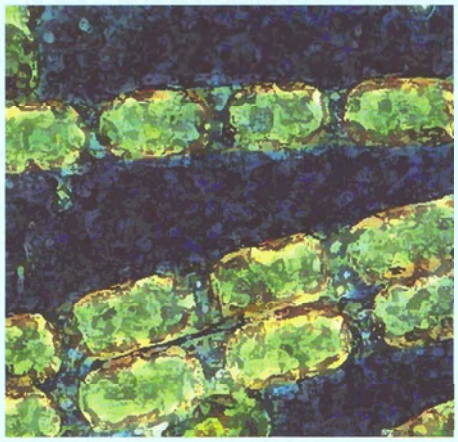

CHAPTER 3

MARINE CULTURE MEDIA

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CONTENTS

Key Index Words: Marine Phytoplankton, Culture Media, Artificial Seawater, Nutrient Enrichments, Trace Metals, Vitamins

1.0. INTRODUCTION

Natural seawater (NW) is a complex medium containing more than 50 known elements and a large and variable number of organic compounds. For algal culture, direct use of NW is seldom acceptable. Without the addition of further nutrients and trace metals, the yield of algae is usually too low for culture maintenance or laboratory experiments, and thus enrichment is normally required. In addition, variations in the quality of NW throughout the year, the need to control nutrient and trace element concentrations, and the limited availability of seawater at inland locations make the option of artificial seawater (AW) attractive (see Section 4.2).

The preparation of NW with an enrichment solution of nutrients, trace metals, and vitamins and the preparation of AW are described in this chapter. For clarity, we use the term *natural seawater* to refer to unenriched NW, and *artificial seawater* for unenriched AW. The term *enrichment solution* refers to the macronutrients, trace elements, and vitamins that must be added to both NW and AW to produce a substantial algal yield. The materials required for preparation of seawater media and the main recipes of stock solutions of macronutrients, trace elements, and vitamins are described. Methods and precautions that are required in media preparation are covered. Recipes for certain algal groups and various natural and synthetic seawater recipes are compared. The recipes have been tested for

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planktonic algae, but reports indicate that the commonly used recipes are also suitable for benthic diatoms and some seaweeds.

Comparing today's marine culture media with those of 30 years ago (McLachlan 1973), it is interesting to see the progress that has been made and that several media are quite broad-spectrum, indicating that most culturable algae can be grown by using only a few different media. The most challenging phytoplankton to culture are still the oceanic species.

1.1. Historical Perspective on Previous Media and Recent Advances

Many basic media concepts that are used today were developed in the late 1800s and early 1900s (for a review see Allen and Nelson 1910; Allen 1914). Early workers soon learned that the ratios of chemicals were not always critical, and subsequently various media recipes were developed with only slight modifications. It was well known that chemicals that were added to seawater contained impurities such as trace elements, and these often improved growth (Allen and Nelson 1910). The importance of culture pH, iron, vitamins for culture growth, and the avoidance of metal toxicity and impurities in distilled water also were established very early (Allen and Nelson 1910; Allen 1914).

NW may be the preferred seawater base if large quantities are required, if a good source is really available, or if open ocean species are being cultured in the laboratory. However, for near-shore sources, the salinity may vary seasonally and large phytoplankton blooms may alter the organic compounds in the seawater. To enhance the algal yield, various additions must be made to the NW. In the early 1900s, boiling water was used to extract unknown, variable amounts of inorganic and organic compounds from soil, and when it was added it produced good growth with few morphological changes during the long-term maintenance of algal cultures. Allen (1914) concluded that organic substances were required in trace amounts. Soil extract, originally introduced by Pringsheim (1912), was established in the marine culture methodology by Foyn's (1934) now famous "Erdschreiber" medium (note that this name was originally written as Erd-Schreiber and that the original recipe was derived from Schreiber [1927]). We now know that soil extract performs numerous functions in culture media, and it has largely been replaced by specific compounds. Soil extract provides various elements and vitamins needed for plant growth, metal complexing by organic compounds that sequester potentially toxic metals, and organic compounds that keep iron in solution. In replacing soil extracts, numerous trace elements and vitamins are usually added to culture media. These include iron, manganese, zinc, cobalt, copper, molybdenum, vitamin B_{12}, thiamine, and biotin. Artificial chelators such as EDTA (ethylenediaminetetraacetic acid) are added to keep iron in solution and to keep free ionic metal concentrations at nontoxic levels.

The history of the development of defined media was largely learning how to dispense with soil extract. The historical development of artificial media has been thoroughly reviewed (Provasoli et al. 1957, Kinne 1976). One of the first attempts to design an AW medium for algae began about 90 years ago (Allen and Nelson 1910). More extensive analyses of NW (Lyman and Fleming 1940) stimulated the development of new recipes (Chu 1946, Levring 1946) that attempted to imitate NW precisely, but they were frequently considered too complex. Because these recipes were similar to NW, they had the same defect, namely the formation of a precipitate during autoclaving when they were enriched. Autoclaving drives carbon dioxide out of the seawater, causing a shift in the carbonate buffer system. The resulting pH of around 10 causes the precipitation of ferric phosphate and ferric hydroxides. The amount and composition of the precipitate varies, often leading to inconsistent growth in the medium. Preoccupation with making a complete medium autoclavable without precipitation led to the following extensive modifications in recipes:

1. Addition of synthetic metal chelators such as EDTA or nitrilotriacetic acid (NTA) to decrease metal precipitates
2. Addition of a pH buffer such as Tris or glycylglycine (7.0–8.5 range), because the amount of precipitate increased as the pH rose during autoclaving
3. Reduction in salinity, thereby reducing the amount of salts available for precipitation
4. Replacement of Mg^{2+} and Ca^{2+} with more soluble univalent salts
5. Replacement of inorganic phosphorus with an organic source (e.g., sodium glycerophosphate) to avoid the precipitation of $Ca_3(PO_4)_2$ (Droop 1969)
6. Introduction of weak solubilizers, which are acids (e.g., citric acid) having highly soluble salts with calcium

As a result of these extensive chemical modifications in ion ratios (Provasoli et al. 1957), few early recipes bore

much resemblance to NW. Various simplified AW recipes were developed (Provasoli et al. 1957, McLachlan 1959, 1964), but many of these recipes could grow only a few species or favored a particular group of algae (Provasoli et al. 1957, Kinne 1976). More recently, several recipes such as the one by Kester et al. (1967) and one commercial one, Instant Ocean (King and Spotte 1974), have ion ratios of the major constituents that are very similar to NW.

