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A REVIEW ON MICROALGAL CULTURE TECHNIQUES

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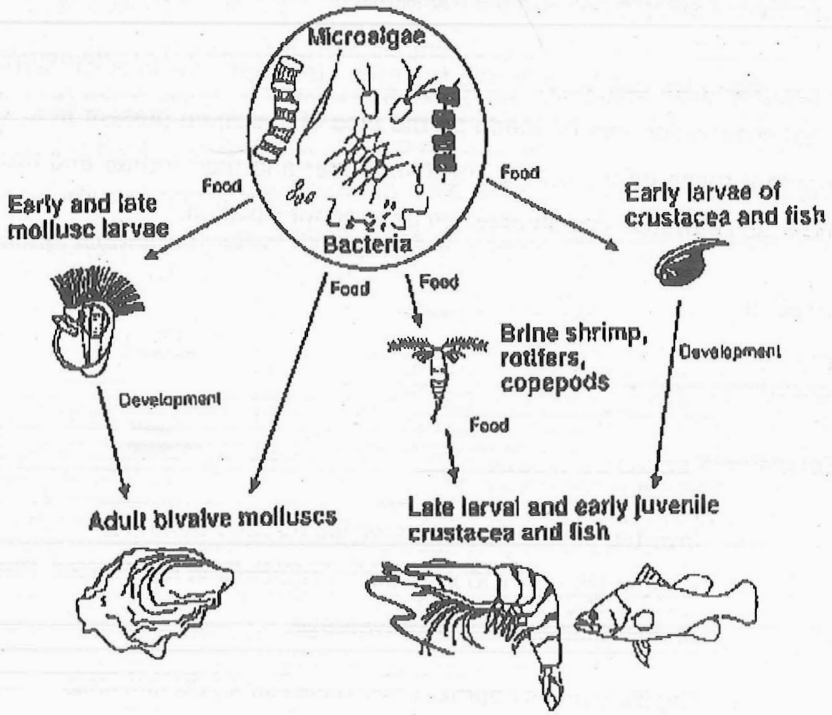
Phytoplankton comprises the base of the food chain in the marine environment Microalgae are an essential food source for marine bivalve molluscs (clams, oysters, scallops) throughout their life cycle, larval stages of some marine Gastropoda (abalone, snails), larvae of some marine fish species, Peneidae shrimp and zooplankton. . Algae are furthermore used to produce mass quantities of zooplankton (rotifers, copepods, brine shrimp) which serve in turn as food for larval and early-juvenile stages of crustaceans and fish. Besides they are believed to play a role in stabilizing the water quality, nutrition of the larvae, and microbial control.

The central role of micro-algae in mariculture (Brown et al., 1989).



Choosing the microalgal specie to be used as food varies according to the nutritional value that the specie has for a specific organism and depending on its cellular size, digestibility, production of toxic compounds and biochemical composition. Another important use for massive phytoplankton culture is obtaining different chemical products of potential marketing interest generated by the metabolic activity of different types of Microalgae, such as vitamins, carotenoids, amino acids, antibiotics, bioflocculants, etc.

A culture can be defined as an artificial simulating environment in which the organism grows. A culture has three distinct components: a) Aseptic controlled environmental condition b) culture medium prepared aseptically in suitable vessel c) Optimal environmental condition for the growth of the algal cells. It involves three steps

- Collection of sample and sample preparation
- Isolation
- Culture

Collection of sample and sample preparation:

Samples can be collected from the identified sites in clean sterilized bottle. Initial observation can be made on the type of specimen present in it. They can be filtered through different pore size filter paper and then further and then diluted in sterilized seawater. This sample can be used for isolation.

Isolation

Micromanipulation

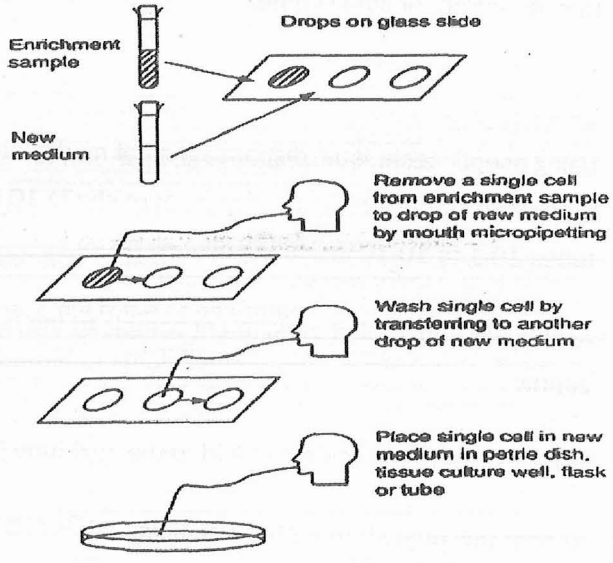
Equipments:

- Inverted microscope or stereo microscope with magnification up to 200 x, although 40–100 x should be sufficient in most cases. Phase contrast or dark field optics is an advantage.
- Capillary tubes approx. 1 mm diameter x 100 mm long.
- Bunsen burner or small flame.

