWEENEY employ is ordinary sea water which has stood in large glass bottles in the dark for months or years. The authors believe that during this period a complete mineralization has occured as a result of

[1] Referred to by GROSS as *Chlamydomonas*.

Table 2. *Sea water enrichments*

	SCHREIBER (1927)	FØYN'S Erd-SCHREIBER (1934a)	BARKER (1935)	SWEENEY (1951)	GOLDBERG *et al.* (1951)	SPENCER (1954)	SWEENEY (1954)	HAXO & SWEENEY (1955)	WILSON & COLLIER (1955)
$NaNO_3$	10 mg.	10 mg.	—	—	—	Varied	—	—	—
KNO_3	—	—	10 mg.	20.2 mg.	20.2 mg.	—	20.2 mg.	20.2 mg.	—
NH_4Cl	—	—	—	—	—	—	—	—	0.1 mg.
$Na_2HPO_4 \cdot 12\ H_2O$	2 mg.	2 mg.	—	—	—	Varied	—	—	—
K_2HPO_4	—	—	1 mg.	3.5 mg.	Varied	—	3.5 mg.	3.5 mg.	—
KH_2PO_4	—	—	—	—	—	—	—	—	0.05 mg.
$FeCl_3$	—	—	0.01 mg.	0.1 mg.	—	—	0.097 mg.	0.097 mg.	—
Fe Citrate	—	—	—	—	0.24 mg.	0.01 mg.	—	—	—
$MnCl_2$	—	—	—	0.0075 mg.	0.013 mg.	0.0045 mg.	0.0075 mg.	0.0075 mg.	—
Na_2HAsO_4	—	—	—	—	0.018 mg.	—	—	—	—
$CoCl_2$	—	—	—	—	0.013 mg.	—	—	—	—
$MgCl_2 \cdot 6\ H_2O$	—	—	—	—	—	—	—	—	0.02 mg.
$NaHCO_3$	—	—	—	—	—	—	—	—	0.1 mg.
$Na_2S \cdot 9\ H_2O$	—	—	—	—	—	—	—	—	0.1 mg.
EDTA	—	—	—	—	—	—	1 mg.	1 mg.	—
B_{12}	—	—	—	—	—	—	1 μg.	—	0.1 μg.
Thiamine HCl	—	—	—	—	—	—	—	—	1.0 mg.
Biotin	—	—	—	—	—	—	—	—	0.05 μg.
Soil extract	—	5 ml.	2 ml.	4 ml.	—	—	—	2 ml.	2 ml.
Sea water	100 ml.	100 ml.	—	—	—	—	—	—	—
Aged sea water	—	—	100 ml.	100 ml.	100 ml.	75 mg.	75 ml.	75 ml.	95 ml.
Distilled water	—	—	—	—	—	25 mg.	25 ml.	25 ml.	5 ml.

bacterial activity. This might be true but it is more probable that the bacterial growth has replenished the water with products of their metabolism and lysis, some of which may be growth factors.

It is to be expected, therefore, that these media may be difficult to reproduce exactly in other laboratories because of the different types of soil, and the different aging and bacterial flora of the "aged" sea water. SWEENEY's 1954 medium (table 2) is an improvement in that it eliminates the soil extract which is replaced by the addition of the active principles for *Gymnodinium splendens*: vitamin B_{12} at ample level and ethylenediamine tetra-acetic acid (EDTA) as a substitute for the metal-chelating activity of the natural humic acids of soil extract. "Aged" sea water, however, is retained in this medium.

Artificial sea water

As ALLEN and NELSON had already expressed clearly, the ideal aim is to grow organisms in artificially prepared solutions. Besides eliminating the difficulties due to the complex and variable composition of natural sea water and permitting the simplification of media by experimental evaluation of the relative needs for the different ions, "synthetic" solutions are necessary to the completion of nutritional studies.

It is understandable that, in tackling afresh this difficult problem, most authors designed artificial media to be as close as possible to the available analyses of sea water, with the consequence that their formulae became more complex as the analysis became more precise. The simple formula of PERRIER (1890) was employed for the great aquarium of oysters at the World Fair of Paris. The formula of HERBST (1897), based on the analysis of FORCHAMMER of the seawater between Naples and Sardinia was employed to rear larvae of sea urchins. PRINGSHEIM (1946a, p. 28) used a solution similar to HERBST's for several euryhaline organisms, among them *Dunaliella salina*. Another type of HERBST's artificial sea water, enriched with MIQUEL solutions and sucrose, was employed by IMAI and HATANAKA (1950) to grow bacterized cultures of a marine *Monas*.

ALLEN and NELSON were the first to try an artificial sea water for *algae*, a solution based on the molecular concentrations of sea water determined by VAN'T HOFF (1905). The mineral solution enriched with MIQUEL's solutions A and B supported the growth of *Coscinodiscus* in four instances (i. e. four isolated flasks); attempts to repeat this result failed. A new mineral solution based on DITTMAR's (1884) analysis of sea water was composed by ALLEN (1914) and served for his experiments on *Thalassiosira gravida*. The artificial sea water was prepared with pure Kahlbaum chemicals, enriched with phosphate, nitrate and iron, and with the p_H adjusted with $NaHCO_3$. Alone it failed to support growth but the addition of a small quantity (1—4 ml./100) of sea water permitted excellent cultures comparable with those in enriched sea water. Employing the addition of 1 ml. of sea water ALLEN made the first analysis of the flexibility of marine organisms toward concentrations of major elements. The salinity could be varied from 17—50‰ without affecting growth [confirmed with other organisms by BRAARUD (1951) and PROVASOLI, MCLAUGHLIN and PINTNER (1954)]; Ca, Mg, and K were necessary but could be varied within very wide limits (confirmed by PROVASOLI *et al.* (1954). The need for an additon of a few milliliters of sea water could not therefore be due to exacting requirements for major elements; nor could it be eliminated by adding bromides, iodides, nitrite, gold, aluminium, strontium and lithium. Several simple experiments pointed to an organic substance active in trace concentrations: a dilute water extract of *Ulva* was active while its ash was inactive; "tank" water was the best source; water from Plymouth Sound was better than the less polluted water of the English Channel.

Putrified peptone was effective while several amino- and organic acids were inactive. ALLEN remarked that these effects may be due to products of the metabolism of bacteria and suggested an organic micronutrient similar to the first vitamin which had just been diseovered by FUNK. How prophetic he was! It is really impressive to read this work and to see his alertness and acuteness in postulating that differences in productivity of coastal and oceanic waters may be due to the auxotrophy of algae and their need for micronutrients. This idea, disregarded by the majority, was followed up vigorously by HARVEY, KYLIN and their followers.

PEACH and DRUMMOND (1924) grew bacteria-free *Phaeodactylum tricornutum* (*Nitzschia closterium* forma *minutissima*) in ALLEN's "DITTMAR" artificial sea water enriched with the ALLEN-MIQUEL nutrients, while BARKER (1935) found that this artificial sea water enriched with nitrate and phosphate with and without the addition of soil extract supported as well bacteria-free growth of a *Peridinium* and *Cryptomonas* sp. as did "aged" sea water; *Prorocentrum micans* and *P. gracile* developed poorly, while *Eutreptia* did not grow, even in the presence of bacteria in a single duplicate experiment. Development was however much more erratic than in "aged" sea water medium, a possible indication that growth in the artificial base was due to carry-over. RYTHER (1954) employed a modification of the artificial sea water of MCCLENDON *et al.* (1917) for growing bacteria-free cultures of *Nannochloris atomus*, *Stichococcus cylindricus* (?), and *Phaeodactylum tricornutum* ("*Nitzschia closterium*").

CHU (1946a), following the extensive sea water analysis of LYMAN and FLEMING (1940) and THOMPSON and ROBINSON 1932) composed a complex artificial sea water (Solution A) in which he could grow in bacterized culture *Chaetoceros decipiens*, *Coscinodiscus excentricus*, *Ditylum brightwellii*, *Fragilaria striata*, *Skeletonema costatum* and *Thalassiosira decipiens*. *Laminaria* ash could substitute for the trace metal solution. LEVRING (1946) employed for growing germlings of *Ulva lactuca* an artificial sea water based on the compilation of the sea water components made by SVERDRUP, JOHNSON and FLEMING (1942) with additions from NODDACK and NODDACK (1939). The same medium was later employed by PETERS (1948) for bacterized cultures of *Enteromorpha intestinalis*. Essentially, CHU's and LEVRING's media are similar and represent a definite improvement over previous synthetic media because they contain trace metals. This was borne out by the knowledge that sea water itself, enriched with nitrates and phosphates is not an adequate medium for the majority of algal species. It is not surprising that artificial media, copied from sea water and similarly enriched, can support the growth of only a few autotrophic and less exacting species such as *Nannochloris* and *Stichococcus* (RYTHER, 1954).

The addition of trace metals became imperative when it was found that some of the growth-promoting substances contained in sea water were inorganic. ALLEN and NELSON (1910) had already noted differences in fertility in sea water collected in different localities and the poor quality of the "offshore" waters. DE VALERA (1940) found that the superficial waters of the *Fucus-Ascophyllum* zone permit the germination and normal development of the zygotes of *Enteromorpha*, while the waters of 30 meter depth allow only a slow and stunted growth. H. KYLIN (1941, 1942, 1943), employing germinating zygotes of *Enteromorpha* and *Ulva* as test organisms and counting the number of cells produced in a fixed time, added nutrients to the infertile waters of 30 meter depth until he obtained a growth equal to that produced by the waters of the littoral zone, making what he termed the biological analysis of the conditions responsible for their fertility.