Although one would expect artificial media to be much more chemically defined than NW, in some respects, artificial media are not completely defined due to the contaminants in reagent grade salts. Because of the large amounts of major salts that must be added, trace contaminants (e.g., copper, zinc, iron) in these salts can result in higher concentrations of some metals in AW than those naturally present in oceanic surface water. The use of ion exchange columns to remove these contaminants, as described by Morel et al. (1979), can greatly reduce this problem (note, however, that Chelex 100 removes only cations), but it is a time-consuming process (see Chapter 4). With the interest in trace metal availability and trace metal toxicity, the development of Aquil by Morel et al. (1979) permitted, for the first time, a complete definition of chemical speciation of various components, as calculated from thermodynamic equilibria, by controlling trace element contamination and precipitate formation. The medium Aquil is useful for trace metal studies of copper, zinc, nickel, cobalt, lead, and cadmium, because they remain in cationic form in seawater. More recent changes in the preparation of Aquil have been the purification of the Chelex column to avoid contamination by the chelating agents, use of alternative sterilization procedures, and an increase in the concentration of trace metal buffers (Price et al. 1988/89).

Medium K (Keller et al. 1987) was developed from culturing fastidious oceanic phytoplankton, and it has been tested on 200 ultraplankton clones representing seven algal classes. It is not recommended for coastal species. This medium includes selenium, both nitrate and ammonium, increased chelation, reduced copper, and a moderate level of pH buffering. The chelation to total metal ratio is 10 : 1, and the EDTA concentration is 10^{-4} M. There is also a synthetic counterpart. The recently formulated MNK medium and Pro99 medium (see later discussion and Appendix A) are also formulated for open ocean phytoplankton, and preliminary results suggest that they are superior to K medium for certain algae (coccolithophores and *Prochlorococcus*, respectively).

A broad-spectrum AW medium (enrichment solution with artificial water [ESAW]) was developed and tested on 83 strains (Harrison et al. 1980). The AW base was taken from Kester et al. (1967), and the ratios of the major ions closely match those found within NW. The enrichment solution was a modified enrichment solution originally developed by Provasoli (1968). The modifications were the omission of Tris (the pH buffer) and the addition of silicate. The omission of Tris was compensated for by adding equimolar amounts of $NaHCO_3$ and HCl to prevent precipitation during autoclaving. During autoclaving CO_2 is lost, and CO_2 can be added indirectly before autoclaving by adding $NaHCO_3$ or by directly bubbling CO_2 through the medium. This medium has a 2.3 : 1 chelator to trace metal ratio, compared to 1 : 1 in 'f' medium (Guillard and Ryther 1962), which may reduce the tendency to form metal precipitates and metal toxicity (Harrison et al. 1980). Over the past 2 decades, further changes were made to ESAW that significantly improved the medium. The forms of phosphate, iron, and silicate were changed and the trace element mixture was altered to include nickel, molybdenum, and selenium (Berges et al. 2001).

2.0. MATERIALS REQUIRED

2.1. Chemicals

Most chemicals required to make marine media are available from various chemical suppliers. Reagent grade salts (e.g., American Chemical Society grade) should be used if possible. The organic chemicals such as vitamins, buffer, and chelators are available from Sigma Chemical Company. If brands of a chemical are changed, this should be noted, because different brands are likely to have different amounts of contaminants or impurities.

2.2. Equipment, Glassware, and Tubing

Most required equipment comprises standard items in laboratories: analytical and top-loading balances, pH meter, hot-plate-magnetic stirrer, and so on. Borosilicate glassware should be used exclusively for all glassware, including stock bottles, beakers, and cultural tubes and flasks (examples of brand names are Pyrex and Kimax). Teflon or plastics are recommended, because they reduce breakage. Check the manufacturer's

specifications for usage, such as autoclaving and storage of concentrated chemicals.

Keep the glassware and plasticware to be used in media preparation separate from general purpose laboratory use. Washing protocols vary, depending on the experiments planned, but in general it is important to be aware that tap water often contains high amounts of nutrients, trace metals, and heavy metals. Therefore, if tap water is used for washing and rinsing, then make sure that deionized water is used for the final rinse. Furthermore, domestic detergents leave a residual film on glassware. The detergents from most large chemical supply companies are satisfactory, but labels should be read to determine if the contents meet your requirements. New glassware and plasticware should be degreased in dilute NaOH, soaked in dilute HCl, and then soaked in deionized water for several days before use. Glassware should not be cleaned in chromic acid, because chromium is toxic to many phytoplankters (McLachlan 1973). Teflon is useful only for stock bottles and is not suitable for culture vessels because of its reduced light-transmission properties. Polycarbonate is good for culture vessels, especially for experiments involving trace-metal limitation. Polypropylene may yield toxins from stocks, notably silicate stocks (Brand et al. 1981). More information on culture vessels is provided in Chapters 2, 4, and 5. Glassware should be autoclaved, clean glassware and plasticware should be stored in closed cupboards, and open vessels should be covered.

One should also be aware of potential toxicity from tubing and other materials. Bernhard was one of the first to call attention to the inhibitory effects of some culture materials by testing more than 50 types on phytoplankton and zooplankton (Bernhard and Zattera 1970, Bernhard 1977). A later study examined latex tubings of rubber, polyvinyl chloride (Tygon), and silicone, and Price et al. (1986) found that latex tubing was surprisingly toxic to phytoplankton, zooplankton, and bacteria. Even using latex tubing to siphon water from one bottle to another one rendered the water toxic for phytoplankton growth. The toxic compound was not identified, but preliminary results indicated that it may be pentachlorophenols and tetrachlorophenols used to preserve the latex tubing (Price et al. 1986). Tygon tubing was generally safe, provided that the powder inside the new tubing was carefully removed by rinsing before use. Silicone tubing was completely safe to use. Colored or black rubber stoppers may be toxic, and therefore silicone stoppers are recommended, especially the ones from Cole-Palmer that are made by injecting small air-bubbles into the polymer and are thus lighter and easier to work with than the solid silicone stoppers.

All containers and tubing used for cultures and media stocks should be carefully selected to avoid toxic compounds. For general purpose culturing, we recommend flasks and test tubes made of borosilicate glass and tissue culture-grade polycarbonate or polystyrene plasticware. Teflon-lined caps are recommended for screw-top glass test tubes, and black caps should be autoclaved several times in changes of seawater, because new caps may release toxic phenolics when heated (McLachlan 1973). For studies on silicon limitation, polycarbonate is recommended. However, borosilicate glassware may be used as long as it has not been rinsed with any acid that causes severe leaching of silicate from the glass.

Likewise, rubber stoppers (or anything that releases volatile compounds when heated) should be autoclaved separately from media. Older autoclaves with copper tubing should be avoided, because excess copper is toxic to algae. The autoclave steam may be contaminated with metals or chemicals used to inhibit corrosion of the autoclave. See Chapter 5 for further information on sterilization procedures.

2.3. Water Sources, Treatment, and Storage

The source of seawater may determine one's success in culturing certain species. To obtain NW free from pollution, it may be necessary to collect offshore water. Oligotrophic open ocean water is ideal, because it is low in nutrients and trace metals, and these components can be added in required amounts in an enrichment solution. In addition, this water contains less sediment and possibly less phytoplankton, making it easier to filter.

