



Microalgal Culture and Maintenance in Marine Hatcheries

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Introduction

Unicellular marine microalgae are widely used as food in the hatchery production of fish and shellfish. Molluscs like oysters, mussels and clams filter them from the seawater in all stages of life. Rotifers and brine shrimp also ingest algae, and these are then used as food for fish and prawn larvae. In hatchery systems algae are added to the larval rearing tanks to improve 'quality' of water as green water systems. The production of algae is very critical in successful hatchery management.

Marine algae are single-celled plants and like all plants, contain chlorophyll, which traps the energy from light and uses it to convert nutrients and carbon dioxide dissolved in the sea water into organic matter. Microalgae are the primary producers of the sea. Among microalgae, flagellate and diatom species, are primary producers at the base of the marine food chain. They are cultured in hatcheries in suitably treated seawater enriched with nutrients, which include nitrates, phosphates, essential trace elements, vitamins and carbon dioxide. Synthetic seawater may be used but it is expensive except for small laboratory scale cultures. The culture microalgae arise because the natural phytoplankton content of seawater is insufficient to support growth of high densities of larvae and juveniles reared. Particularly in the hatchery, the water treatments will remove almost all of the natural phytoplankton which then needs to be replaced from cultures of preferred, high food value species. In this context, few of the naturally occurring algae of good food value are amenable to artificial culture.

Major classes and genera of cultured algal species

The major classes of cultured algae currently used to feed different groups of commercially important aquatic organisms include species of diatoms, flagellated and green algae, and filamentous blue-green algae, ranging in size from a few micrometers to more than 100 μ . The most frequently used species in commercial mariculture operations are the diatoms *Skeletonema costatum*, *Thalassiosira pseudonana*, *Chaetoceros gracilis*, *C. calcitrans*, the flagellates *Isochrysis galbana*, *Tetraselmis suecica*, *Pavlova lutheri* and *Chlorella* spp. The basic methods of algal culture have changed little over the years. Hatcheries have either opted for indoor, intensive culture with artificial illumination, usually external to the culture vessels, or outdoor, extensive culture in large tanks or ponds utilizing natural light. The intensive techniques are satisfactory in terms of reliability and productivity but are expensive in terms of capital outlay and labour, while the extensive methods tend to be less reliable and, sometimes not very productive.

Isolation of pure algal strains

Sterile cultures of micro-algae used for aquaculture purposes may be obtained from specialized culture collections. A list of culture collections is provided by Vonshak (1986) and Smith *et al.* (1993a). Alternatively, the isolation of endemic strains could be considered because of their ability to grow under the local environmental conditions. Isolation of algal species is not simple because of the small cell size and the association with other epiphytic species. Several laboratory techniques are available for isolating individual cells, such as serial dilution and successive plating on agar media, and separation using capillary pipettes. Bacteria can be eliminated from the phytoplankton culture by washing or plating in the presence of antibiotics. The sterility of the culture can be checked with a test tube containing seawater with 1 g.l^{-1} bactopectone. After sterilization, a drop of the culture to be tested is added and any residual bacteria will turn the bactopectone solution turbid. The collection of algal strains should be carefully protected against contamination during handling and poor temperature regulations. To reduce risks, two series of stocks are often retained, one which supplies the starter cultures for the production system and the other which is only subjected to the handling necessary for maintenance. Stock cultures are kept in test tubes at a light intensity of about 1000 lux and a temperature of 16 to 19°C. Constant illumination is suitable for the maintenance of flagellates, but may result in decreased cell size in diatom stock cultures. Stock cultures are maintained for about a month and then transferred to create a new culture line

Agar plating

Agar plating technique can be used to isolate algal strains from raw seawater and for the maintenance of existing strains. The procedure is as follows:

- prepare 0.9% agar medium.
- streak the algal sample onto the agar surface.
- Incubate for 5 - 21 days.
- select the best colonies and transfer them into a test tube.
- incubation on an illuminated glass rack when a colour change is observed in the tube, check the isolated algal strain under microscope.

Serial dilution

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.

- 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 from each 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

**Physical and chemical conditions**

The important parameters regulating algal growth are nutrient quantity and quality, light intensity, pH, turbulence, salinity and temperature. The optimal parameters as well as the tolerated ranges are species specific and a broad generalization is given in Table 1. Also, the various factors may be interdependent and a parameter that is optimal for one set of conditions is not necessarily optimal for another.