It can be concluded from his results that the waters of 30 meter depth are poor in nitrates, phosphates, iron and manganese. A. KYLIN (1943, 1945), employing the same techniques found that an addition of Zn, Mn, Fe and Co stimulated growth

while nickel, aluminium and cadmium were inert; aluminium and cadmium became toxic at concentrations above 10^{-4} and 10^{-5} respectively.

HARVEY (1947) concluded that Ni, Ga, Co, Zr, Mo, V, Zn and Cu are present in sufficient concentration in the inshore waters because their addition did not affect the growth rate of a marine *Dunaliella*[1]. These waters contained less N, P, and Fe than is required for maximal growth and manganese was the limiting factor for *Dunaliella*. Manganese was not, however, the limiting factor for *Coscinodiscus excentricus* in the same waters.

Later H. KYLIN found that waters of 70 meter depth are even poorer in trace metals than those of 30 meter depth and concluded that the fertility of the inshore waters is due to their relative richness in nitrates, phosphates and trace metals and that these important elements for plant growth diminish progressively with depth and distance from the shore (H. KYLIN, 1946).

Unfortunately biological assays performed with bacterized cultures cannot admit of precise interpretation, particularly when organic substances but also when mineral and trace constituents are in question. To give an extreme example, the action of glucose, some organic and amino-acids, and also some growth factors on *Ulva* were studied by KYLIN (1942) and LEVRING (1946). Since these substances induce heavy growth of bacteria, increased growth of the alga cannot be interpreted as a direct action on the algae. Any product of bacterial metabolism might be responsible, from growth factors to KREBS cycle intermediates, or more simply, observed effects may be due merely to the creation of a reducing environment rich in carbon dioxide.

The beneficial action of soil or algal extracts may reflect the need for trace metals or might be interpreted as a way of providing growth factors, or even p_H and r_H buffering. Soil extract is a wonderful trace element solution chelated with humic acids, while sea weeds have been found to effect a manifold concentration of trace metals from sea water (BLACK and MITCHELL, 1952). B_{12} was synthesized by 70% of soil bacteria examined by LOCHEAD and THEXTON (1951) and many algae contain B_{12} (ROBBINS, HERVEY and STEBBINS, 1951; HASHIMOTO, 1954). But these extracts contain many other biologically active substances, the nucleic acid components, amino-acids, etc. which can be responsible for increased growth of an appropriate organism.

A recent example may illustrate the unreliability of results obtained with bacterized cultures. It is now established by one of us (DROOP, 1955b) that *Skeletonema costatum* in bacteria-free culture requires vitamin B_{12}. LEVRING (1945) had found that *Enteromorpha intestinalis, Fucus vesiculosus, Ascophyllum nodosum,* and *Ceramium rubrum,* were among the seaweeds whose extracts stimulated the growth of *Skeletonema* in bacterized cultures, while *Chondrus crispus* and *Scytosyphon lamentaria* were not. According to the analyses of ROBBINS *et al.* (1951) only *Ceramium rubrum* and *Enteromorpha intestinalis* contain good quantities of B_{12} (0.05—0.1 μg. per gram of dry alga) while *Fucus* and *Ascophyllum* are poor in this vitamin. The *Skeletonema*-inactive *Chondrus* contains 0.01—0.04 μg.

Results obtained by adding small quantities of vitamins to bacterized cultures are more indicative and more, though not completely, reliable. HARVEY (1955, p. 107) found that bacterized cultures of *Chaetoceros decipiens, Nitzschia seriata* and *Skeletonema costatum* gave no or only limited growth in enriched natural sea water, and that the addition of B_{12} gave abundant cultures. These results indicate that the particular sample of sea water was poor in vitamin B_{12} and suggest, but do not, because of the presence of bacteria, definitely establish a B_{12} requirement on the part of the diatoms.

[1] Referred to by HARVEY (1947) as *Chlamydomonas.*

Other attempts to find the nature of the growth-promoting substances contained in sea water were made by HARVEY (1939) who found that two different types of organic substances were necessary for continued growth of *Ditylum brightwellii* in bacterized cultures. One could be extracted from sea water, soil or algal extracts and is a sulphur-containing organic acid whose effect could be replaced by several organic compounds containing divalent sulphur, such as cysteine, methionine, glutathione, thiamine and biotin, and also by sodium sulphide. MATUDAIRA (1942) confirmed the effect of sulphides and cysteine with bacterized cultures of *Skeletonema*. LEVRING (1945) found cystine and asparagine stimulating for *Skeletonema*. R. A. LEWIN (personal communication) finds in aerated cultures that sulphides are responsible for extremely good growth of a strain of *Skeletonema* even in the absence of bacteria; on the other hand, in still cultures growth of the Millport strain of this diatom is obtained without divalent sulphur unless the medium (S 36) has been allowed to stand 10 days or more between autoclaving and inoculating. The effects observed with divalent sulphur will probably be interpreted more easily as a physical than as a vitaminological phenomenon.

Development of marine media in New York

At the beginning of our studies in New York the best available media were (1) sea water enriched with soil extract (FØYN's Erd-SCHREIBER) and (2) LEVRING's artificial sea water, which represented the most complete sea water substitute of chemically known composition. Erd-SCHREIBER was used extensively both as an isolation and a conservation medium. In it were isolated *Gyrodinium californicum*, *Syracosphaera carterae*, *Phormidium persicinum*, and several *Nitzschia* sp. The medium however forms an abundant precipitate during autoclaving and the consistency of results is very poor; the inconsistency depends both on the type of soil extract employed and the length and temperature of autoclaving.

In 1951 several attempts were made to improve this medium; these resulted in the formulation of ASW III (table 3) which is in effect Erd-SCHREIBER enriched with manganese, iron, glutamic acid, glycine, a mixture of known B-vitamins, and liver extract. This is the most successful formula of a series of all-purpose media rich in favourable substances yet non-toxic and of reliable performance. Both the organic and the inorganic enrichments were added with the intention of equating differences in activity of soil extracts of various origin. Manganese was added because HARVEY's (1947) results point to the possibility of encountering marine organisms with a high requirement for Mn like his *Dunaliella tertiolecta*[1]. Glycine and glutamic acid are two carbon sources utilized by many fresh-water algae (HUTNER and PROVASOLI, 1951), and their addition in relatively high concentrations was intended to serve also as metal chelating and stabilizing agents. The vitamin mixture and the liver extract were added as a source of the known and possibly unknown water-soluble vitamins.

[1] Referred to by HARVEY (1947) as *Chlamydomonas*.

This formula proved very valuable for the isolation of *Amphora perpusilla* and *Gomphonema parvulum* and has since proved useful for maintaining a number of species. On re-examining the medium in the light of knowledge of the needs and tolerance of several marine algae, it would appear that the concentrations of amino acids and vitamins are near to

Table 3. *Sea water organic enrichments* (Haskins Laboratories and Millport

	ASW III	STP	E 3	E 13	E 6
Sea water	100 ml.	80 ml.	50 ml.[1]	75 ml[1]	50 ml.[1]
Distilled water	—	15 ml.	50 ml.	25 ml.	50 ml.
Soil extract	4 ml.	5 ml.	0.5 ml.[2]	0.25 ml.[2]	0.25 ml.[2]
KNO_3	20 mg.	20 mg.	10 mg.	5 mg.	5 mg.
K_2HPO_4	2 mg.	1.0 mg.	1.0 mg.	0.5 mg.	0.5 mg.
Mn (as Cl^-)	0.04 mg.	—	—	—	—
Fe (as Cl^-)	0.01 mg.	—	—	—	—
NaH glutamate	50 mg.	50 mg.	—	—	—
Glycine	50 mg.	10 mg.	—	—	—
D, L-alanine	—	10 mg.	—	—	—
Vitamins 8[3]	0.1 ml.	0.1 ml.	—	—	—
Liver 1 : 20[4]	1.0 mg.	—	—	—	—
Liver (OXO L25)	—	—	—	—	25 mg.
Trypticase	—	20 mg.	—	—	—
Bacto Tryptone	—	—	—	—	25 mg.
Yeast autolysate	—	20 mg.	—	—	—
Sucrose	—	100 mg.	—	—	—
Glucose	—	—	—	—	25 mg.
pH	7.5	7.5—7.6			

[1] Autoclaved apart from the nutrients.
[2] Of a concentrated extract (see p. 411).
[3] See note [1] Table 4.
[4] Nutritional Biochemicals Corporation, Cleveland, U.S.A.

the high limit of tolerance, but the presence of these enrichments makes it also a very useful sterility test medium, especially if made in a semi-solid consistency by the addition of 0.3% agar.

The effect of concentration is even more evident with another sterility test medium STP shown in table 3. It is more complete and more concentrated than ASW III and stimulates luxuriant growth of marine bacteria but it is less favourable to the majority of algae, though *Prymnesium parvum*, *Monochrysis lutheri*, *Syracosphaera carterae* and a *Coccolithophora* do well in it. These results were fully explained when, in attempts to speed up growth, it was found that, even in bacteria-free culture, various peptones (tryptic digest of muscle, blood, casein, soya bean, etc.), yeast extract, liver extract, and other organic substances were inhibitory at 5—50 mg.% and become toxic above these values. A mixture of B-vitamins of suitable strength for protists such as *Herpetomonas* (COWPERTHWAITE, WEBER, PACKER and HUTNER, 1953),

Tetrahymena, soil amoebae (HUTNER, unpublished) and *Peranema* (STORM and HUTNER, 1953) is too concentrated for more sensitive planktonic fresh-water and marine organisms. Riboflavin stands out for its toxicity at 0.01 mg. %. On the other hand, there is some evidence of antagonistic (detoxifying) action between vitamins, for, while dissecting the diluted and now innocuous mixture to determine its active components, we found some of the component vitamins still toxic in these concentrations *if used singly.*

STP besides being employed as a sterility test medium has been used advantageously as an aseptic enriching addition (0.5, 1.0, 2.0 ml./10 ml. of medium) to mineral media when isolating in bacteria-free culture organisms suspected of having complex organic requirements.