Nearshore water may be seasonally variable due to rainfall and runoff inputs, which may have elevated nutrients and sediments and decreased salinity. If inshore water is used, then water below the photic zone or pycnocline is likely to have less sediments and algal biomass to remove by filtration. Water should not be collected during blooms, especially when noxious species are present. Various pumps or large water bottles may be used to obtain the water. Water should not be collected with a water bottle that uses latex tubing for the rubber spring that closes off the ends of the water bottle, because the latex rubber tubing renders the water toxic for some algae (Price et al. 1986). Because filtration may be a slow process, plastic containers or carboys are usually filled and brought back to the laboratory for filtration. Usually a large-scale filtration apparatus is used, such as 147- or 293-mm-

diameter membrane filters contained in a plastic holder (e.g., Millipore or Pall-Gelman). A prefilter may be placed on top of the membrane filter to slow down the clogging of the membrane filter. Alternatively, filter cartridges (e.g., Pall-Gelman Acropak capsules) are relatively inexpensive, do not require any special filter holders, and do not clog as readily as membrane filters. The choice of filter pore size is determined by the source of the seawater, its intended use, and the volume needed. Normally, water should be filtered to 0.45 μm with membrane filters or, in special cases, down to 0.2 μm. If glass fiber filters are used, which are much faster and clog less quickly, then a GF/F filter (e.g., Whatman) with a nominal pore size of 0.7 μm is recommended.

Occasionally, dissolved organic matter removal may be required for special projects or because of suspected contamination. Dissolved organics may be removed by adsorption onto activated charcoal. The charcoal is prepared by washing with benzene, methanol, or 50% ethanol and distilled water (Craigie and McLachlan 1964). Dissolved organics in small volumes of seawater may be removed by adding 2 g of powdered, washed charcoal per liter of seawater, stirring for 1 hour and then filtering. Large volumes of seawater are passed through charcoal in a glass column. The charcoal may be washed as described previously, but it must not be allowed to dry before the addition of seawater. There is no "best method" for cleaning charcoal, and often a simple washing with seawater or distilled water may be acceptable (Guillard, personal communication). One should adjust the cleaning to suit the organism and purpose of the experiments planned. Dissolved organics may also be removed by exposing the seawater to high-intensity ultraviolet light (Armstrong et al. 1966). This destroys most dissolved organics and sterilizes the seawater.

Depending on where the seawater is collected, the salinity varies, especially with different seasons. The salinity should be noted for each collection. Offshore seawater salinity normally ranges 32 to 35 psu, whereas inshore water may often be <30 psu. Most algae grow well between 30 and 35 psu, but some species do not tolerate reduced salinities. If a lower salinity is desired, the salinity must be decreased by adding deionized water before any nutrients, trace metals, or vitamins are added, to avoid dilution of these components.

Filtered seawater can be stored in either glass or plastic carboys, often 20 liters for ease of handling. Rectangular containers require minimal storage and can be stacked. New containers should be leached for several days with diluted (i.e., 10%) HCl and then rinsed thoroughly. The seawater should be kept cool (refrigerated if possible) and in the dark (or covered with black plastic).

Filtered seawater is traditionally sterilized by steam autoclaving for 15 minutes at 121°C and 15 lb in^{-2} or longer, depending on the volume. After autoclaving, leave the media for 24 hours to equilibrate, so that gases such as CO_2 are allowed to diffuse into the medium. To avoid formation of a precipitate during autoclaving, the following treatments are helpful:

1. Adding 1.44 mL of 1N HCl and 0.12 g of $NaHCO_3$ per liter. These additions indirectly add CO_2 and lower the pH, which helps to reduce the formation of a precipitate during autoclaving (Harrison et al. 1980). Carbon dioxide may be added directly by bubbling the medium before autoclaving (Morel, personal communication).
2. Cooling the seawater quickly after autoclaving by standing it in cold tap water in a sink helps to prevent precipitation.
3. Sterilizing by filtration with a 0.22-μm membrane filter.
4. Pasteurizing by heating the seawater to 90°C–95°C for 24 hours. Sometimes this heating is done for a shorter time but repeated twice or three times, with cooling between heating periods (tyndallization).
5. Adding pH buffers such as 4–5 mM Tris or glycylglycine. Note, however, that these buffers are organic compounds and may encourage bacterial growth (Fabregas et al. 1993). In addition, Tris may be toxic to some phytoplankton species (McLachlan 1973, Blankley 1973).
6. Adding high concentrations of EDTA (e.g., 10^{-4} M in medium K; Keller et al. 1988) for algae that tolerate it.
7. Sterilizing smaller volumes of seawater with a microwave (Keller et al. 1988).
8. Lowering the concentration of iron.

3.0. STOCK SOLUTIONS

3.1. Macronutrients (Nitrogen, Phosphorus, and Silicon)

Macronutrients are generally considered to be nitrogen, phosphorus, and silicon. However, silicon is required only for diatoms, silicoflagellates, and some chrysophytes. These macronutrients are generally required in

a ratio of 16N:16Si:1P (Parsons et al. 1984, Brzezinski 1985), and the ambient ratio in NW is often similar to the ratio required by the algae, except in some estuaries where there are large inputs of nitrogen and phosphorus. Most media do not balance the relative concentrations of macronutrients needed for algal growth. Several popular media (e.g., f/2 medium) have nitrogen : phosphorus ratios >16 : 1, indicating that the phytoplankton would be phosphorus-limited in senescent phase (Berges et al. 2001).

Unfortunately, experimentalists usually pay little attention to the nitrogen : phosphorus or nitrogen : silicon ratios in the medium that they are using, which will ultimately determine which nutrient limits growth and influences the chemical composition and physiological rates when the cells become senescent. Similarly, carbon concentrations and carbon : nitrogen ratios are rarely considered. Many media have a bicarbonate concentration of about 2 mM and nitrogen (nitrate) of about 500 μM or higher, which yields a carbon : nitrogen ratio of about 4 : 1. According to the Redfield ratio, the chemical composition of the average phytoplankter is 106C : 16N : 1P, or 6.7C : 1N. Therefore, most media are nitrogen-rich relative to carbon, and carbon could become limiting, depending on the growth rate of the phytoplankton and the surface area of the medium through which atmospheric CO_2 can diffuse (Riebesell et al. 1993). When culture pH rises quickly to 9 or higher, this may be an indication that carbon may be limiting. Species that can readily use bicarbonate may be able to grow, whereas other species that are more dependent on CO_2 may exhibit a reduced growth rate or cell yield. Depending on the use of the seawater, one may consider bubbling with CO_2 or adding more bicarbonate in late exponential phase to ensure that carbon is not limiting algal growth. This is especially important in physiological experiments where it is essential to know which nutrient is limiting during senescent phase.

To simplify routine media preparation, recipes are usually divided into working stock solutions. Direct combinations of several stock solutions without dilution in water may result in undesirable precipitation. To make a working solution of several stocks, add one stock to a certain volume of water and mix thoroughly before adding the next stock solution. Nitrate and phosphate are normally added as $NaNO_3$ and $NaHPO_4 \cdot H_2O$. In some media, phosphate is added as sodium glycerophosphate to make the trace metal salts less prone to precipitation; however, sodium glycerophosphate may precipitate as a calcium salt at elevated temperatures (Provasoli 1971).