- Silicone tubing to fit over end of capillary tube. Length approximately 300–400 mm
- Hot plate with beaker containing distilled water
- Clean Glass slides
- Agar plates (1.5% Bacto-Agar, eg Difco Cat. No. 0140–01) made up in petri dishes (disposable, 90 mm diam.)
- Tissue culture plates.
- Pasteur pipettes (sterile), rubber or silicon teat.
- Sterile media, usually at dilute nutrient concentrations; e.g. f_{20}, f_{50}
- Sterile tissue culture multi-well plates or sterile disposable petri dishes (e.g. 33 or 55 mm diam or sterile culture tubes.

Method

- ❖ Exposing to the flame from a bunsen burner heat and draw out two micropipettes from the capillary tube. The narrow end should be about twice the diameter of the cell to be micromanipulated.
- ❖ Heat distilled water to simmering point on hot plate. This is used for sterilizing the micropipette between each transfer.
- ❖ Place drops of sterile medium onto 1.5% agar plates with a sterile pasteur pipette. Alternatively place three drops on a glass slide.



- With silicone tubing attached to micropipette suck up and blow out with mouth a small amount of hot distilled water. This sterilizes the micropipette.
- Locate algal cell to be isolated in drop of enrichment sample. While observing the cell, suck up into the micropipette.
- Transfer the cell to a drop of sterile medium on agar plate or glass slide.
- Sterilize the micropipette.

Repeat this process to "wash" the cell. The more times a cell is washed the less likely is bacterial contamination. However, the risk of cell damage increases with the number of times a cell is handled. The optimum number of washes will depend on the type of algae. Transfer the cell to dilute medium in a tissue culture plate, petri dish or culture tube. Place culture vessel under controlled light and temperature. Check microscopically for growth or wait until macroscopic growth can be detected. clonal uni-algal culture should result from this method.

Serial dilution

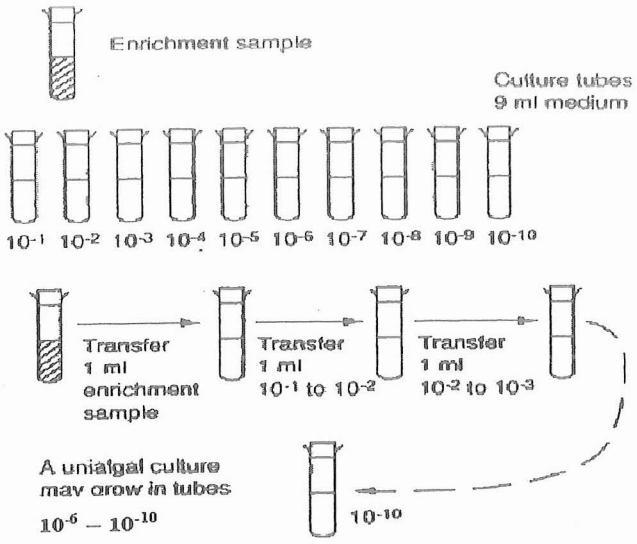
Equipments

- Culture tubes, (sterile) screw-capped or steristoppered (see Note below)
- Test-tube racks, open mesh.
- Media usually dilute e.g. f10, f20.
- Automatic dispenser (sterile) or 10 ml sterile glass pipettes
- Glass pipettes 1 ml or pasteur (sterile) with rubber or silicone.
- Bunsen burner or small flame.

Method

- Using aseptic technique, dispense 9 ml of media into each of ten test tubes with sterile automatic dispenser or sterile 10 ml pipettes. Label tubes 10-1 to 10-10 indicating dilution factor.
- Aseptically add 1 ml of enrichment sample to the first tube (10^{-1}) and mix gently.
- Take 1 ml of this dilution and add to the next tube (10^{-2}), mix gently.
- Repeat this procedure for the remaining tubes (10^{-3} to 10^{-10}).

❖ Incubate test-tubes under controlled temperature and light conditions



Examine cultures microscopically after 2–4 weeks by withdrawing a small sample aseptically from each dilution tube. A unialgal culture may grow in one of the higher dilution tubes e.g. 10^{-6} to 10^{-10}. If tubes contain two or three different species then micromanipulation can be used to obtain unialgal cultures.

Streak plating

This is a suitable method for small species (<10μm) or algae that grow well on a substrate.