Early experiments on the replacement of sea water by chemically defined media were full of difficulties; media such as that formulated for *Phaeodactylum tricornutum (Nitzschia closterium* forma *minutissima)* (HUTNER, 1948) did not support the growth of *Gyrodinium* nor *Amphora,* probably because they are high in phosphates and citrate and low in calcium and magnesium.

The LEVRING solution had the same defects as sea water, the easy formation of precipitates during sterilization and the inconsistency of results. The addition after sterilization of sodium bicarbonate to bring the p_H back to the right zone complicated matters.

For a fresh start we tried various possibilities to construct a non-precipitating yet adequate medium of general use for marine organisms. This goal was sought by the following means:

a) metal-buffering by the addition of metal chelators to create a non-toxic reservoir of trace metals;

b) p_H buffering;

c) determination of the lowest p_H compatible with growth;

d) reduction of the general salinity of the medium;

e) determination of the lowest optimal concentration of Mg and Ca and their optimal ratio;

f) investigation into the use of glycerophosphoric acid as a source of phosphorus. The salts of this acid are quite soluble;

g) introduction of other weak solubilizers, i. e. acids having highly soluble salts, such as lactic and tartaric acids;

h) determination of the lowest optimal concentration of phosphates.

Our first efforts were applied to *Gyrodinium californicum.* The quantities of Ca, Mg, and phosphates, the obvious causes of precipitates, were reduced as much as possible and 10 mg. % of ethylenediamine tetra-acetic acid (EDTA), a metal chelator and solubilizer, were added to

avoid further precipitation. Addition of EDTA necessitated the study of the needs for trace metals and, when a first adequate level of EDTA-trace metals + soil extract was found, we could investigate the lowest levels of Ca, Mg and K compatible with growth and formulate a less precipitatable medium. The elimination of soil extract was made possible by adding to the mineral base a mixture of amino-acids and known vitamins and by raising the concentration of the trace metal mixture and, later, by finding a new metal level for a higher percentage of EDTA (20 mg. %). The work of 'tailoring' a medium for *Gyrodinium* was extremely protracted because optimal growth of this organism was reached only after a month or more and because we had of necessity to control an extensive list of variables.

The resultant medium (PROVASOLI and HOWELL, 1952; PROVASOLI and PINTNER, 1953) still formed occasionally small amounts of precipitate and did not always allow consistent growth; it did however permit an analysis of the vitamin requirements (PROVASOLI and PINTNER, 1953). Maximal growth, when a large liquid-air surface is provided, is 30—50,000 cells of *Gyrodinium* per ml. This medium, however, would not support the growth of the other marine organisms which were growing in Erd-SCHREIBER or ASW III.

The attempts to find an artificial sea water suitable for many algae continued along two convergent lines: (*a*) the study of the mineral nutrition of single marine species as test organisms, in the hope of finding common requirements or some guiding principles for formulating a common medium; (*b*) the gradual simplification of sea water-like solutions. Progress along either line was applied towards the advancement of the other.

An extremely important finding in the early and slow study of the nutrition of *Gyrodinium californicum*, a colourless flagellate, and *Amphora perpusilla* (HUTNER and PROVASOLI, 1953) was that for each of them soil extract could be replaced by a mixture of vitamins and trace elements. This opened the way to the discovery of the extent to which auxotrophy is common in algae (PROVASOLI and PINTNER, 1953; LEWIN, R. A., 1954; DROOP, 1954b, 1955a, 1955b; SWEENEY, 1954): chrysomonads, cryptomonads, dinoflagellates, and diatoms frequently needed vitamins. LWOFF and DÜSI (1938) were the first to find auxotrophy in algae in the form of a need for thiamine (or for its thiazole or pyrimidine moieties) of a few colourless flagellates (*Polytoma*, *Polytomella*, and *Chilomonas*). Ten years later B_{12} (HUTNER *et al.* 1949) was found to be a growth factor for *Euglena gracilis* var. *bacillaris* and soon after many new algae were isolated belonging to phyla not yet explored nutritionally, most of them proving auxotrophic. Auxotrophy is present in organisms of every algal group and of all ecological environments. B_{12} and thiamine are the

most commonly required vitamins; others are required by certain forms.

With this knowledge, we tried again other variations of the LEVRING solution, one of them, ASP (table 4), proved quite useful and served in general, when suitably diluted, to isolate in bacteria-free culture some brackish dinoflagellates: *Peridinium balticum*, *P. chattoni*, and *Exuviaella cassubica*. The major elements, compounded according to LEVRING's formula, were enriched with a new trace metal mixture, vitamin B_{12}, and a mixture of known B-vitamins.

We modified LEVRING's trace metal solution which employed values found optimal by A. KYLIN, with iron increased 3-fold and cobalt added. We based our calculations on the values given by LEVRING for anhydrous salts (PETERS 1948 referring to the LEVRING formula employed the same values but for hydrated salts). The trace metals were compounded in a stock solution such that 1 ml. of it added to 100 ml. of medium gave the desired values. The other important modification was the addition of EDTA acid in a concentration about 2—3 times larger than the one necessary to form a 1 : 1 chelate with the trace metals of the mixture. The final concentration of EDTA in the medium is 1 mg. % an extremely small quantity in comparison with the 10—20 mg. % employed in the *Gyrodinium* medium. Yet this quantity is enough to bind the trace metal carried in with the large quantities of "pure" chemicals employed in marine media. With this quantity of chelator, the omission of sodium bicarbonate, and the lowering of the p_H to 7.4—7.6, the precipitate was reduced to almost nothing.

Different proportions of EDTA are not as useful: the quantity for a 1 : 1 chelate causes larger precipitates in the medium and the chelation above the 3 : 1 ratio often gives metal deficiencies. This metal-mix (P1) became very useful and, in employing it widely, we found that it could be added without harm even at high concentrations, *i. e.* 20 ml./100 ml. Its lack of toxicity pointed to a happy selection with balanced ratios of trace metals and chelator, making the whole a satisfactory metal buffer which could be employed at different levels; the best value for most species was later found to be 3 ml./100 ml.

Through evaluating the action of this mixture we at last understand some of the difficulties met when we were using higher concentrations of EDTA in marine media. At p_H's above 8.0 EDTA actively binds calcium and magnesium and, in some conditions attendant upon sterilization, probably forms double Ca or Mg salts less soluble than Na-Ca-EDTA salts. Adding therefore more chelator to marine media is not an advantage and the difficulty can be overcome only by employing quantities far above the nutritional need of elements which are, like Co, Cu and Zn, more avidly chelated and therefore able to displace most of Ca and Mg from the EDTA chelates (see also Discussion). In other words,

we had created a difficulty which we had of necessity to resolve in a manner which, while offering no physiological advantage, had the drawback of introducing such levels of metals as to be toxic to many species.

The ASP medium, appropriately enriched, will support the growth of *Gyrodinium californicum*, a *Platymonas*, *Syracosphaera carterae*, the aforementioned colourless flagellate and bacterized cultures of *Gymnodinium splendens*. Cultivating a few species in a common medium was a most encouraging achievement. But the major elements of the base, made to imitate a simplified sea water, were still prone to cause precipitates. The real achievement had been the discovery of a method of adding trace metals in a non-toxic way for several species.

Another way to avoid precipitates is to lower the p_H; originally we had started with media at p_H 8.0—8.2 to simulate the p_H of sea water, and our first experiments with *Gyrodinium* were aimed at finding the right conditions for lowering the p_H. Later this study was extended to *Amphora*, to the colourless flagellate, and to *Syracosphaera*, and it was found that some marine organisms can be grown at p_H 7.2—7.6 with certain balances of trace metals and chelator; chelation intensity varies with p_H. The P1 metal mix has the advantage of being perfectly suitable for this p_H range; some species however, as will be seen later, need an increased chelation for full growth.

Meanwhile we were searching for a good buffer for the zone 7.0—8.0. It was evident that we could not employ phosphates because they become rapidly toxic at concentrations far below those needed for buffering; moreover, phosphates form abundant precipitates. At first, we tried triethanolamine and tris(hydroxymethyl)aminomethane (TRIS), with the colourless flagellate. For this organism both triethanolamine and TRIS were adequate but TRIS became inhibitory at about 0.3%. Subsequently *Rhodomonas* was found not to be inhibited by 1.0% TRIS (the maximum tried), while *Gyrodinium* was inhibited by triethanolamine: the dinoflagellates are in general more sensitive to amines. We are now employing TRIS routinely as the p_H buffer for marine organisms, the 0.1% level not being toxic for any of the organisms tried. It buffers well between p_H 7.5 and 8.5 and at 0.4% successfully holds the p_H constant while a 4% CO_2 mixture in air is bubbled through the medium.

TRIS, however, like other amines, is mildly antibacterial owing to its tendency to antagonize potassium (MACLEOD and ONOFREY, 1954). This antagonism can be shown well with *Phormidium persicinum* (PINTNER and PROVASOLI, unpublished); in the absence of TRIS, the K requirement is already satisfied at 0.5 mg. % (the minimal quantity tried); the addition of 100 mg.% TRIS raises to 4—5 mg. %, the amount of K needed for full growth. The use of TRIS in our marine media at

the level of 0.1% is quite safe because we add a large quantity of KCl (50—60 mg. %); the abundance of Ca and Mg in sea water media helps to increase the margin of safety in using TRIS.