Ammonium may be an alternative nitrogen source and may be added as NH_4Cl. At the typical pH of seawater (8.2), there is about 90% NH_4 and 10% NH_3 (ammonia). Because considerable quantities of ammonia may be lost from the medium through volatilization during autoclaving, ammonium should be added aseptically after autoclaving. As the pH of the culture medium increases during algal growth, the ratio of $NH_4 : NH_3$ increases and reaches 1 : 1 at a pH 9.3. Therefore, substantial amounts of ammonium may be lost from the culture if the algal culture is kept mixed by bubbling with air. Ammonium, at concentrations of 100 to 250 μM, may be inhibitory to some coastal species, but most coastal species tolerate concentrations as high as 1,000 μM (McLachlan 1973). Similarly, some oceanic species show toxicity to ammonium at only 25 μM (Keller et al. 1987), whereas others (e.g., *Prochlorococcus*, *Bolidomonas*) tolerate concentrations >500 μM. In some special cases, urea is another form of nitrogen to consider, but it decomposes when heated (McLachlan 1973). In experiments in which different forms of nitrogen are compared, it is important to note that 1 μM urea provides 2 μM $N \cdot L^{-1}$, because the urea molecule contains two atoms of nitrogen.

Silicate is added as $Na_2SiO_3 \cdot 9H_2O$. Because silicic acid enhances precipitation, it is useful to omit it from the medium if one is culturing species that do not require silicate (e.g., most flagellates). If concentrated stock solutions (e.g., 100 mM) are prepared in deionized water and acidified to pH 2 (McLachlan 1973), silicate polymerizes. When this stock is added to seawater, it may take several days before the entire concentration of silicon that was added is available for uptake and growth (Suttle et al. 1986). It is recommended to store the 100-mM stock solution of Na_2SiO_3 at its pH of dissolution in deionized water (pH = 12.6) at 4°C in the dark (Suttle et al. 1986). When the silicon stock is added to seawater, it should be added slowly with rapid stirring. Autoclaving the Na_2SiO_3 stock solution in a glass container may result in etching of the glass and precipitation, and therefore it is recommended to prepare it in a Teflon-coated bottle (Suttle et al. 1986).

To help control contamination with bacteria or fungi, autoclave all nutrient stocks and then use sterile techniques subsequently. Another possibility is to sterilize the stocks through a 0.2-μm filter. Generally, nutrient enrichments should be added aseptically after autoclaving, but they may also be added before autoclaving, except in the case of ammonium.

For concentrations of the various macronutrient stock solutions for the various media, see Appendix A.

3.2. Trace Metals

Trace metals and vitamins are usually prepared as "primary" stocks of high concentrations to permit weighing of reasonable amounts. These are used to make "working" solutions from which the final medium is made (see Appendix A for examples). Because some primary or working solutions are kept for very long periods, evaporation through ground glass stoppers, screw caps, or plastic bottles may be significant. Mark the liquid level on the bottle and keep it cold or wrapped in laboratory film.

Typical trace metal stock solutions may consist of chloride or sulphate salts of zinc, cobalt, manganese, selenium, and nickel, and they are kept in a solution containing the chelator EDTA. Iron is usually kept as a separate solution, and it should be chelated or kept in 10^{-2} M HCl to avoid precipitation. It may be added as ferric chloride, ferrous sulphate, or ferrous ammonium sulphate, but the latter compound contains ammonium, and this may be a problem if one is conducting nitrogen uptake studies. There is sufficient boron in NW and therefore it is not necessary to add it, but boron should be added to AW. The stock solutions for various recipes are given in Appendix A. For a more detailed discussion of trace metals, see Chapter 4.

3.3. Vitamins

Usually three vitamins—vitamin B_{12} (cyanocobalamin), thiamine, and biotin—are added, but very few algae need all three vitamins (Provasoli and Carlucci 1974). The general order of vitamin requirements for algae is vitamin B_{12} > thiamine > biotin. If large-scale culturing of a single species is the goal, check what vitamins this species requires. It may be possible to omit the addition of two of the three vitamins, because most species require only one or two. Vitamins are normally added aseptically (through a 0.2-μm filter) after the medium has been autoclaved. Vitamins maintain maximum potency if they are filter sterilized (0.2-μm filtration) rather than autoclaved. Autoclaving may cause decomposition of some vitamins, but it is thought that some algae may be able to use some of these decomposition products (Provasoli and Carlucci 1974). Vitamin stocks may be frozen for long periods without noticeable degradation, and the stocks may be refrozen after each use. These three vitamin stocks may be combined into a single working solution for a 1,000-fold dilution.

3.4. pH Buffers

Two common pH buffers are used to prevent or reduce precipitation: Tris (2-amino-2-[hydroxymethyl]-1-3-propanediol) and glycylglycine (McLachlan 1973). Mix Tris base and Tris : HCl as per Sigma instructions and make a stock to be added as desired; for example, 1 mg L^{-1} for a Tris concentration of 10^{-3}M. Adjust the pH with concentrated HCl to obtain the desired pH of the medium. Glycylglycine is readily soluble in water, and the powder can be added directly to seawater. It is slightly acidic, and it may be necessary to make a small adjustment to the pH with several drops of 1N NaOH. Tris may be toxic to some species (McLachlan 1973; use no more than 1–5 mM), whereas glycylglycine is nontoxic. Apparently, neither Tris nor glycylglycine can serve as a nitrogen source for algal growth, but they can serve as a carbon source for bacteria (Fabregas et al. 1993). They may also interfere with the analysis of dissolved organic nitrogen and ammonium.

HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]) and MOPS (3-N-morpholino propane sulfonic acid) are used extensively in freshwater media (McFadden and Melkonian 1986), but they are not commonly used in marine media. Loeblich (1975) compared the growth of a marine dinoflagellate in several buffers (including MOPS, HEPES, Tris, glycylglycine, and TAPS N-Tris [hydroxymethyl] methyl-3-aminopropanesulfonic acid), and concluded that Tris and TAPS provided maximal growth with minimal pH change.

3.5. Chelators

Chelate : metal ratios of 1.5 : 1 to 3 : 1 are commonly used. EDTA is the most common chelator and is usually purchased as the disodium salt ($Na_2EDTA.2H_2O$) that is readily soluble in water. However, EDTA has been noted to inhibit the growth of some oceanic species (Muggli and Harrison 1996). For routine use in culture media, nitrilotriacetic acid (NTA) and citric acid are less effective than EDTA, but they are used sometimes in experimental work (Brand et al. 1986). Chelators are discussed extensively in Chapter 4.

3.6. Soil Extracts

In the simplest media, only nitrogen and phosphorus and soil extract are added to NW. Erdschreiber is an

example of such a medium, and it has been used successfully to grow various planktonic and benthic species (McLachlan 1973). Some culture collections still use soil extract to maintain some species (see Plymouth Erdscheiber in Appendix A), but it is seldom used in physiological experiments when a defined medium is preferred.