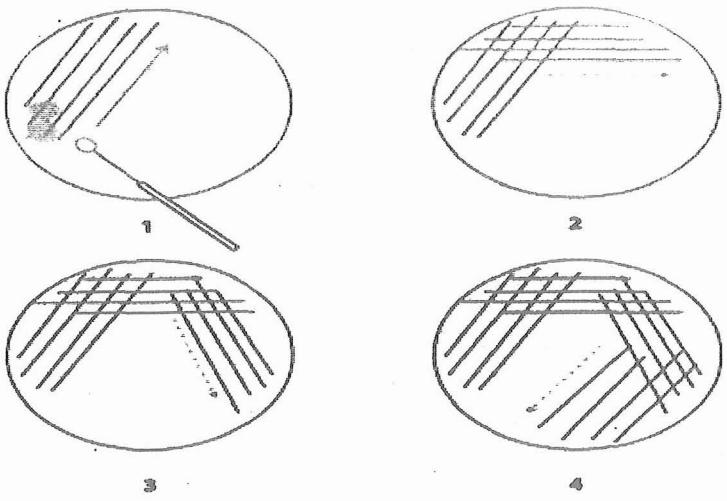
Equipments

- Petri dish sterile, disposable, 90 mm diameter
- Media f2
- Agar Bacto-Agar, Difco,
- Wire loops nichcrome or platinum
- Bunsen burner or small flame
- Parafilm

Method

- Prepare petri dishes containing growth medium solidified with 1-1.5% agar medium. The agar should be ½-2/3 the depth of the dish.

- Place 1–2 drops of mixed phytoplankton sample near the periphery of



the agar. Flame sterilize a wire loop.

- Using aseptic technique use the sterile loop to make parallel streaks of the suspension on the agar. Note that there are 16 streaks (4 sets of 4) to be made and the whole surface of the agar plate is used (see fig below).

- Cover and seal plate with parafilm. Invert and incubate under low light at constant temperature.

- Select colonies that are free of other organisms for further isolation. Remove a sample using a sterilized wire loop and place in a drop of sterile culture medium on a glass slide. Check microscopically that the desired species has been isolated and is unialgal.

- Repeat the streaking procedure with the algal cells from a single colony and again allow colonies to develop. This second streaking reduces the possibility of bacterial contamination and of colonies containing more than one algal species.

- ❖ Transfer selected colonies to liquid or agar.

Density centrifugation

In preparation: use of silicon colloids such as Percoll gradient centrifugation

Based on their growth characteristics, two kinds of cultures can be defined.

a. **Static or Bach Cultures.** Consist on only one cell inoculation in a culture medium or fertilized water followed by a period of several days growth and finally harvesting when the microalgal population reaches maximum density. In limited volume (batch) cultures, resources are finite. When the resources present in the culture medium are abundant, growth occurs according to a sigmoid curve, but once the resources have been utilised by the cells, the cultures die unless supplied with new medium. In practise this is done by subculturing, i.e. transferring a small volume of existing culture to a large volume of fresh culture medium at regular intervals.

    - o 1 and 2 L flasks
    - o 18 L carboys
    - o 40 L vinyl bags
    - o 400L fiber glass columns
    - o 2000 L tanks

Semi-continuous cultures. Semi-continuous culture technique extends the use of culture tanks due to partial harvesting followed by immediate sea water addition with nutrients with a volume same as the one harvested. This type of culture can be carried out both in interior as in exterior even though duration is uncertain as competitors might be developed (bacteria, and other microalgal species) and/or accumulation of metabolites of the same microalgae. Considering that the culture is not completely harvested, better microalgal productions are obtained with the semi-continuous method than by the static method. In continuous cultures, resources are potentially infinite: cultures are maintained at a chosen point on the growth curve by the regulated addition of fresh culture medium. In practise, a volume of fresh culture medium is added automatically at a rate proportional to the growth rate of the alga, while an equal volume of culture is removed.

- 18 L carboys
- 200 L glass fiber columns

Physical parameters

Temperature

The temperature at which cultures are maintained should ideally be as close as possible to the temperature at which the organisms were collected. Temperature controlled incubators or a stock culture room with optimum temperature level are used for maintaining the culture. It is preferably 26 to 28°C for tropical country.

Light

Natural light is usually sufficient to maintain cultures in the laboratory. Cultures should never be exposed to direct sunlight. Artificial lighting by fluorescent bulbs is often employed for maintenance of culture. Light intensity should range between 0.2-50% of full daylight (= 1660 µE/s/m^2), with 5-10% (c. 80-160µE/s/m^2) most often employed. Light quality (spectrum) depends on type of bulb used (see manufacturers technical data), the most common types being 'cool white' or 'daylight' bulbs. Light intensity and quality can be manipulated with filters. Many microalgal species do not grow well under constant illumination, and hence a light/dark (LD) cycle is used (maximum 16:8 LD, usually 14:10 or 12:12).