It was the application of these findings, the use of the P1 metal mix and TRIS, that permitted the construction of a synthetic mineral base for *Rhodomonas lens* (table 4, RC); the other important factors were the Ca/Mg ratio and the total concentrations needed by this organism. Ca and Mg are widely interchangeable so long as each element is present in a minimal quantity; equally good growth can be obtained with 8 mg. of Ca and 40 mg. of Mg or with 4—6 mg. Ca and 70 mg. of Mg; only 1 or 2 mg. of Ca are needed with Mg 100 mg. %. Similar results were obtained with *Phormidium persicinum*, which also seems to need very little calcium when magnesium is present in very high concentrations. It might be noted now that a small variation in Ca is reflected in a far larger one in Mg; this fact had escaped us previously and might have been one of the causes for poor growth in the old media. These experiments pointed to the possibility of growing marine organisms at concentrations of Ca and Mg entirely different from sea water; it was an invitation to inquire a little more extensively into the ability of marine and brackish organisms to grow at different salinities and at different monovalent/divalent ion and Ca/Mg ratios. Obviously non-precipitating media can be formulated more easily if one can replace the bulk of the divalent salts with the more soluble monovalent salts and reduce the overall salinity.

Experiments were set up to investigate the behaviour toward total salinity, Ca/Mg, and mono/divalent ion ratios of *Peridinium balticum* and *Exuviaella cassubica* as representatives of brackish water organisms and *Syracosphaera carterae* and *Rhodomonas lens* of the marine. Our results confirm BRAARUD's (1951) conclusions that most investigated marine and brackish organisms are euryhaline, being able to withstand wide variations in salinities and that salinities of 20—24 per thousand are often better than sea water. Even more encouraging was the flexibility shown toward the monovalent/divalent ratio: if the other conditions are right one can increase the monovalent ions to 6 and even 10 times the sea water ratio. In general Ca and Mg are both needed and, when both are present, they are widely interchangeable but the utilization of very different Ca/Mg ratios (from 24 : 1 to 1 : 24) seems to be dependent upon the salinity and mono/divalent ratios. The experiments, though too sketchy and based on too few species to permit more detailed conclusions, indicate the great physiological flexibility of the organisms in respect to certain parameters.

Simultaneously, the study of the nutritional requirements of *Amphora perpusilla* resulted in an artificial medium (DC in table 4) different from

the *Rhodomonas* conservation medium (RC). This medium is interesting in having high levels of glycerophosphate and lactate. These acids have the property of forming very soluble salts with Ca and heavy metals generally, and glycerophosphate, if utilized by the organism, serves as a most convenient and non-precipitating source of P and C. Glycerophosphate is utilized very well by *Phaeodactylum tricornutum (Nitzschia closterium* f. *minutissima)* (CHU 1946, b; HARVEY, 1953), by *Tabellaria flocculosa*, *Asterionella formosa*, and *Fragilaria capucina* (PROVASOLI and PINTNER, unpublished) and several purple bacteria (HUTNER, unpublished).

Calcium glycerophosphate is extremely soluble at room temperature but quite insoluble at high temperatures: therefore, to avoid precipitates during sterilization, glycerophosphate should be employed together with another acid whose chelated calcium salts are soluble, such as citric or nitrilotriacetic acids. Lactic acid, a weak chelator, has so far been found to be a good carbon source only for *Amphora*. As a rule of thumb, equal weights of glycerophosphoric and one of the other acids avoid precipitation. DC is an example of this combination; it does not precipitate and contains lactic acid.

The other alternative is the use of inorganic phosphorus (where glycerophosphate is not utilized). It is advisable to add it in minimal quantities even in the presence of the slight over-chelation introduced by the P1 mix. because precipitates are prone to form. Fortunately, very low concentrations suffice for most organisms: full growth is attained at 0.1—1.0 mg. % of phosphate. Furthermore, higher concentrations quickly become toxic, often at 10—20 mg. %. If more abundant growth is obtained by shaking and by bubbling CO_2 through, then P may become limiting and may have to be added intermittently. Another possibility is to add it as a known quantity of a poorly soluble salt of P such as Ca triphosphate. The concentration of 0.5 mg. % of phosphate is perfectly adequate for stagnant cultures and the possibility of precipitation is limited.

As a result of these convergent findings, new media like ASP 2 (table 4) were formulated to embody all the advantages of previous media: low salinity, high concentration of monovalent and low concentration of divalent ions; buffering with TRIS at 100 mg.% — a value low enough to avoid toxicity from competition with K yet high enough to ensure a good buffering action; addition of the P 1 metal mix at 3 ml./100 ml.— a relatively large and non-toxic reservoir of trace metals; p_H 7.4—7.6 to minimize formation of precipitates during sterilization; and a low concentration of phosphate. A Mg/Ca ratio of 5 was selected because this value serves for *Rhodomonas*, *Phormidium* and *Amphora*; we selected a relatively high value of Ca (10 mg. %) because a low level of Ca seems to

Table 4. *Selected synthetic marine media* (Haskins Laboratories)

	ASP	RC	DC	ASP 2	ASP 6
NaCl	2.4 g.	2.1 g.	1.8 g.	1.8 g.	2.4 g.
$MgSO_4 \cdot 7\ H_2O$	0.6 g.	—	0.5 g.	0.5 g.	0.8 g.
$MgCl_2 \cdot 6\ H_2O$	0.45 g.	0.5 g.	—	—	—
KCl	0.06 g.	0.06 g.	0.06 g.	0.06 g.	0.07 g.
Ca (as Cl^-)	40 mg.	7 mg.	10 mg.	10 mg.	15 mg.
$Na_2SO_4 \cdot 10\ H_2O$	—	0.3 g.	—	—	—
$NaNO_3$	—	—	50 mg.	5 mg.	30 mg.
KNO_3	10 mg.	10 mg.	—	—	—
K_2HPO_4	2 mg.	1 mg.	—	0.5 mg.	—
K_2 glycero-phosphate	—	—	40 mg.	—	10 mg.
$Na_2SiO_3 \cdot 9\ H_2O$	2.5 mg.	—	20 mg.	15 mg.	7 mg.
"TRIS"	—	0.5 g.	0.5 g.	0.1 g.	0.1 g.
Thiamine	—	0.1 mg.	—	—	—
Biotin	—	0.05 μg	—	—	—
B_{12}	0.02 μg.	0.1 μg.	0.3 μg.	0.2 μg.	0.05 μg.
Vitamin Mix 8[1]	0.05 ml.	0.02 ml.	0.1 ml.	—	0.1 ml.
Vitamin Mix S3[2]	—	—	—	1.0 ml.	—
Na taurocholate	—	0.3 mg.	—	—	—
Na lactate	—	—	0.05 g.	—	—
Na acetate $\cdot 3\ H_2O$	—	0.02 g.	0.05 g.	—	—
Na H glutamate	—	0.05 g.	0.05 g.	—	—
D,L-glycine	—	—	0.05 g.	—	—
Sucrose	—	0.07 g.	0.05 g.	—	—
Na_2 EDTA	1.0 mg.	1.0 mg.	3.0 mg.	3.0 mg.	—
Na_3 Versenol	—	—	—	—	3.0 mg.
Fe (as Cl^-)	0.01 mg.	0.01 mg.	0.08 mg.	0.08 mg.	0.2 mg.
Zn (as Cl^-)	5.0 μg.	5.0 μg.	15.0 μg.	15.0 μg.	0.05 mg.
Mn (as Cl^-)	0.04 mg.	0.04 mg.	0.12 mg.	0.12 mg.	0.1 mg.
Co (as Cl^-)	0.1 μg.	0.1 μg.	0.3 μg.	0.3 μg.	1.0 μg.
Cu (as Cl^-)	0.04 μg.	0.04 μg.	0.12 μg.	0.12 μg.	2.0 μg.
B (as H_3BO_3)	0.2 mg.	0.2 mg.	0.6 mg.	0.6 mg.	0.2 mg
"1 S" Metals[3]	—	1.0 ml.	—	—	—
Mo (as Na Salt)	—	—	—	—	0.05 mg.
H_2O	100 ml.	100 ml.	100 ml.	100 ml.	100 ml.
pH	7.6	7.2—7.4	7.6—8.0	7.6—7.8	7.4—7.6

[1] 1 ml. of Vitamin Mix 8 contains: thiamine · HCl, 0.2 mg.; nicotinic acid, 0.1 mg.; putrescine · 2 HCl, 0.04 mg.; Ca pantothenate, 0.1 mg.; riboflavin, 5.0 μg.; pyridoxine · 2 HCl, 0.04 mg.; pyridoxamine · 2 HCl, 0.02 mg.; *p*-aminobenzoic acid 0.01 mg.; biotin, 0.5 μg.; choline · H_2 citrate, 0.5 mg.; inositol, 1.0 mg.; thymine, 0.8 mg.; orotic acid, 0.26 mg.; B_{12}, 0.05 μg.; folinic acid, 0.2 μg.; folic acid, 2.5 μg.

[2] 1 ml. of vitamin Mix S3 contains: Thiamine · HCl, 0.05 mg.; nicotinic acid, 0.01 mg.; Ca pantothenate, 0.01 mg.; *p*-aminobenzoic acid, 1.0 μg.; biotin, 0.1 μg.; inositol, 0.5 mg.; folic acid, 0.2 μg.; thymine, 0.3 mg.