3.7. Germanium Dioxide

In special cases, germanium dioxide may be added to prevent the growth of diatoms (Lewin 1966), but other algae may also be affected. For example, the addition of 100 mg L^{-1} of GeO_2 prevents diatom growth in macroalgal cultures (Markham and Hagmeier 1982), but McLachlan et al. (1971) found that GeO_2 inhibited brown algal growth. Thomas et al. (1978) added germanium dioxide to natural phytoplankton samples in an attempt to separate flagellate productivity from diatom productivity and noted that dinoflagellate photosynthesis was inhibited as well. Therefore, it seems unwise to add germanium dioxide during physiological experiments.

4.0. GENERAL METHODS OF MEDIA PREPARATION

The various aspects and precautions involved in media preparation have already been covered in the sections on materials required (Section 2) and stock solutions (Section 3).

4.1. Natural Seawater

The general steps are as follows:

1. Obtain good–quality NW with salinity >30, if possible.
2. Filter as soon as possible, and store the seawater in the dark and cold (4°C).
3. Sterilize as needed by filtration (0.2 μm), autoclaving, ultraviolet light treatment, or pasteurization.
4. Add macronutrients, Fe-EDTA, trace metals, and vitamins aseptically after autoclaving. Mix thoroughly after the addition of each stock.

4.2. Artificial Seawater

Artificial or synthetic seawater consists of two parts: the basal (main) salts that form the "basal seawater" and the enrichment solution (often the same as the enrichment solution that is added to NW). In the early AW recipes, calcium and magnesium salts were reduced to avoid precipitation. Because precipitation can now be avoided (Harrison et al. 1980), the more recent recipes ensure that the ratio of the major ions is identical to NW. For example, in ESAW (see Appendix A), the basic salts (usually 10 or 11) are divided into the anhydrous sodium or potassium salts (i.e., NaCl, Na_2SO_4, KCl, $NaHCO_3$, KBr, NaF) and the hydrated chloride or sulphate salts (i.e., $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot H_2O$, and $SrCl_2 \cdot 6H_2O$). The anhydrous salts must be dissolved separately from the hydrated salts, and then the two solutions can be mixed together and the enrichment solution components (macronutrient, trace metals, and vitamins) can be added after the salt solution is autoclaved.

Studies of the physiology of marine phytoplankton have been greatly facilitated by the ability to culture these species in a defined medium (i.e., AW). One of the great advantages of AW is that the composition of the seawater is relatively constant over several decades, unless the impurities in the major salts (e.g., NaCl, Na_2SO_4, and KCl) change either by a change in brands or a change in the manufacturing process. In 1985, we suddenly were unable to grow the common diatom, *Thalassiosira pseudonana* (Hust.) Hasle et Heimdal, despite the fact that we were using the same ESAW recipe that we used for the previous 7 years. We later discovered that selenium was previously an impurity in sufficient quantity in our basal salts, and therefore it had not been necessary to add it (Price et al. 1987, Harrison et al. 1988). We assume that a change in the process of producing one of the basal salts reduced selenium contamination. When we added selenium to the new enrichment solution, *T. pseudonana* grew well. Therefore, selenium is routinely added to our medium now (Berges et al. 2001).

Another advantage of AW for nutrient limitation studies is that one can control the amount of the limiting nutrient because there is little or none in the AW, unlike NW. Similarly, one can precisely control the nutrient ratios. However, cleanly collected oligotrophic ocean water may also be used, because nutrients and trace metals are also very low.

Commercial preparations are synthetic mixes and can be purchased in various amounts. One of the best is Instant Ocean, produced by Aquarium Systems, Inc. (King and Spotte 1974). The ion ratio of the major salts

is very close to NW. Macronutrients, trace metals, and vitamins must be added. If large-scale preparation is required, this may be an economical alternative to buying individual salts and weighing and mixing the salts.

For all the AW basal salt recipes, if contamination is a concern, then we recommend that you analyze the basal medium for copper, zinc, cobalt, lead, manganese, nickel, chromium, etc. For example, we measured 2.5, 4.0, and 0.13 μM lead, copper, and cadmium, respectively, in ESAW, which uses reagent grade salts. This concentration of copper is sufficient for growth, depending on the concentration of the other nutrients, trace metals, and chelation. Brand et al. (1986) found that 4.0 μM copper is toxic in the absence of a chelator for some species.

5.0. MEDIA

Comparisons between media are complicated by many factors. McLachlan (1973) observed: "Numerous enriched and synthetic media have been formulated, which together with generally trivial modifications, almost equal the number of investigators." This has certainly remained true. Many modifications result from a desire to increase the flexibility of a medium (i.e., creating multiple nutrient stocks so that individual macronutrient and micronutrient concentrations may be manipulated) or to reduce the number of stocks necessary in cases where various different media are being used in a single laboratory. (In Appendix A note the different recipes that incorporate f/2 trace metals stock.) In many cases, minor modifications to the original recipes have been made (e.g., changing a nitrogen source or adding a single trace metal) and an entirely new name has been given to the medium with this minor modification. In other cases, fairly extensive modifications have been made, yet a medium name has been retained or simply designated as "modified." In still other cases, subsequent modifications have been made in addition to a name change to honor the originator (e.g., "Grund" versus "von Stosch"; von Stosch 1963, Guiry and Cunningham 1984). More commonly used media such as f/2 and ESAW/ESNW have not only evolved over time (Berges et al. 2001), but they can change substantially in different publications (e.g., compare the recipe for f medium given in Guillard and Ryther [1962] with that for f/2 medium in Guillard [1975] and with those given on the Web sites of major culture collections). When compiling the recipes in Appendix A, we used both original recipes and those provided by major culture collection Web sites. The reader is cautioned that the Web site recipes are subject to change; indeed, Berges et al. (2001) recommended that Web-based recipes may have real advantages for phycological research.

In both AW and NW recipes, there is some variation in salinity. McLachlan (1973) considered that salinity was not an "inherent feature" of media recipes. Although salinities of 35 are often considered normal, most artificial media tend to produce somewhat lower salinities (27 to 30). Many coastal phytoplankton species can be cultured at a much lower salinity (cf. McLachlan 1973).

Sometimes it is necessary to grow axenic cultures. Details for preparing test media for bacteria and fungal contamination are provided in Chapter 8.

5.1. Artificial Seawater Media

Relatively few artificial water media are commonly used. Most are based on the ASM and ASP formulations of Provasoli and McLachlan, with minor variations (the AWs described by Goldman and McCarthy [1978] and Keller et al. [1987] both fall into this category). There are numerous species-specific artificial media that have been developed; for example, YBC-II, designed for nitrogen-fixing strains of *Trichodesmium* (Chen et al. 1996), was derived from an earlier medium by Ohki et al. (1992). As noted previously, the ion ratios of these media have diverged from that of NW (Table 3.1), particularly with respect to sulfate and magnesium (Kester et al. 1967). ESAW was formulated on the basis of Kester et al. (1967) and is thus quite similar to NW.

TABLE 3.1 Comparison of the ratio (normalized to K) of selected major ions in different artificial seawater recipes and natural seawater.