Mixing

Mixing of microalgal cultures may be necessary under certain circumstances. Cells must be kept in suspension in order to grow to prevent limiting effect of nutrient and light. Mixing should be gentle either by bubbling with air (may damage cells) or plankton wheel or roller table (about 1 rpm) by gentle manual swirling. Most cultures do well without mixing, particularly when not too concentrated, but when possible gentle manual swirling (once each day) is recommended.

Culture Flask

Culture flasks should be non toxic (chemically inert, transparent to light can be easily cleaned and sterilized and should provide a large surface to volume ratio (depending on organism). Certain synthetic materials may leach chemicals which

might have a deleterious effect on algal growth into the medium. The use of chemically inert materials is particularly important when culturing oceanic plankton and during isolation. A list of materials which should be safe, inhibitory, or toxic to algal cultures is given by Stein (1973). Recommended materials for culture vessels and media preparation include:

- Teflon (very expensive, used for media preparation);
- Polycarbonate (expensive and becomes cloudy and cracks with repeated autoclaving);
- Polystyrene (cheaper alternative to Teflon and polycarbonate, not autoclavable);
- Borosilicate glass (has been shown to inhibit growth of some species).
- Erlenmeyer flasks (glass or polycarbonate) with cotton, glass, polypropylene, or metal covers.
- Tubes (glass or polycarbonate) with cotton, glass, polypropylene or metal caps.
- Polystyrene tissue culture flasks, purchased as single-use sterile units (eg. Iwaki, Nunc, Corning).

Cleaning/sterilization of culture materials

Cleaning procedure

- Scrub (abrasive brushes not appropriate for most plastics) and soak with warm detergent (not domestic detergents, which leave a residual film on culture ware – use laboratory detergent such as phosphate-free Decon);
- Rinse extensively with tap water;
- Soak in 10% HCl for 1 day-1 week (not routinely necessary, but particularly important for new glass and polycarbonate material);
- Rinse extensively with distilled and finally distilled water;
- Leave inverted to dry in a clean, dust-free place.

Sterilization

Sterilization can be defined as a process, which ensures total inactivation of microbial life. The primary purpose of sterilization is to prevent contamination by

unwanted organisms, but it may also serve to eliminate unwanted chemicals. There are several sterilization methods and the choice depends on the purpose and material used:

Gas (ethylene oxide, often used for disposable plastic material);

Autoclaving: Autoclave or pressure cooker the glassware under 121 °C (at 15 lb pressure in pure saturated steam). Never use close vessels. It should be capped loosely

Dry heat

Oven dry the glasswares for at least 2 hours at 160°C. Cover neck of vessel with aluminium foil to maintain sterility on removal from oven.

Flame heating is used for the for metal equipments while transferring the samples during inoculation

Radiation

X ray and Gamma radiation is used for industrial applications for disposable plastics that cannot stand more then 60°C, not always effective. UV radiation 240-280 nm (not often used for culture materials).

Transfer protocol

The following procedures should always be used when preparing media or transferring cultures:

- Work place preferably in a laminar flow cabinet. Cabinet with UV lamps must be turned on at least 30 minutes before transfer.
- Clean working surface with 70% alcohol (ethanol/isopropanol) prior to and after use.
- Clean hands with disinfecting soap and rinse with 70% alcohol prior to all operations.
- Pipettes, autoclavable tips pre-wrapped sterile plastic/glass pipettes were exposed to UV lamps under laminar flow.
- Sterilise the neck of vessel under flame before and after transfer.

Culture media

Media for the culture of marine phytoplankton consist of a seawater base (natural or artificial) which may be supplemented by various substances essential for microalgal growth, including nutrients, trace metals and chelators, vitamins, soil extract and buffer compounds.

Seawater

Natural seawater

- The quality of water used in media preparation is very important. Natural seawater can be collected nearshore, but its salinity and quality is often quite variable and unpredictable. Thus offshore water is most preferable. The quality of coastal water may be improved by ageing for a few months (allowing bacterial degradation of inhibitory substances), by autoclaving (heat may denature inhibitory substances), or by filtering through acid-washed charcoal (which absorbs toxic organic compounds). Most coastal waters contain significant quantities of inorganic and organic particulate matter, and therefore must be filtered before use (eg Whatman no. 1 filter paper).

- The low biomass and continual depletion of many trace elements from the surface waters of the open ocean by biogeochemical processes makes this water much cleaner, and therefore preferable for culturing purposes. Collect seawater from the front of the boat (or pump from subsurface) to avoid contamination. Seawater can be stored in polyethylene carboys, and should be stored in cool dark conditions.

Artificial seawater

- Artificial seawater, made by mixing various salts with deionized water, has the advantage of being entirely defined from the chemical point of view, but they are very laborious to prepare, and often do not support satisfactory algal growth. Trace contaminants in the salts used are at rather high concentrations in artificial seawater because so much salt must be added to achieve the salinity of full strength seawater. Some of the more successful artificial seawater media

that have been developed include the ESAW medium of Harrison *et al.* (1980), and the AK medium of Keller *et al.* (1987).