[3] 1 ml. of "1 S" metals contains; Sr, 1.3 mg.; Al, 0.05 mg.; Rb, 0.02 mg.; Li, 0.01 mg.; I, 0.005 mg.; Br, 6.5 mg.

substitute for a greater quantity by weight of Mg and because it was noted that the minimal quantity of Ca needed for 50 mg. % of Mg varies from species to species (from 3 to 10 mg. %).

The ASP 2 medium was designed to serve both for bacterized and pure cultures of photosynthetic marine algae; the values of nitrate and phosphate are kept low to suppress excessive bacterial growth and if needed they can be reduced even further to simulate the conditions found in nature. No carbon sources were added because most of the photosynthetic marine algae so far isolated seem to be strict phototrophs; only *Amphora perpusilla*, *Prymnesium parvum* and *Monochrysis lutheri* have thus far been shown to utilize preformed organic carbon sources.

The addition of vitamins was restricted to those most likely to be needed: B_{12} seems to be the most prominent; thiamine and biotin are seldom needed. The other vitamins included in the S 3 mixture were added as a precaution; this mixture proved to be less toxic than the more complete vitamin mix normally employed at the Haskins Laboratories (COWPERTHWAITE *et al.* 1953, p. 974). The ASP 2 medium, because of its low content in N and P, and its lack of carbon sources, does not favour bacterial growth, and it can be made to favour the preferential growth of algae even more by lowering the temperature to 10—15° C and by using the optimal light intensity for the algal species. Intense light is often needed but there are species, e. g. *Phormidium persicinum*, which are inhibited by more than 300 ft. candles (PROVASOLI and PINTNER, 1954). By keeping the bacter iato a minimum, bacterized cultures can be further improved by modifying the mineral base and purification procedures can be started with more chance of success. The silicate is generally added only when culturing diatoms; if it is added, to avoid precipitation, the p_H should be lowered to 7.2 after the addition of silicate and before adding the phosphate.

ASP 2 is only one of the typical media employed for culturing marine algae. It allows some growth of several diatoms, chrysomonads, cryptomonads, dinoflagellates, blue-green algae and chlorophytes and is a very good medium for *Phormidium persicinum*, *Gyrodinium californicum* and two other species of *Gyrodinium*, *Amphidinium klebsii*, *Prymnesium parvum*, *Rhodomonas lens*, *Stephanopyxis turris* and *Pilinia*. It has been found, however, that some organisms may need more trace metals or more metal chelators or both. The following experimental procedure often proved successful:

a) keeping constant the ASP 2 basal medium, add nitrilotriacetic acid (NTA), (a weaker metal chelator whose Ca and Mg salts are more soluble) in different quantities: 2, 4, 6, 8, and 10 mg. per 100 ml. of medium

(dissolve the NTA in a dilute solution of NaOH and bring to the final p_H of 7.5).

b) modify the ASP 2 base by adding the P1 metal mixture at different levels: 1, 3, 10 or 20 ml. per 100 ml. of medium.

c) vary simultaneously the quantities of NTA and P1 metals.

ASP 2 + 10 mg. % of NTA is a very good medium for *Eutreptia* sp., *Gymnodinium splendens*, *Isochrysis galbana*, *Syracosphaera elongata*, *Monochrysis lutheri*, and *Exuviaella cassubica* (medium diluted 1/3). Some species seem to prefer richer media: *Prorocentrum micans* and an *Eutreptia* grow better in RC and *Hemiselmis virescens* prefers media rich in amino-acids like S 46. The amino-acids can be beneficial as metal chelators, as carbon or nitrogen sources, and p_H buffers, and, in general are very useful when the mineral part of the base is not well balanced. The need in the case of *Hemiselmis* however is a specific one; one of us (M. R. D) finds that glycine is the best N source; apparently amino nitrogen is needed. Other substances that often seem to help growth are alanine, glutamic acid, asparagine, uric acid, guanine, guanylic acid, and thymine, though their need is not absolute or specific.

Another medium, ASP 6, (table 4) proved useful: it has a higher salinity, and makes easier the direct transfer from sea water of osmotically sensitive species. The increased salinity was obtained by raising proportionally the quantity of Na, K, Mg, and Ca of ASP 2. The metal mix is an entirely new one, more concentrated in trace metals and adjusted to Versenol (Na_3 hydroxyethylethylenediamine triacetate). Versenol has the advantage of forming metal chelates which do not hydrolyze easily at high p_H, thus avoiding precipitates at p_H 8.0—8.4. The raising to p_H 8.4 would be beneficial because at this p_H CO_2 is avidly absorbed and forms a reservoir of bicarbonate. This could be a means of providing increased CO_2 (which favours the growth of photoautotrophic algae) without having to employ bubbling or shaking — a difficulty when experimenting with the many variables to be considered in the study of nutritional requirements. Little work has been done yet with this metal mixture but ASP 6 is a good medium for *Syracosphaera elongata*, *Skeletonema costatum*, *Rhodomonas lens*, and *Amphidinium klebsii*.

Development of marine media at Millport

Identity of aims has naturally resulted in many problems and difficulties being common to the New York and Millport laboratories. Since we have discussed these in the previous section it will suffice in the present merely to annotate the Millport media given in the tables and to point out differences and similarities in our solution to the problems.

Modifications of the Erd-SCHREIBER medium have been used with success at Millport for the purpose of isolation and for conservation. In these solutions (e. g. E 3, E 13, E 6 in table 3) the hazards of precipitation are avoided by autoclaving fresh sea water apart from the nutrients and mixing them when cold. Autoclaving is kept down to a minimum: pressure is just brought to 15 lb. in a *small* pressure cooker. The soil extract recipe differs from others as it involves alkaline extraction, resulting in a very concentrated neutral extract which can be standardized by acid precipitation[1]. E 3 formed the base of a series of media enriched further with various organic extracts and peptones, E 6 being a variant most widely and successfully used. E 6, is, in effect, PRINGSHEIM's (1946a) 'soil-extract and salts', PRINGSHEIM's (1952) '*Ochromonas*' medium and natural sea water in the proportion 1 : 1 : 2 by volume, while E 3 is the first and last of these in equal proportions and E 13 in the proportion 1 : 3. Media of the E series were used in isolating in bacteria-free culture a number of supra-littoral, littoral and pelagic species (DROOP, 1954a, 1954b, 1955a, 1955b): *Monochrysis lutheri, Syracosphaera elongata, Prymnesium parvum, Mallomonas epithalattia, Microglena arenicola, Glenodinium foliaceum, Peridinium trochoideum, Hemiselmis virescens, Skeletonema costatum, Coscinoscira polychorda, Waerniella lucifuga,* and a number of Chlorophyceae. The presence of glucose, tryptone and liver extract in E 6 renders it very suitable for sterility testing. It has also been used for making minute aseptic enrichments (1—20 ml./100 ml.) to membrane filter-sterilized sea water. The recent tendency has been to decrease the quantity of organic enrichments, particulary for pelagic organisms.

Attempts at synthetic marine media at Millport have mostly been based on a sea water substitute (DROOP 1955a: SW 1) inherited from the Culture Collection of Algae and Protozoa, Cambridge, and which is PRINGSHEIM's (1946a) formula with magnesium sulphate omitted. Calcium is added as sulphate and the mixture differs from sea water in being low in Mg, Ca, and sulphate. In the media (e. g. table 5, S 22, S 32 etc.) this mixture is generally attenuated 4 times to give a salinity of $16^0/_{00}$. The solution then has 11.6 mg. % Ca, about a quarter of the amount present in sea water.

Experiments, rather similar to those referred to on p. 406, to determine the optimum concentrations of Na, Mg and Ca indicated values of 3—6, 12 and 12—$24^0/_{00}$. salinity respectively for *Monochrysis, Phaeodactylum,* and *Skeletonema* and Ca optima of 1.5—3, 1—10, and 10 to 20 mg. % respectively. Unlike Ca, Mg could be varied within very wide

[1] Two parts by volume of water to one of soil with 2—3 g. NaOH per l. of water steamed for two hours gives a neutral extract yielding 5 to 6 g. of acid precipitatable humic substance per l. of extract. The extract is filtered clear.

limits (0.1—100 mg. %) particularly when Ca was optimal. There was no indication that Ca was replaceable by Mg in these experiments, in which respect they differ from the New York experiments.

SW 1 would seem, therefore, to present a fairly good compromise, though, of course, one could hardly expect to meet the requirements of every organism with a single solution.

More exact imitations of natural sea water have been used instead of SW 1 in some of the media (e. g. U 22, U 32, U 36 etc.) but without any general advantage. Their use is limited to instances when a more naturalistic artificial sea water is desired for reasons other than the requirements of the alga. The low salinity medium V 37 was designed for *Monochrysis*, on the results of the experiments referred to above, and has been used for *Mallomonas epithalattia* and other supra-littoral species.

With the mixture of major elements has been added a rather arbitrary mixture of minor elements down to lithium, but with boron and fluorine omitted (table 5; SW 2 in Droop, 1955a) but there is no evidence to suggest that any of them is generally essential.

Attempts at heavy metal control were at first directed towards finding a mineral substitute for soil extract for the freshwater non-auxotrophic *Haematococcus pluvialis*. Presentation of iron was the main problem. RODHE's (1948) citric acid-iron citrate mixture was not efficient in the alkaline media necessary for the cultivation of this organism. The first successful defined media contained an unusually high addition (1—10 mg. %) of iron citrate which functioned as a reservoir after being hydrolyzed and precipitated by autoclaving. Later, iron citrate was replaced by smaller amounts (0.01—0.1 mg. %) of an iron chelate of EDTA[1], with even better results. Manganese and calcium were the only other trace metals added.