Recipe	Na^+	Cl^-	Mg^{2+}	SO_4^{2-}	K^+
Aquil	60	51	7.0	3.1	1.0
ASNIII	64	69	3.6	2.0	1.0
ASP-M	40	47	4.0	2.0	1.0
ESAW	52	59	5.1	3.1	1.0
YBC-II	42	45	5.0	2.5	1.0
NW	47	55	5.3	2.8	1.0

Several commercially available AWs are also available. Instant Ocean is one of the oldest and best-evaluated (discussed previously), but Tropic Marin Sea Salt (Tropic Marin) is also well-regarded by European aquarists, and Aus Aqua Pty Ltd (www.algaboost.com) sells various AW formulations, including ESAW, and enrichment solutions such as f/2. In published research, it appears that relatively few phycologists use ready-made salts, but at least one recipe available on the Culture Collection of Algae and Protozoa (CCAP) Web site uses a commercial sea salt preparation (Ultramarine Synthetica, Waterlife Research Industries Ltd.). More recently, Sigma-Aldrich has begun producing a dry sea salt mixture (S9883). When using these preparations, it is important to note that the dry mixture may not be homogenous; subsamples from a large package may vary considerably, especially if the salts have hydrated during storage.

5.2. Natural Seawater Media

Among the major NW enrichment media recommended for a broad spectrum of algae, f/2 medium and variations, different versions of Erdschreiber medium (e.g., Plymouth Erdschreiber medium), and ESNW appear to dominate citations (Berges et al. 2001, see Appendix A). The K-medium family has been specifically developed for oceanic species (Keller et al. 1987), and two new oceanic media, MNK and Pro99 media, offer promising improvements. More species-specific media that are frequently used include the L-medium family (Guillard and Hargraves 1993; Guillard 1995), GPM and variants (Sweeney et al. 1959; modified by Loeblich 1975 and Blackburn et al. 1989), and numerous media for oceanic cyanobacteria, including SN (Waterbury et al. 1986), PC (Keller in Andersen et al. 1997), PRO99 (Chisholm, unpublished), and ASNIII (Rippka et al. 1979); BG medium (Rippka et al. 1979) and variations are also recommended for marine cyanobacteria but are less commonly used with oceanic species. For macroalgal cultures, enrichments based on the "Grund" medium of von Stosch (1963) are most commonly used. Provasoli's ES medium and its modifications are also used (McLachlin 1973, West and McBride 1999).

It is difficult to provide a comprehensive set of recommendations as to which media are best for certain species. A good starting point is the media in which the species are being maintained in the culture collections from which they are obtained (see culture collection Web sites). A variety of detailed recipes are now available on culture collection Web sites, but the reader is cautioned that there is considerable variation in recipe details and even in the names used to describe recipes between sites. However, some generalizations about media recipes can be made.

In terms of vitamins there is remarkable consistency in media: thiamine, biotin, and B_{12} all are normally added, at quite comparable concentrations, although they may not be required (see Appendix A). Some media specify that autoclaving should be avoided, because it can lead to decomposition of vitamins, but species that require vitamins are usually able to grow well on the decomposition products (see McLachlan 1973).

Trace metals stock solutions are more variable, but it appears that most recipes have basic similarities (see Appendix A). Elements such as iron, zinc, manganese, cobalt, copper, and molybdenum are almost always included. Less commonly found, but critical to certain species, is selenium (Harrison et al. 1988). Nickel is required by most algae if urea is the nitrogen source, because nickel is a component of the enzyme unease (Syrett 1981). For oceanic species, a widely variable set of metals has been added, including the potentially toxic vanadium and chromium. There are also numerous metals found in some of Provasoli's early media, such as aluminum, ruthenium, lithium, and iodine (Provasoli et al. 1957), that are rarely found in media currently used. As noted earlier, the trace metal mixture is more critical when AW is used, particularly if water of very high purity (i.e., >18.2 MΩ water, such as that provided by MilliQ systems) is used.

Concentrations and forms of macronutrients (e.g., f, f/2, and f/50) in media commonly vary. Typically, macronutrients are in great excess in comparison with natural concentrations, particularly in the case of media intended for aquaculture. For example, Walne's medium (Appendix A) contains >1 mM nitrate, or about 40 times the maximum levels found in coastal waters. As noted above, the ratios of nutrients relative to algal requirements often appear to make little sense (Berges et al. 2001). Lower nutrients have been found to support growth of oceanic species, but often it is only the major nutrients that have been reduced in the recipe. McLachlan (1973) noted that ammonium can become toxic to some species of phytoplankton at concentrations of 100 µM or greater, and Berges et al. (2001) speculated that this may be the reason that dilutions of media in which ammonium is included may improve growth of some species.

It is also worth noting that there are commercially available premade enrichment stocks. Some major

culture collections sell nutrient stock solutions for various culture media as well as premixed culture media and seawater (e.g., CCAP, at www.ife.ac.uk/ccap, and CCMP, at http://ccmp.bigelow.org). Sigma-Aldrich sells f/2 medium (dry salt mixture G1775 or liquid G9903), and AusAqua Pty. Ltd. sells f/2 medium under the name AlgaBoost and ES enrichment stocks.

6.0. COMPARING RECIPES BASED ON ALGAL RESPONSE

Growing phytoplankton remains an art as well as a science, and in our experience, most investigators tend to settle on media that "work" for the species they are growing, rather than engage in wholesale comparisons to determine which media are the best. McLachlan (1973) observed that media preferences were "rarely supported by comprehensive, qualitative comparisons."

Any media comparison should be based on the use of the medium by the investigator. There are three broad categories of general use: culture maintenance, algal biomass yield, and physiological (growth rate) experiments. It is strongly advised that the growth rate in NW and the medium be compared, because growth rate is a general index of algal health in the medium. Some species grow better if there is a large surface area/volume ratio to allow the diffusion of gases into the medium.

In principle, the simplest comparison to make is whether the algae grow. Consult the culture collection Web sites for the most recent information on recipes and also to find the most appropriate medium for a particular strain. In practice, this is considerably complicated by the culture methods. For example, some investigators may maintain their culture collections quite conscientiously, transferring cultures weekly or more often. In this case, species that grow quickly (i.e., a high exponential growth rate, μ) could be favored. Under these conditions, a medium that was relatively poorly buffered or contained relatively low levels of macronutrients might be regarded as superior. On the other hand, cultures are often transferred far less frequently, and consequently they can persist in stationary phase for especially long periods if they are kept in dim light. In these cases, a medium that supports a higher growth rate might not be regarded as superior, but media that are more strongly buffered or have nutrients in excess may perform much better. Berges et al. (2001) compared several variations on ESAW with ESNW and found that assessment based on growth rate and final biomass did not always agree. For example, *Phaeocystis pouchetti* (Hariot) Lagerh. and *Karlodinium micrum* (Leadbeater et Dodge) Larsen (*Gymnodinium galatheanum* [Lohm.] Kofoid & Swezy) both grew at equal rates (μ) in ESAW and ESNW, but whereas *P. pouchetti* achieved a threefold higher biomass in ESAW versus ESNW, *K. micrum* cultures were almost twice as dense in stationary phase in ESNW as in ESAW.