Nutrients, trace metals, and chelators

The term 'nutrient' is colloquially applied to a chemical required in relatively large quantities, but can be used for any element or compound necessary for algal growth. It is catagorized into three groups.

- Group A: concentrations of these constituents exhibit essentially no variation in seawater, and high algal biomass cannot deplete them in culture media. These constituents do not, therefore, have to be added to culture media using natural seawater, but do need to be added to deionized water when making artificial seawater media (Brand, 1990).

Group A	Average Molar concentration (range in brackets)
Na^+	4.7×10^{-1}
K^+	1.02×10^{-2}
Mg^{2+}	5.3×10^{-2}
Ca^{2+}	1.03×10^{-2}
Cl^-	5.5×10^{-1}
SO_4^{2-}	2.8×10^{-2}
HCO_3^-	2.3×10^{-3}
BO_3^{3-}	4.2×10^{-4}

Group B: They are also found in a constant proportion in seawater. So it need not be added to natural seawater media. Standard artificial media (and some natural seawater media) add molybdenum (as molybdate), an essential nutrient for algae, selenium (as selenite), which has been demonstrated to be needed by some algae, as well as strontium, bromide and flouride, all of which occur at relatively high concentrations in seawater, but none of which have been shown to be essential for microalgal growth.

Group B	Average Molar concentration (range in brackets)
Br^-	8.4×10^{-4}
F^-	6.8×10^{-5}
IO_3^-	4.4×10^{-7}
Li^+	2.5×10^{-5}
Rb^+	1.4×10^{-6}
Sr^{2+}	8.7×10^{-5}
Ba^{2+}	1×10^{-7}
MoO_4^{2-}	1.1×10^{-7}
VO_4^{3-}	2.3×10^{-8}
CrO_4^{2-}	4×10^{-9}
AsO_4^{3-}	2.3×10^{-8}
SeO_4^{2-}	1.7×10^{-9}

Group C: All known to be needed by microalgae (silicon is needed only by diatoms and some chrysophytes, and nickel is only known to be needed to form urease when algae are using urea as a nitrogen source). These nutrients are generally present at low concentrations in natural. All of these nutrients (except silicon and nickel in some circumstances) generally need to be added to culture media in order to generate significant microalgal biomass.

Group C	Average Molar concentration (range in brackets)
NO_3^-	3×10^{-5} (10^{-8} to 4.5×10^{-5})
PO_4^{3-}	2.3×10^{-6} (10^{-7} to 3.5×10^{-6})
Fe^{3+}	1×10^{-9} (10^{-10} to 10^{-7})
Zn^{2+}	6×10^{-9} (5×10^{-11} to 10^{-7})
Mn^{2+}	5×10^{-10} (2×10^{-10} to 10^{-6})
Cu^{2+}	4×10^{-9} (5×10^{-10} to 6×10^{-9})
Co^{2+}	2×10^{-11} (10^{-11} to 10^{-10})
SiO_4^{4-}	1×10^{-4} (10^{-7} to 1.8×10^{-4})
Ni^{2+}	8×10^{-9} (2×10^{-9} to 1.2×10^{-8})

The trace metals which are essential for microalgal growth are incorporated into essential organic molecules, particularly a variety of coenzyme factors which enter into photosynthetic reactions. Of these metals, the concentrations (or more accurately the biologically available concentrations) of Fe, Mn, Zn, Cu and Co (and sometimes Mo and Se) in natural waters may be limiting to algal growth.

Chelators act as trace metal buffers, maintaining constant concentrations of free ionic metal. It is the free ionic metal, not the chelated metal, which influences microalgae, either as a nutrient or as a toxin. The most widely used chelator in culture media additions is EDTA (ethylenediaminetetraacetic acid), which must be present at high concentrations since most complexes with Ca and Mg, present in large amounts in seawater. EDTA may have an additional benefit of reducing precipitation during autoclaving. High concentrations have, however, occasionally been reported to be toxic to microalgae. The ratio of chelator: metal in culture medium ranges from 1:1 in f/2 to 10:1 in K medium.

Vitamins

Roughly ½ of all microalgal species tested have been shown to have a requirement for vitamin B_{12}, which appears to be important in transferring methyl groups and methylating toxic elements such as arsenic, mercury, tin, thallium, platinum, gold, and tellurium (Brand, 1986), around 20% need thiamine, and less than 5% need biotin. It is recommended that these vitamins are routinely added to seawater media.