HUTNER and PROVASOLI's early formula of an EDTA chelated metal mixture (HUTNER and PROVASOLI, 1951) was very much too concentrated for either *H. pluvialis* or the majority of the euryhaline organisms in culture at Millport. However, when copper and cobalt were omitted the mixture was satisfactory when the heavy metals and EDTA were presented at 1/25 of the concentration suggested. Finally, following PROVASOLI and PINTNER (1953) the re-introduction of small amounts of copper and cobalt led to the metal mixture TM 2 (table 8; DROOP, 1955a) which has since been used extensively in both fresh water and marine media at Millport. The original mixture has survived several attempts of improvement by increasing or decreasing the chelate ratio. TM 2 is very similar to the P1 mixture of the Haskins Laboratories as regards

[1] $FeCl_3$ precipitated from solution with NaOH, washed, and dissolved to saturation in a known solution of Na_2 EDTA.

overall concentration, but the high Zn concentration causes the chelate ratio to be as low as 6 : 5. TM 2 stock solution is prepared at one hundred-fold the concentration at which it is used in the media; if prepared with sulphates it does not throw a precipitate on standing.

The three mineral solutions, SW 1, SW 2, and TM 2, described above constitute the base of all the synthetic media in routine use at Millport (Series S media). With appropriate defined enrichments (tables 5, 9) they have allowed very heavy bacteria-free cultures of *Monochrysis lutheri, Prymnesium parvum, Syracosphaera elongata, Isochrysis galbana, Hemiselmis virescens, Nannochloris oculata, Phaeodactylum tricornutum* and *Skeletonema costatum* and have been used to maintain *Prorocentrum micans, Oxyrrhis marina, Microglena arenicola, Mallomonas epithalattia, Waerniella lucifuga* and *Ectocarpus parasitica.*

The useful p_H range of culture media prepared from these solutions has been found to be from 8.8 (for *Hemiselmis virescens*) to 7.2; and throughout this range precipitates do not form with SW 1 even at double the normal strength (salinity $32^0/_{00}$) when the solutions are autoclaved with 1 mg. % K_2HPO_4. It should be noted, however, that the methods of avoiding precipitates differ from those in use at the Haskins Laboratories. It has been the practice at Millport to autoclave all solutions as lightly as is consistent with efficiency. Opinions differ as to what the minimum permissible pressure and time are, but at Millport we have found that contamination never occurs in media autoclaved in test tubes in a small pressure cooker (capacity 5 litres) when the pressure is brought up to 15 lb./in^2 and left there for no longer than one minute (in contrast to 15 lb. for 20 mins.). With light autoclaving such as this the problems of precipitation are much less and the latitude in permissible formulae consequently greater. This practice also obviates having to lower p_H merely in order to avoid precipitates, a consideration of some importance when an ecological p_H is demanded.

In the course of investigations into the nutrition of chrysomonads it was found that soil and liver extracts were not always completely replaceable by known B vitamins and the mineral solutions described above. There frequently remained a residual beneficial effect derived from the addition of various natural substances which did not seem at all specific. Sometimes mixtures of amino- and aliphatic-acids and purines could complete the replacement and one had the impression that all that was needed was to make the medium as complex as possible. It was eventually realized that the effect of these substances was more often physical than nutritional and that p_H buffering was chiefly concerned. The first buffered media to be used at Millport contained a glycine-guanine-uric acid mixture (S 22) which was adequate for *Nannochloris, Hemiselmis* and the chrysomonads; but, following the practice of the

Table 5. *Selected synthetic marine media* (Millport)

1) Sea Water Substitutes

Serial letter of medium	S (SW 1 attenuated × 4)	T	U	V
NaCl	1.5 g.	1.23 g.	2.45 g.	0.5 g.
$MgCl_2 \cdot 6\,H_2O$	0.25 g.	0.49 g.	0.98 g.	0.075 g.
KCl	0.04 g.	—	—	—
Ca (as Cl^-)	—	0.02 g.	0.04 g.	0.0024 g.
Ca (as $SO_4^=$)	0.012 g.	—	—	—
$Na_2SO_4 \cdot 10\,H_2O$	—	0.36 g.	0.73 g.	—
K_2SO_4	—	0.043 g.	0.085 g.	0.013 g.

2) Minor Elements (SW2 attenuated × 200) common to all media		3) Trace Metals (TM2 attenuated × 100) common to all media	
Br (as K^+)	2.2 mg.	Na_2 EDTA	2.0 mg.
Sr (as Cl^-)	0.38 mg.	Fe (as EDTA)[1]	0.01 mg.
Al (as Cl^-)	0.0028 mg.	Zn (as $SO_4^=$)	0.23 mg.
Rb (as Cl^-)	0.0061 mg.	Mn (as $SO_4^=$)	0.065 mg.
Li (as Cl^-)	0.0006 mg.	Mo (as $NaMoO_4$)	0.02 mg.
I (as K^+)	0.002 mg.	Co (as $SO_4^=$)	0.00063 mg.
		Cu (as $SO_4^=$)	0.00013 mg.

4) Enrichments

Serial Number of medium	22	32	36	37	46
TRIS	—	50 mg.	50 mg.	50 mg.	50 mg.
KNO_3	10 mg.	10 mg.	10 mg.	10 mg.	10 mg.
K_2HPO_4	1.0 mg.	1.0 mg.	1.0 mg.	1.0 mg.	1.0 mg.
Thiamine	0.1 mg.	0.1 mg.	0.1 mg.	0.1 mg.	0.1 mg.
B_{12}	10 mμg.	10 mμg.	10 mμg.	10 mμg.	10 mμg.
Glycine	4.0 mg.	—	—	4.0 mg.	30 mg.
Glutamic acid	—	—	—	—	30 mg.
Asparagine	—	—	—	—	30 mg.
Guanine	4.0 mg.	—	—	1.0 mg.	—
Uric acid	0.4 mg.	—	—	0.4 mg.	—
Glucose	—	—	—	—	30 mg.
Na acetate (anhyd.)	—	—	—	—	30 mg.
$Na_2SiO_3 \cdot 9\,H_2O$	—	—	10 mg.	—	—

Quantities given are amounts per 100 ml. of medium (prepared with glass distilled water). The sea water substitutes, Minor Elements and Trace Metals are conveniently dispensed from concentrated stock solutions. (e. g. The Sea Water Substitute of Series S media is the solution referred to on p. 411 as SW 1 attenuated 4 times, the chelated Trace Metals those of solution TM2 attenuated 100 times, and the Minor Elements solution SW2 attenuated 200 times.) p_H is adjusted with HCl or NaOH as required.

[1] The sulphate or chloride may be used.

Haskins Laboratories, it has since been replaced by the more efficient TRIS (in S 32, S 36 etc.). Uric acid and guanine had originally been included among the organics in our defined media because the species then being studied came from a habitat often rich in uric acid and related compounds in the form of gull excrement (DROOP, 1953). TRIS enabled *Skeletonema* to be cultured in a defined medium, S 36 (DROOP, 1955b).

S 36 is equivalent to the Haskins ASP 2 medium in both composition and application. Since nutritional studies have indicated a frequent requirement for thiamine and B_{12}, table 5 has included only versions of the media containing these vitamins. Mixtures of B vitamins of which there are numerous examples in the literature (and two at the base of table 4) can be usefully added to these media during the early stages of nutritional investigations. Medium S 46 was designed to meet the high glycine requirement of *Hemiselmis virescens* and was further enriched to make it more generally useful for species preferring a high organic content; it is very similar to medium DC of the Haskins Laboratories, and is used to maintain *Prymnesium parvum*. S 37 is, however a more generally useful medium as it allows adequate growth of the species preferring S 46 and also of those, like *Monochrysis lutheri* and *Syracosphaera elongata*, which give heaviest growth in relatively poor media such as S 32.

Discussion

We have described how several similar media have been developed independently in New York and at Millport. Another marine medium, developed by VISHNIAC (1955a, 1955b) for *Labyrinthula* (table 6), is also a good all-purpose medium: besides supporting growth of three species of *Labyrinthula* it has been used for several marine fungi. It is, therefore, of interest to find that this fungal medium has points of similarity to our media for algae.

The ratio between Mg and Ca is extremely close to ours (6 : 1); the description of a wide interchangeability between the two ions and the ability of a little Ca to substitute for a larger quantity of Mg (VISHNIAC, 1955b) parallel almost exactly our observations on *Phormidium* and *Rhodomonas*. The only differences are the lower minimal requirement in Mg and Ca and the ability of one isolate of *Labyrinthula* to grow without added Ca and with very low Mg when the other ion is present in high quantities.