Growth rates are relatively straightforward to measure by performing cell counts, but this is quite labor-intensive, even when using electronic particle counters (see Chapters 16–18). Measurements of fluorescence are much quicker and agree very well with cell numbers in most species, so long as cultures remain in exponential growth phase and are measured at the same time of day, if grown on a light : dark cycle. If culturing is done in 25 × 150–mm screw-capped culture tubes, then repeated measurements of cultures can easily be made with a Turner Designs fluorometer. For further information on measuring growth rates, see Chapter 18.

Determining the biomass of stationary phase cultures requires cell counts, and it is probably wise to measure cell volumes as well; in this case, fluorescence is unsuitable (see Berges et al. 2001). Although such comparisons are rarely done quantitatively, assessing media on the basis of the length of time a culture can remain at a stationary biomass is a criterion that is probably quite relevant to culture collections.

Another criterion for media evaluation is whether the original morphology of the cells is maintained, but this is seldom evaluated (Harrison et al. 1980) and can be impractical for small species. Many recipes that include soil water extract justify the addition on the basis that it maintains the original cell morphology during long-term culturing.

7.0. REFERENCES

Allen, E. J. 1914. On the culture of the plankton diatom *Thalassiosira gravida* Cleve, in artificial sea-water. *J. Mar. Biol. Assoc. U.K.* 10:417–39.

Allen, E. J., and Nelson, E. W. 1910. On the artificial culture of marine plankton organisms. *J. Mar. Biol. Assoc. U.K.* 8: 421–74.

Armstrong, F. A. J., Williams, P. M., and Strickland, J. D. H. 1966. Photo-oxidation of organic matter in seawater by ultraviolet radiation, analytical and other applications. *Nature* 211:481–3.

Berges, J. A., Franklin, D. J., and Harrison, P. J. 2001. Evolution of an artificial seawater medium: Improvements in enriched seawater, artificial water over the last two decades. *J. Phycol.* 37:1138–45.

Bernhard, M., and Zattera, A. 1970. The importance of avoiding chemical contamination for a successful cultivation of marine organisms. *Helgolander wiss Meeresunters* 20: 655–75.

Bernhard, M. 1977. Chemical contamination of culture media: Assessment, avoidance and control. In: Kinne, O., ed. *Marine Ecology. Vol III. Cultivation, Part 3.* Wiley & Sons, Chichester, UK, pp. 1459–99.

Blackburn, S., Hallegraeff, G., and Bolch, C. J. S. 1989. Vegetative reproduction and sexual life cycle of the toxic dinoflagellate *Gymnodinium catenatum* from Tasmania, Australia. *J. Phycol.* 25:577–90.

Blankley, W. 1973. Toxic and inhibitory materials associated with culturing. In: Stein, J., ed. *Handbook of Phycological Methods: Culture Methods and Growth Measurements.* Cambridge University Press, Cambridge, UK, pp. 207–29.

Brand, L. E., Guillard, R. R. L., and Murphy, L. S. 1981. A method for a rapid and precise determination of acclimated phytoplankton reproduction rates. *J. Plankton Res.* 3:93–101.

Brand, L. E., Sunda, W. G., and Guillard, R. R. L. 1986. Reduction of marine phytoplankton reproduction rates by copper and cadmium. *J. Exp. Mar. Biol. Ecol.* 96:225–50.

Brzezinski, M. A. 1985. The Si-C-N ratio of marine diatoms: Interspecific variability and the effect of some environmental variables. *J. Phycol.* 21:347–57.

Chen, Y.-B., Zehr, J. P., and Mellon, M. 1996. Growth and nitrogen fixation of the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. IMS 101 in defined media: Evidence for a circadian rhythm. *J. Phycol.* 32:916–23.

Chu, S. P. 1946. Note on the technique of making bacteria-free cultures of marine diatoms. *J. Mar. Biol. Assoc. U.K.* 26:296–302.

Craigie, J. S., and McLachlan, J. 1964. Excretion of colored ultraviolet-absorbing substances by marine algae. *Can. J. Bot.* 42:23–3.

Droop, M. R. 1969. Algae. In: Norris, J. R., and Ribbon, D. W., eds. *Methods in Microbiology* (Vol. 3B). Academic Press, New York, pp. 1–324.

Fabregas, J., Vazquez, V., Cabezas, B., and Otero, A. 1993. TRIS not only controls the pH in microalgal cultures, but also feeds bacteria. *J. Appl. Phycol.* 5:543–5.

Foyn, B. 1934. Lebenszyklus, cytology und sexualitat der Chlorophyceae *Cladophora subriana* Kützing. *Arch. Protistenk* 83:1–56.

Goldman, J. C., and McCarthy, J. J. 1978. Steady state growth and ammonium uptake of a fast growing marine diatom. *Limnol. Oceanogr.* 23:695–703.

Guillard, R. R. L., and Ryther, J. H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* 8:229–39.

Guillard, R. R. L. 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W. L., and Chanley M. H., eds. *Culture of Marine Invertebrate Animals.* Plenum Press, New York, pp. 26–60.

Guillard, R. R. L. 1995. Culture methods. In: Hallegraeff, G. M., Anderson, D. M., and Cembella, A. D., eds. *Manual on Harmful Marine Microalgae. IOC Manual and Guides No. 33.* UNESCO, Paris, pp. 45–62.

Guillard, R. R. L., and Hargraves, P. E. 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. *Phycologia* 32:234–36.

Guiry, M., and Cunningham, E. 1984. Photoperiodic and temperature responses in the reproduction of the northeastern Atlantic *Gigartina acicularis* (Rhodophyta: Gigartinales). *Phycologia* 23:357–67.

Harrison, P. J., Waters, R. E., and Taylor, F. J. R. 1980. A broad spectrum artificial seawater medium for coastal and open ocean phytoplankon. *J. Phycol.* 16:28–35.

Harrison, P. J., Yu, P. W., Thompson, P. A., Price, N. M., and Phillips, D. J. 1988. Survey of selenium requirements in marine phytoplankton. *Mar. Ecol. Prog. Ser.* 47:89–96.

Keller, M. D., Bellows, W. K., and Guillard, R. R. L. 1988. Microwave treatment for sterilization of phytoplankton culture media. *J. Exp. Mar. Biol. Ecol.* 117:279–83.

Keller, M. D., Selvin, R. C., Claus, W., and Guillard, R. R. L. 1987. Media for the culture of oceanic ultraphytoplankton. *J. Phycol.* 23:633–8.

Kester, D., Duedall, I., Connors, D., and Pytkowicz, R. 1967. Preparation of artificial seawater. *Limnol. Oceanogr.* 12:176–9.

King, J. M., and Spotte, S. H. 1974. *Marine Aquariums in the Research Laboratory.* Aquarium Systems, Inc., Eastlake, Ohio.