Soil extract

Prepared by heating, boiling, or autoclaving 5 to 30% slurry of soil in fresh water or seawater and subsequently filtering out the soil. Soil extract has historically been an important component of culture media. The solution provides macronutrients, micronutrients, vitamins, and trace metal chelators in undefined quantities, each batch being different, and hence having unpredictable effects on microalgae. With increasing understanding of the importance of various constituents of culture media, soil extract is less frequently used. Soil extract should only be used on a non-experimental basis.

Buffers

The control of pH in culture media is important since certain algae grow only within narrowly defined pH ranges, and in order to prevent the formation of precipitates. Except under unusual conditions, the pH of natural seawater is around 8. Because of the large buffering capacity of natural seawater (due to a bicarbonate buffering system, HCO_3 being present at c. 2.2M) it is quite easy to maintain the pH of marine culture media.

- The buffer system is changed only during autoclaving, when high temperatures drive out CO_2 from the medium or in very dense cultures of microalgae, when enough CO_2 is taken up to produce a similar effect. Thus the pH of seawater may be lowered prior to autoclaving (adjustment to pH 7-7.5 with 1M HCl) to compensate for subsequent increases. Certain media recipes include additions of extra buffer, either as bicarbonate, Tris (Tris-hydroxymethyl-aminomethane), or glycylglycine to supplement the natural buffering system. Tris may also act as a Cu buffer, but has occasionally been cited for its toxic properties to microalgae. Glycylglycine is rapidly metabolized by bacteria and hence can only be used with axenic cultures. These additions are generally not necessary if media are filter sterilized, unless very high cell densities are expected.

- The problem of CO_2 depletion in dense cultures may be reduced by having a large surface area of media exposed to the atmosphere relative to the volume of the culture, or by bubbling with either air (CO_2 concentration c.0.03%) or air with increased CO_2 concentrations (0.5 to 5%). Unless there is a large amount of biomass taking up the CO_2, the higher concentrations could actually cause a significant decline in pH. When bubbling is employed, the gas must first pass through an in line 0.2µm filter unit (eg. Millipore Millex GS) to maintain sterile conditions. For many microalgal species, aeration is not an option since the physical disturbance may inhibit growth or kill cells.

Media preparation

- The salinity of the seawater base should first be checked (30-35‰ for marine phytoplankton), and any necessary adjustments (addition of fresh water/evaporation) made before addition of enrichments.

- Always use reagent grade chemicals and de-ionized distilled water to make stock solutions of enrichments. Gentle heating and/or magnetic stirring of stock solutions can be used to ensure complete dissolution. When preparing a stock solution containing a mixture of compounds, dissolve each individually in a minimal volume of water before mixing, then combine and dilute to volume.

- Seawater, stock solutions of enrichments and the final media must be sterile in order to prevent (or more realistically minimize) biological contamination of unialgal cultures. Several methods are available for sterilization:

Autoclaving is the most widely used technique for sterilizing culture media, and is the ultimate guarantee of sterility. Bottles containing media should be no more than ¾ full, and should be left partially open or plugged with cotton wool or covered with aluminium foil.

Pasteurization (heating to 90-95°C for 30 minutes) of media in Teflon or polycarbonate bottles is a potential alternative, reducing the problems of trace metal contamination and alteration of organic molecules inherent with autoclaving. Pasteurization does not, however, completely sterilize the seawater; it kills all eukaryotes and most bacteria, but some bacterial spores probably survive.

Ultraviolet radiation can be used to sterilize seawater, but very high intensities are needed to kill everything in the seawater (1200 W lamp, 2-4h for culture media in quartz tubes). Such intense UV light necessarily alters and destroys the organic molecules in seawater and generates many long lived free radicals and other toxic reactive chemical species (Brand, 1986). Seawater exposed to intense UV light must, therefore, be stored for several days prior to use to allow the level of these highly reactive chemical species to decline.

Microwave sterilization has not, as yet, been widely employed in culture media preparation due to uncertainties about sterilization efficiency. Trials should be conducted before use of this method to ensure sterility of seawater.

Sterile filtration is probably the best method of sterilizing seawater without altering the chemistry of the seawater, as long as care is taken not to contaminate the seawater with dirty filter apparatus. Sterilization efficiency is, however, to some extent reduced compared with heat sterilization methods. Membrane filters of 0.2µm porosity are generally considered to yield water free of bacteria, but not viruses. Vitamin stock solutions are routinely filtered through 0.2µm single use filter units (eg. Millipore Millex GS), since heat sterilization will denature these organic compounds.