The interesting ecological situation of the Salton Sea formed by the irrigation drainage of the Imperial Valley, California, lends weight to data on flexibility of a number of organisms in respect to Ca/Mg ratios. The waters of the Colorado River which form the Salton Sea, through intense evaporation have reached salinities similar to sea water but,

Table 6. *Other Synthetic marine media*

	Hutner (1948) *Phaeodactylum*	Hutner *et al.* (1950) *Dunaliella*	Vishniac & Watson (1953) *Labyrinthula*	Vishniac (1955a, 1955b) marine fungi	Provasoli & Pintner (1953) *Gyrodinium*	Lewin (1954) *Stichococcus*	Lewin (1955) *Prasiola*	Ryther (1954) *Nannochloris, Stichococcus*
NaCl	0.2 g.	0.25—4.0 g.	2.5 g.	2.5 g.	2.4 g.	2.3 g.	2.6 g.	2.67 g.
$MgCl_2 \cdot 6\,H_2O$	—	—	—	—	0.3 g.	1.1 g.	—	0.22 g.
$MgSO_4 \cdot 7\,H_2O$	0.25 g.	0.25 g	0.5 g.	0.5 g.	30 mg.	—	0.6 g.	0.32 g.
KCl	—	—	—	0.1 g.	30 mg.	—	—	73 mg.
Na_2SO_4	—	—	—	—	—	0.4 g.	—	—
$NaHCO_3$	—	—	—	—	—	0.2 g.	20 mg.	20 mg.
KNO_3	—	—	—	—	10 mg.	—	—	—
$Ca(NO_3)_2 \cdot 4\,H_2O$	—	15 mg.	—	—	—	10 mg.	100 mg.	—
NH_4NO_3	50 mg.	—	—	—	—	—	—	—
NH_4Cl	—	—	—	—	—	—	—	5.3 mg.
$(NH_4)_2CO_3$	—	—	—	20 mg.	—	—	—	—
$CaCO_3$	14 mg.	—	25 mg.	20 mg.	20 mg.	—	—	—
$CaCl_2$	—	—	—	—	—	—	—	0.15 g.
K_2HPO_4	40 mg.	20 mg.	10 mg.	—	—	2 mg.	20 mg.	—
$Na_2HPO_4 \cdot 12\,H_2O$	—	—	—	—	—	—	—	2.0 mg.
KH_2PO_4	—	—	—	10 mg.	2 mg.	—	—	—
$Na_2SiO_3 \cdot 9\,H_2O$	5.0 mg.	—	—	—	—	—	—	2.0 mg.
EDTA[1]	—	50 mg.	50 mg.	50 mg.	20 mg.	—	10 mg.	—
Na_3 citrate $\cdot 2\,H_2O$	100 mg.	—	—	—	—	—	—	—
Fe	0.5 mg.	0.6 mg.	0.2 mg.	0.2 mg.	0.3 mg.	0.05 mg.	0.05 mg.	0.02 mg.[3]
Mn	0.05 μg.	1.0 mg.	2.0 mg.	2.0 mg.	1.0 mg.	0.01 mg.	0.01 mg.	—
Mo	5.0 μg.	1.0 mg.	—	0.02 mg.	0.05 mg.	0.01 mg.	0.01 mg.	—
Zn	5.0 μg.	3.0 mg.	2.0 mg.	2.0 mg.	0.4 mg.	0.03 mg.	0.03 mg.	—
Co	—	—	—	0.02 mg.	3.0 μg.	—	—	—
Cu	5.0 μg.	0.5 mg.	—	0.002 mg.	0.3 μg.	0.01 mg.	0.01 mg.	—
B	0.05 mg.	4.0 mg.	—	0.02 mg.	0.2 mg.	0.01 mg.	0.01 mg.	1.0 mg.
KBr	—	—	—	—	—	—	—	5.8 mg.

Table 6 (Continued)

Other additions .	K acetate 0.2 g., Glycine 0.25 g.	Gelatin hydrol. 100 mg., PABA, folic, biotin, nicotinic, pantothenate, pyridoxine, pyridoxamine, B_{12}, thiamine.	Gelatin hydrol. 200 mg.[2]	1 S metals[4] 0.5 ml., NaH glutamate, lysine, leucine, B_{12}	B_{12}	Asparagine 100 mg.
pH	7.2—7.5	8.0—8.2		7.5	8.0	

Amounts are per 100 ml. of medium.

[1] Ethylenediamine tetraacetic acid. [2] Added in VISHNIAC 1955b for 3 isolates of *Labyrinthula*. [3] As citrate. [4] See note [3] table 4.

because of their origin, have a different ionic composition. Compared with sea water (table 7) they are high in SO_4, Ca and Na and lower in Mg and K: the Mg/Ca ratio is roughly 1 : 1, that of sea water 3 : 1.

During the last few years many artificial introductions of marine organisms have resulted in the establishment of a peculiar community in which the dominant species are the plankton-feeding fish, *Bardiella icistius*, a barnacle, *Balanus amphitrite*, an anellid, *Neanthes suecinea*, many marine phytoplanktonts, including *Exuviaella*, *Peridinium*, *Amphidinium*, *Gyrodinium*, many diatoms, and a blue-green alga (BOYD CARPELAN and WHITNEY, 1954). We have here a whole series of typically marine organisms belonging to both animal and plant kingdoms which are evidently able to tolerate ionic ratios of the major elemente far removed from those of their normal habitat. The Salton Sea is instructive for the very reason that its evolution has not proceeded as far as that of, for instance, the Great Salt Lake or the Dead Sea, whose inhabitants are so specialized or tolerant as to be useless for purposes of comparison.

It is interesting to compare our trace metal solutions with those of VISHNIAC. In order to do this, in table 8 the P1 and TM 2 mixtures have been entered at, respectively, 50 and 30 times their normal concentration to bring the amounts of chelator up to the level used in VISHNIAC's media. The ratios between chelator and trace metals (by equivalents) are respectively 3 : 1 (P 1) 2 : 1 (VISHNIAC and *Gyrodinium*) and 1.2 : 1 (TM 2). The difference is caused mainly by the Zn content of the mixtures: in

the P1 mixture the order of concentrations follows the MELLOR-MALEY series (MELLOR and MALEY, 1948), Zn being in its rightful place between Fe and Co. It is difficult to see what advantage is gained by the displacement of Zn in the other three mixtures; though possibly it has the effect that Mn and Fe are rather more loosely held and therefore more readily available while Co and Cu are not affected. In the case of TM 2 no obvious benefit was derived from reducing Zn. These mixtures are to a great

Table 7. *Comparison of the ionic composition of Salton Sea and ocean water* (BOYD, CARPELAN and WHITNEY, 1954)

Ion	Ocean Water (Concentration per 1000)	Salton Sea Water (Concentration per 1000)
Cl^-	18.98	17.011
$SO_4^=$	2.65	7.45
HCO_3^-	0.14	0.17
Na^+	10.55	11.82
Mg^{++}	1.27	0.99
Ca^{++}	0.40	0.80
K^+	0.38	0.19
Total	34.37	38.43

Table 8. *Comparison of chelated trace metal mixtures*

	VISHNIAC (1955) (mg.)	P 1 concentrated[1] 60 × (mg.)	TM 2 concentrated[1] 30 × (mg.)	*Gyrodinium* concentrated[1] 2.5 × (mg.)
EDTA	50	50	50	50
Zn	2	0.30	6.7	1.0
Mn	2	2.4	1.9	2.5
Fe	0.2	0.6	0.3	0.75
Co	0.02	0.006	0.02	0.0075
Cu	0.002	0.0024	0.004	0.00075
Mo	0.02	—	0.66	0.125

[1] Relative to concentration used in medium.

extent empirical (P1 is the most logical) and it is not easy to justify them theoretically. But it does appear that provided the MELLOR-MALEY order is adhered to, the most useful chelation ratio lies between 3 : 1 and 2 : 1. As might be expected, greater ratios lead to over-chelation and metal deficiencies while smaller ones cause precipitation. Some deviations from the MELLOR-MALEY order are tolerated, however, or may even be advantageous, if they alter slightly the permitted limits of the chelation ratio.

We have already remarked on the relative lack of toxicity of P1 and TM 2 toward most algae, some tolerated P1 up to 20 or even 40 ml./100 (i. e. 2/5 or 4/5 of VISHNIAC's concentrations). We have chosen

to employ for our media low values to avoid the addition of high quantities of chelator. VISHNIAC employs a large quantity of trace metals in her media, and it might well be that, in general, marine fungi withstand, or indeed, prefer higher concentrations of trace metals than algae. On the other hand P1 mixture (in ASP 2) permits growth of several marine ascomycetes and of *Halophiobolus* sp., *Ceriosporopsis* sp. and *Peritrichospora* sp., (S. MEYER, personal communication), and VISHNIAC's S 3 strain (probably an *Ectrogella*) also grows in an ASP 2 type of medium if glycerol and thiamine are added (VISHNIAC, 1955a).

It will now be apparent that all the more recent synthetic media discussed in this paper[1] are built according to a common plan which may be shortly stated:

1) An artificial sea water mixture of low salinity and low calcium content.

2) A chelated trace metal solution with a ratio of chelator to trace metals of not less than 1 : 1 and not more than 3 : 1, usually employed at great dilution.

3) A p_H buffering system.

4) Inorganic macro-nutrients, and organic macro- and micro-nutrients as required.

The success of culture media built on this plan is by and large considerable. Indeed it appears that our knowledge now enables us to undertake with some confidence culture and nutritional studies on any photosynthetic littoral, supra-littoral or estuarine organism. There is no such confidence, however, where pelagic species are concerned. Whereas the chrysomonads probably do not present a nutritional obstacle to their pure culture and several have been cultured satisfactorily, not a single *Chrysochromulina* is represented. Pelagic members of other groups are limited to two or three dinoflagellates, a diatom or two and a single cryptomonad. No oceanic species has yet been obtained in pure culture.

In spite of the ubiquity and abundance of μ-flagellates, the bulk of phytoplankton is considered to be composed of centric diatoms, and to a lesser extent, at any rate in northern waters, of dinoflagellates. It is precisely with this class of organisms that least progress has been made and, although the fact may not be surprising, the exact reasons are not at all clear.

In the first place it can be assumed that further refinements of culture media along present lines, particularly as regards heavy-metal control, would extend the range of organism it is possible to culture axenically;

[1] The latter part of table 9 summarises the general characteristics and uses of these media.

but there may well be other quite unrelated factors of which we have no knowledge at present.

The oceans are of course a relatively constant environment in many respects. Considerably smaller variations in constitution, temperature and CO_2 content than are encountered in supra-littoral, littoral and estuarine waters are very likely reflected in narrower tolerances of oceanic organisms. With the exception of the micro-chemical constitution all these factors can be controlled in the laboratory, although very likely insufficient attention is normally given to avoiding temperature shock and like hazards during collection and initial manipulation of material destined for cultures.