Kinne, O. 1976. Cultivation of marine organisms. In: Kinne, O., ed. *Marine Ecology, Vol III. Cultivation.* John Wiley & Sons, Toronto, pp. 29–37.

Levring, T. 1946. Some culture experiments with *Ulva lactuca. Kgl. Fysiogr. Sallsk. Hdl.* 16:45–56.

Lewin, J. 1966. Silicon metabolism in diatoms. V. Germanium dioxide, a specific inhibitor of diatom growth. *Phycologia* 6:1–12.

Loeblich, A. 1975. A seawater medium for dinoflagellates and the nutrition of *Cachonina niei. J. Phycol.* 11:80–86.

Lyman, J., and Fleming, R. H. 1940. Composition of seawater. *J. Mar. Res.* 3:134–46.

Markham, J. W., and Hagmeier. 1982. Observations on the effects of germanium dioxide on the growth of macro-algae and diatoms. *Phycologia* 21:125–30.

McFadden, G. I., and Melkonian, M. 1986. Use of HEPES buffer for microalgal culture media and fixation for electron microscopy. *Phycologia* 25:551–7.

McLachlan, J. 1959. The growth of unicellular algae in artificial and enriched seawater media. *Can. J. Microbiol.* 5:9–15.

McLachlan, J. 1964. Some considerations of the growth of marine algae in artificial media. *Can. J. Microbiol.* 10:769–82.

McLachlan, J. 1973. Growth media—marine. In: Stein, J., ed. *Handbook of Phycological Methods: Culture Methods and Growth Measurements.* Cambridge University Press, Cambridge, UK, pp. 25–51.

McLachlan, J., Chen, L. C.-M., and Edelstein, T. 1971. The culture of four species of *Fucus* under laboratory conditions. *Can. J. Bot.* 49:1463–69.

Morel, F. M. M., Rueter, J. G., Anderson, D. M., and Guillard, R. R. L. 1979. Aquil: A chemically defined phytoplankton culture medium for trace metal studies. *J. Phycol.* 15:135–41.

Muggli, D. L., and Harrison, P. J. 1996. EDTA suppresses the growth of oceanic phytoplankton from the northeast subarctic Pacific. *J. Exp. Mar. Biol. Ecol.* 205: 221–7.

Ohki, K., Zehr, J. P., and Fujita, Y. 1992. *Trichodesmium*: establishment of culture and characteristics of N-fixation. In: Carpenter, E. J., Capone, D. G., and Rueter, J. G., eds. *Marine Pelagic Cyanobacteria: Trichodesmium and other diazotrophs.* Kluwer Academic Publishers, Dordrecht, Germany, pp. 307–18.

Parsons, T. R., Takahashi, M., and Hargrave, B. 1984. *Biological Oceanographic Processes.* 3rd ed. Pergamon Press, New York, 342 pp.

Price, N. M., Harrison, G. I., Hering, J. G., Hudson, R. J., Nirel, P. M. V., Palenik, B., and Morel, F. M. M. 1988/89. Preparation and chemistry of the artificial culture medium Aquil. *Biol. Oceanogr.* 6:443–61.

Price, N. M., Harrison, P., Landry, M. R., Azam, F., and Hall, K. 1986. Toxic effects of latex and Tygon tubing on marine phytoplankton, zooplankton and bacteria. *Mar. Ecol. Prog. Ser.* 34:41–9.

Price, N. M., Thompson, P. A., and Harrison, P. J. 1987. Selenium: An essential element for growth of the coastal marine diatom *Thalassiosira pseudonana* (Bacillariophyceae). *J. Phycol.* 23:1–9.

Pringsheim, E. C. 1912. Die kultur von algen in agar. *Beitr. Biol. Pflanz.* 11:305–33.

Pringsheim, E. G. 1946. *Pure Cultures of Algae.* Cambridge Univ. Press, London, 119 pp.

Provasoli, L. 1958. Nutrition and ecology of protozoa and algae. *Annu. Rev. Microbiol.* 12:279–308.

Provasoli, L. 1968. Media and prospects for the cultivation of marine algae. In: Watanabe, H., and Hattori, A., eds. *Culture and Collection of Algae. Proceedings, U. S.-Japan Conference.* Japanese Society of Plant Physiology, Hakone, Japan, pp. 63–75.

Provasoli, L. 1971. Media and prospects for the cultivation of algae. In: Rosowski, J. R., and Parker, B. C., eds. *Selected Papers in Phycology.* Department of Botany, University of Nebraska, Lincoln, Nebraska, 876 pp.

Provasoli, L., and Carlucci, A. F. 1974. Vitamins and growth regulators. In: Stewart, W. D. P., ed. *Algal Physiology and Biochemistry.* Blackwell Scientific, UK, pp. 741–87.

Provasoli, L., McLaughlin, J. J. A., and Droop, M. R. 1957. The development of artificial media for marine algae. *Arch. Mikrobiol.* 25:392–428.

Riebesell, U., Wolfgladrow, D. A., and Smetacek, V. 1993. Carbon dioxide limitation of marine phytoplankton growth rates. *Nature* 361:249–51.

Rippka, R., Derulles, J., Waterbury, J. B., Herdman, M., and Stainer, R. Y. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* 111:1–61.

Schreiber, E. 1927. Die Reinkultur von marinen Phytoplankton und deren Bedeutung für die Erforschung der Produktionsfähigkeit des Meerwassers. *Wiss. Meeresuntersuch., N.F.* 10:1–34.

Suttle, C., Price, N., Harrison, P., and Thompson, P. 1986. Polymerization of silica in acidic solutions: A note of caution to phycologists. *J. Phycol.* 22:234–7.

Sweeney, B., Haxo, F., and Hastings, J. 1959. Action spectra for two effects of light on luminescence in *Gonyaulax polyedra. J. Gen. Physiol.* 43:285–99.

Syrett, P.J 1981. Nitrogen metabolism of microalgae. *Can. Bull Fish. Aquatic Sci.* 210:182–210.

Thomas, W. H., Dodson, A. W., and Reid, F. M. H. 1978. Diatom productivity compared to other algae in natural marine assemblages. *J. Phycol.* 14:250–3.

Turner, M. F. 1979. Nutrition of some marine microalgae with special reference to vitamin requirements and utilization of nitrogen and carbon sources. *J. Mar. Biol. Assoc. U.K.* 59:535–52.

von Stosch, H. 1963. Wirkungen von jod un arsenit auf meeresalgen in kultur. *Proc. Int. Seaweed Symp.* 4: 142–50.

Waterbury, J. B., Watson, S. W., Valois, F. W., and Franks, D. G. 1986. Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus.* In: Platt, T., and Li, W. K. W., eds. *Photosynthetic Picoplankton. Can. Bull. Fish. Aquatic Sci.*, 214. Department of Fisheries and Oceans, Ottawa, Canada, pp. 71–120.

West, J. A., and McBride, D. L. 1999. Long-term and diurnal carpospore discharge patterns in the *Ceramiaceae, Rhodomelaceae* and *Delesseriaceae* (Rhodophyta). *Hydrobiologia* 298/299:101–13.