Culture medium recipes

A variety of alternative marine culture media recipes are given by Stein (1975) and on the web pages of the major culture collections (eg. CCMP, Utex).

f/2-Si (Guillard, 1975)

To 996ml of sterile seawater aseptically add:

Quantity	Compound	Stock solution (sterile)	Final conc. in medium
1.0ml	$NaNO_3$	75.0g/litre H_2O	884µM
1.0ml	$NaH_2PO_4.H_2O$	5.0g/litre H_2O	36µM
1.0ml	f/2 trace metal solution	(see recipe below)	(see below)
1.0ml	f/2 vitamin solution	(see recipe below)	(see below)

f/2 trace metal solution

To 950ml distilled H_2O add:

Quantity	Compound	Stock solution	Final conc. in medium
3.15g	$FeCl_3.6H_2O$	-	11.7µM
4.36g	$Na_2EDTA.2H_2O$	-	12µM
1.0ml	$CuSO_4.5H_2O$	9.8g/litre H_2O	0.04µM
1.0ml	$Na_2MoO_4.2H_2O$	6.3g/litre H_2O	0.03µM
1.0ml	$ZnSO_4.7H_2O$	22.0g/litre H_2O	0.08µM
1.0ml	$CoCl_2.6H_2O$	11.9g/litre H_2O	0.05µM
1.0ml	$MnCl_2.4H_2O$	178.2g/litre H_2O	0.9µM

Make up to 1 litre with distilled H_2O, sterilize (autoclave or filter) and store in fridge.

f/2 Vitamin solution

To 950ml distilled H_2O add:

Quantity	Compound	Stock solution	Final conc. in medium
1.0ml	Vit. B_{12} (cyanocobalamin)	0.5g/litre H_2O	0.37nM
1.0ml	Biotin	5.0mg/litre H_2O	2.0nM
100.0mg	Thiamine HCl	-	0.3µM

Make up to 1 litre with distilled H20, filter sterilize into plastic vials and store in freezer.

K (-Si) (Keller *et al.*, 1987)

To 994 ml of sterile seawater aseptically add:

Quantity	Compound	Stock solution (sterile)	Final conc. in medium
1.0ml	$NaNO_3$	75.0g/litre H_2O	884µM
1.0ml	NH_4Cl *	0.535g/litre H_2O	10µM
1.0ml	Na_2glycerophosphate **	2.16g/litre H_2O	10µM
1.0ml	TRIS-base (pH7.2) ***	121.1g/litre H_2O	1mM
1.0ml	K trace metal solution	(see recipe below)	(see below)
1.0ml	f/2 vitamin solution	(see recipe below)	(see below)

* should not be autoclaved (volatile when heated)

** inorganic orthophosphate can be substituted (particularly if not autoclaving)

*** can be omitted (particularly if not autoclaving, or if cell density will not be very high)

K trace metal solution

To 950ml distilled H_2O add:

Quantity	Compound	Stock solution	Final conc. in medium
4.3g	(Na)FeEDTA	-	11.7µM
37.22g	$Na_2EDTA.2H_2O$	-	100µM
0.5ml	$CuSO_4.5H_2O$	9.8g/litre H_2O	0.02µM
1.0ml	$Na_2MoO_4.2H_2O$	6.3g/litre H_2O	0.03µM
1.0ml	$ZnSO_4.7H_2O$	22.0g/litre H_2O	0.08µM
1.0ml	$CoCl_2.6H_2O$	11.9g/litre H_2O	0.05µM
1.0ml	$MnCl_2.4H_2O$	178.2g/litre H_2O	0.9µM
1.0ml	H_2SeO_3	1.29mg/litre H_2O	0.01µM

Make up to 1 litre with distilled H_2O, sterilize (autoclave or filter) and store in fridge.

f/2 Vitamin solution

To 950ml distilled H_2O add:

Quantity	Compound	Stock solution	Final conc. in medium
1.0ml	Vit. B_{12} (cyanocobalamin)	0.5g/litre H_2O	0.37nM
1.0ml	Biotin	5.0mg/litre H_2O	2.0nM
100.0mg	Thiamine HCl	-	0.3µM

Make up to 1 litre with distilled H20, filter sterilize into plastic vials and store in freezer.

A.Guillard and Ryther's Modified F Medium		**B. Walne's Medium**	
$NaNO_3$	84.148 mg	$NaNO_3$	100.0000 mg
$NaH_2PO_4.H_2O$	10.000 mg	Na_2EDTA	45.0000 mg
$FeCl_3.6H_2O$	2.900 mg	H_3BO_3	33.600 mg
Na_2EDTA	10.000 mg	$NaH_2PO_4.2H_2O$	20.000 mg
$Na_2SiO_3.9H_2O$	50.000 mg	$FeCl_3.6H_2O$	1.3000 mg
Vitamins:		$MnCl_2.4H_2O$	0.360 mg
B_1 (Thiamin HCl)	00.200 mg	**Vitamins:**	
B_{12} (Cobalamine)	1.000 ug	B_1	0.1 mg
Biotin	1.000 ug	B_{12}	0.005 mg
Trace metals:		**Trace metals:**	