Oceanic diatoms such as *Chaetoceros decipiens* have been maintained in unialgal culture with comparative ease but have defied all attempts to culture them free of bacteria—not, it should be emphasized, because they could not be freed from bacteria, which in the case of these diatoms is a simple thing to do, but because they tend not to thrive in our media upon isolation. This suggests, in the first place, requirements for micro-nutrients not supplied by the range of organic enrichments in the media, a possibility which should at all times be kept in mind. But it is possible to over-emphasise the role of micro-nutrients, and, although a mixed population of bacteria is likely to make available the whole gamut of growth factors, it also may have a pronounced stabilizing influence on the physical state of the medium; on, for instance, heavy metal solubility, buffering and poising of the medium.

Indeed, the work of HARVEY (1939), MATUDAIRA (1942) and J. C. LEWIN (1954) on the role of divalent sulphur in diatom nutrition does suggest the existence for diatoms of a critical oxidation-reduction potential. Hitherto little attention has been given to this aspect of culture technique in the algal field for the obvious reason that heavy algal cultures become oxygen-rich. Such conditions, however, do not necessarily prevail in recently transferred cultures which, on the contrary, may be very sensitive to the potential during the lag and early phases of growth. According to J. C. LEWIN (1954) reducing conditions are necessary for silicon utilization in washed cells of *Navicula pelliculosa*. Some recent unpublished experiments on *Skeletonema* by one of us (M. R. D.), however, point to the existence of a rather sharp optimum in this respect, for it has been extremely easy to poison the medium with as little as 0.3 mg. % sulphide or cysteine, unless the medium has been allowed to stand for some days before making the addition.

Factors such as these very likely contribute to the difficulty of establishing axenic cultures of pelagic and oceanic diatoms, and, although there is little as yet to suggest it, the same may be true of dinoflagellates.

Table 9. *Summary of the development of marine media*

a) Sea Water Media

Medium	Composition	Defects	Advantages	Use
ALLEN and NELSON "MIQUEL Sea Water"	Sea water and mineral enrichment	Precipitates — inconsistent results		Bacterized cultures of algae
FØYN "ERD-SCHREIBER"	Aged sea water + minerals + soil extract	Precipitates — reproducibility depends on type of aged sea water and soil extract	More reliable, richer in vitamins and trace metals	Bacterized cultures of more exacting algae
BARKER Medium . .	Same	Same	Same	Pure cultures of dinoflagellates
SWEENEY Media . . .	Aged sea water + minerals + Fe, Mn, + chelator + B_{12}	Precipitates — reproducibility depends on type of aged sea water	More reliable because of added trace metals & B_{12}	Pure cultures of dinoflagellates
ASW III . .	Sea water + minerals + Fe, Mn + vitamins + liver extract + glutamate + glycine + soil extract	Precipitates — Some inconsistent results. Too concentrated?	Less precipitate. Less dependent on age of sea water	Conservation medium for pure cultures of exacting species. Sterility test medium
E 6	Fresh sea water + minerals + liver extract + tryptone + glucose + soil extract. Sea water autoclaved apart from nutrients	Too concentrated for some species, insufficient organics for others	No precipitate. Age of sea water immaterial	Conservation medium for pure cultures of exacting species. Sterility test medium (*Skeletonema* conservation)

b) Artificial Media

RYTHER . .	Sea water-like basal medium	Precipitates — Lacks vitamins and trace metals		Bacteria-free cultures of two Volvocales

Table 9 (Continued)

Medium	Composition	Defects	Advantages	Use
LEVRING . . .	Sea water-like basal medium + trace metals	Precipitates — Lacks vitamins	Less dependent on impurities of major elements as source of trace metals	Bacterized cultures of *Ulva* and *Enteromorpha*
ASP	Sea water-like basal medium + 1 ml of chelator-trace metal mixture + vitamins + low p_H	Still prone to precipitate because of its sea water-like basal medium	Introduction of a chelated mixture of trace metals-addition of vitamins + low p_H	A few bacteria-free cultures of algae
RC	A new mineral base with reduced Mg, Ca, K + p_H buffer + organics + vitamins + chelator/trace metal mixture	Too much organic (?). Too much p_H buffer	Addition of p_H buffer — no precipitation due to lower Mg and Ca	Developed for *Rhodomonas lens*. Also good for a few other algae like *Prorocentrum*
DC	Mineral base with reduced salinity and Mg/Ca ratio of 5 + high N, P, and organics. Higher level of chelator /trace metal mixture (3 ml /100) + vitamins	Too high organic for many species. Too much p_H buffer	Higher chelated metal mix. No precipitation at high P concentration due to solubilizing action of glycerophosphate/lactate	Developed for *Amphora perpusilla*. Useful where high organics are needed
ASP 2. . . .	Low salinity mineral base + low p_H buffer + Mg/Ca ratio of 5 + Ca 10 mg. % + high level chelator/trace metal mixture + low N and P + vitamins	Occasional precipitate	Embodies the favourable conditions for the largest number of marine algae	Bacteria-free cultures of chrysomonads, cryptomonads, dinoflagellates, diatoms and blue green algae
ASP 2 NTA .	Same + 10 mg. % nitrilotriacetic acid	Same	More chelation	More favourable for some algae than ASP 2

Table 9 (Continued)

ASP 6. . . .	Same as ASP 2 but with a higher salinity though keeping the same ratio for Na, K, Mg, Ca + a different metal mixture and chelator	Same	A higher salinity might be necessary for some marine species, especially for isolation purposes	More favourable for some algae
S 22	Half strength sea water equivalent. Reduced Mg, Ca, SO_4. EDTA chelated trace metals. Some p_H buffering. B_{12} and thiamine	Insufficient p_H buffering. Some guanine precipitates	Some organics	Developed for chrysomonads, now mostly replaced by S 37
S 32	As S 22 but with more efficient p_H buffering (TRIS)	Lacks organics	No precipitate. Efficient p_H buffering	Chrysomonads
S 36	As S 32 with added silicon, B_{12} and thiamine but no organics	Lacks organics. Too much silicon? Possibly too low salinity for oceanic species	Same	Developed for *Skeletonema*
S 37	As S 32 with additional organics		Some organics	Chrysomonad conservation medium
S 46	As S 32 with organics further increased	Organics too high for many species	High organics; can be used at p_H as high as 8.8	Developed for *Hemiselmis*. Useful where high organics are needed
V 37	Low salinity ($5^0/_{00}$) sea water substitute, otherwise as S 37	Too low in salinity for most marine species	Low calcium	Developed for *Monochrysis*. Supra-littoral species
U 36	Full strength ($32^0/_{00}$) naturalistic sea water substitute. Otherwise as S 36	Requires light autoclaving to avoid precipitates at p_H 8. Salinity too high for most purposes	High salinity	A diatom medium imitating natural sea water

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Summary

The development of culture media for marine algae is traced from the time of MIQUEL (1890) to the present. The principles involved in designing synthetic media suitable for bacteria-free cultures of a variety of exacting organisms are discussed and an account given of their application in the United States and United Kingdom.

Enriched sea water of the ALLEN-MIQUEL and Erd-SCHREIBER (soil-extract) types have been extensively used. The former are suitable for unialgal (bacterized) cultures of diatoms and some flagellates, the latter for pure cultures of some species. The further addition of organic extracts and the use of sea water which has been allowed to age increases the range of organisms. The principal defects of these media are that they are prone to form precipitates upon sterilization and are difficult to reproduce exactly.

The literature shows that sea water substitutes based on analyses of sea water retain the defects of the latter and are unsuitable for most species even when enriched with essential trace elements. Small additions of extracts of natural substances improve them. There has thus been ample evidence of the need for organic as well as inorganic micro-nutrients.

The introduction of artificial chelating agents permitted sufficient heavy metal control for these sea water substitutes to be used for the identification in many cases of the essential organic micro-nutrients. For a number of species mixtures of the known B vitamins (particularly

thiamine and B_{12}) and chelated trace metals satisfactorily substituted for soil extract and natural sea water.

Refinement of the trace metal mixtures involved an overall attenuation of the earliest published formulae and a proper balancing of the metals against the chelator. The most generally successful solutions contain from 1—3 mg. % chelator (ethylenediamine tetraacetic acid) with a chelation ratio of from 3 : 1 to 1 : 1. Other chelators, such as trisodium hydroxyethylethylenediamine triacetate and nitrilotriacetic acid, are under trial and show some promise.

Deviations from natural sea water of the quantities of major ions have been found not only to be tolerated but beneficial, with the consequence that it has been possible to design sea water substitutes of greater flexibility, having low salinity and low calcium content. Such mixtures are less prone to precipitate during autoclaving. Greater flexibility is also obtained by lowering p_H where possible which lessens the chances of precipitation and makes more available some organic compounds. Glycerophosphate is a useful non-precipitating phosphorus source.

The incorporation of p_H buffering in the form of tris(hydroxymethyl)-aminomethane further improved synthetic media. It is now possible to hold p_H relatively constant at any desired value between 7 and 9 both during autoclaving and for the duration of a culture even while 4% CO_2 is being administered.

Synthetic solutions suitable for the greatest variety of organisms, therefore, contain the following: *(a)* a low salinity sea water substitute with low calcium content, *(b)* chelated trace metals at low concentration with a chelation ratio lying between 3 : 1 and 1 : 1, *(c)* p_H buffering, *(d)* in addition to macro-nutrients, mixtures of most commonly required growth factors and, occasionally, KREBS cycle intermediates, amino-acids and purines.

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