$CuSO_4.5H_2O$	0.0196 mg	$ZnCl_2$	0.021 mg
$ZnSO_4.7H_2O$	0.0440 mg	$CoCl_2.6H_2O$	0.020 mg
$CoCl_2.6H_2O$	0.2000 mg	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.009 mg
$MnCl_2.4H_2O$	3.6000 mg	$CuSO_4.5H_2O$	0.020 mg
$NaMoO_4.2H_2O$	0.0126 mg		
Seawater to 1 lit		Seawater to 1 lit	

	Liao and Huang's Modified Medium	
	KNO_3	100.000 mg
	$Na_2HPO_4.H_2O$	10.000 mg
	$FeCl_3.6H_2O$	3.000 mg
	$Na_2SiO_3.9H_2O$	1.000 mg
	Seawater	to 1 liter

Stock solutions Guillard and Ryther's Modified F Medium

1.	$NaNO_3$ and $NaH_2PO_4.H_2O$ Stock (500x)	
	$NaNO_3$	42.074 g
	$NaH_2PO_4.H_2O$	5.000 g
	Distilled water	1 liter
	Utilization	2 ml/L
2.	$Na_2SiO_3.9H_2O$ Stock (500x)	
	$Na_2SiO_3.9H_2O$	16.50 g
	Distilled water	1 liter
	Utilization	2 ml/L
3.	$FeCl_3.6H_2O$ Stock (500x)	
	$FeCl_3.6H_2O$	1.45 g
Distilled water 1 L : Utilization 2 ml/L		
4.	NaEDTA stock (1000x)	
	Na_2EDTA	10.0 g
Distilled water 1 L : Utilization 1 ml/L		

5.	Vitamin stock (1000x)	
	B_1	0.2 g
	B_{12}primary stock	10 g
	Biotin primary stock	10 ml
Distilled water 1 L : Utilization 1 ml/L		
6.	Trace metal stock (1000x)	
	Trace metals primary stocks; A,B,C, and D	1 ml
Distilled water 1 L : Utilization 1 ml/L		
7.	Biotin primary stock	
	Biotin	0.1 g
	Distilled water	1 L
8.	B_{12} primary stock	
	B_{12}	0.1 g
	Distilled water	1 L
9.	Trace metals primary stock A	
	$CuSO_4.5H_2O$	1.96 g
	$ZnSO_4.7H_2O$	4.40 g
	Distilled water	100 ml
10.	**Trace metal primary stock B**	
	$Na_2MoO_4.2H_2O$	1.26 g
	or	
	$(NH_4)_6Mo_7O_{24}.4H_2O$	6.43 g
	Distilled water	100 ml
11.	Trace metal primary stock C	
	$MnCl_2.4H_2O$	36.00 g
	Distilled water	100 ml
12.	Trace metal primary stock D	
	$CoCl_2.6H_2O$	2.0 g
	Distilled water	100.0 ml
Walne's Medium (1000 x)		

1.	$NaNO_3$	100.0 g
	Na_2 EDTA	45.0 g
	H_3BO_3	33.6 g
	$NaH_2PO_4.H_2O$	20.0 g
	$FeCl_3.6H_2O$	1.30 g
	$MnCl_2.4H_2O$	0.36 g
	Vitamins primary stock	100.0 ml
	Trace metals primary stock	1.0 ml
Distilled water 1 L : Utilization 1 ml/L		
2.	Vitamins primary stock	
	B_1	200.0 mg
	B_{12}	10.0 mg
	Distilled water	**200.00 ml**
3.	Trace metals primary stock	
	$ZnCl_2$	2.1 g
	$CoCl_2.6H_2O$	2.0 g
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.9 g
	$CuSo_4.5H_2O$	2.0 g
	Distilled water	**100.0 ml**
Liao and Huang's Modified Medium (1000x)		
	KNO_3	100.00 g
	$Na_2HPO_4.12H_2O$	10.00 g
	$FeCl_3.6H_2O$	3.00 g
	$Na_2SiO_3.9H_2O$	1.00 g
Distilled water 1 L : Utilization 1 ml/L		

** can be substituted for inorganic orthophosphate

* optional

Solid Substrate medium

Some aquatic microalgae grow well on solid substrate. A 3% high grade agar can be used for the solid substrate. The agar and culture medium should not be

autoclaved together, because toxic breakdown products can be generated. The best procedure is to autoclave 30% agar in deionized water in one container and nine times as much seawater base in another. After removing from the autoclave, sterile nutrients are added aseptically to the water, which is then mixed with the molten agar. After mixing, the warm fluid is poured into sterile petri dishes, where it solidifies when it cools. The plate is inoculated by placing a drop of water containing the algae on the surface of the agar, and streaking with a sterile implement. The plates are then maintained under standard culture conditions. This method may be particularly effective for cleaning cultures infected with bacteria, clean colonies of the algal species being isolated from the plate into fresh liquid medium.

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