Table 1 A generalized set of conditions for culturing micro-algae (modified Anonymous, 1991)

| Parameters | Range | Optimum |
|--|--------------|--------------|
| Temperature (°C) | 16-27 | 18-24 |
| Salinity (g.l-1) | 12-40 | 20-24 |
| Light intensity (lux) (depends on volume and density) | 1,000-10,000 | 2,500- 5,000 |
| Photoperiod (light:dark, hours) | 6:8 (min) | 24:0 (max) |
| pH | 7-9 | 8.2-8.7 |

Maintenance of stock and starter cultures

Stock cultures, otherwise known as master cultures, of the preferred species are the basic foundation of culture. They are normally supplied as monospecific cultures from reputed culture collections. Stock cultures are used as inocula when required. Every effort should be made to minimize the risk of contaminating the stock and starter cultures with competing microorganisms. The sterile procedures described below should be followed to ensure that contamination does not occur. Stock cultures are kept in small, transparent, autoclavable containers. For example, 500 ml borosilicate glass, flat-bottomed boiling or conical flasks fitted with a cotton wool plug at the neck, suitable for containing 250 ml of sterile, autoclaved medium, are ideal. The composition and preparation of Guillard's F/2 medium is given in Table 2.

Table 2: Guillard's F/2 media used for culturing algae in bivalve hatcheries from Guillard (1975)

| Nutrient | wt (g l ⁻¹) |
|--|------------------------------|
| Nitrate NaNO ₃ | 75.0 |
| Phosphate NaH ₂ PO ₄ .H ₂ O | 5.0 |
| Silicate Na ₂ SiO ₃ .9H ₂ O | 30.0 |
| FeCl ₃ .6H ₂ O | 3.5 |
| Na ₂ EDTA | 4.36 |
| Dissolve in 900 ml distilled H ₂ O | |
| Add 1 ml of each of the following trace metal solutions | |
| Trace metal | wt (g 100 ml ⁻¹) |
| CuSO ₄ .5 H ₂ O | 0.98 |
| ZnSO ₄ .7 H ₂ O | 2.20 |
| CoCl ₂ .6 H ₂ O | 1.00 |
| MnCl ₂ .4 H ₂ O | 18.00 |
| Na ₂ MoO ₄ .2 H ₂ O | 0.63 |
| Make up the volume to 1 l with distilled H ₂ O (pH ca. 2.0) | |
| Add 1 ml per litre FSW of the above solutions (#1-4). | |

| Vitamins | wt (mg l ⁻¹) |
|---|--------------------------|
| Biotin | 1.0 mg |
| B-12 | 1.0 mg |
| Thiamine HCl | 20.0 mg |
| Dissolve in 1 l distilled H ₂ O. Store under refrigeration | |
| Add 1/2 ml of vitamin solution for every 1 l of FSW. | |

Stock solutions and salts

The culture media are referred to “working stocks” and “primary stocks”. Working stocks are those whose aliquots contribute directly to making the final media. Primary stocks are normally made where several single substance solutions are then combined to form the working stock, eg. CuSO₄.5H₂O and ZnSO₄.7H₂O are two of the primary stocks used to make up the Trace Metal working stock in F/2 medium. It is suggested that all stock or starter cultures be grown with AR grade chemicals it is understandable that in mass culture applications (> 20 - 50 L), particularly for aquaculture, these chemicals may be too expensive when bought in bulk quantities. Stock solutions are made up by accurately weighing the prescribed amount of nutrient and dissolving in a specified volume of distilled water, if possible in a volumetric flask. Some nutrients will readily dissolve; others need heat and stirring to fully dissolve. In contrast vitamin stocks are heat sensitive and should not be subjected to heat treatment and should also be kept in the dark. Failure to fully dissolve the primary stocks of some nutrients such as EDTA can lead to gross precipitation when these stocks are combined to make the media.

Nutrients come with different salts and hydration. For example, while copper and zinc may be two desired active constituents they are readily obtained from suppliers with either SO₄ or Cl₂ salts (i.e. CuSO₄ or CuCl₂ and ZnSO₄ or ZnCl₂). Some nutrients also come with different hydrations, i.e. the nH₂O suffix. Substituting one form for another may have no effect on the growth of some microalgae species, but it can lead to poor growth in others and also lead to unwanted and time consuming precipitation problems as the overall ratio of salts in the medium has changed. Therefore deviating from the prescribed recipes is to be avoided and ordering the correct form is recommended.

Procedure for transferring algal cultures from flask to flask

- Wipe all inner surfaces of inoculating booth with 85% ethanol.
- Place all flasks that will be required in the booth; i.e. all flasks to be transferred from (the transfer flask) and flasks containing sterilized media to be transferred into (new flasks).
- Close booth and switch on ultra-violet lamp. Leave for at least 20 minutes. (It is not safe to look directly at ultraviolet light, so a dark cover should be placed over the plexi-glass (transparent acrylic plastic) viewing plate when the light is on.)
- Switch off lamp. Ignite small burner.
- Remove foil caps from one transfer and one new flask. Flame the neck of each flask by slowly rotating the neck through the flame.
- Tilt the neck of the transfer flask toward the new flask. In one motion, remove both stoppers and pour an inoculum into the new flask. Transfer approximately 50 ml for diatom species and 100 ml for flagellates.



Avoid touching the necks of the two flasks. Never touch the portion of the stopper that is inserted into the flask. Once the inoculum is added, replace the stopper in the transfer flask. Slowly flame the neck of the new flask before replacing its stopper.

- (g) Replace foil cap over the neck of the new flask. Using a waterproof marker pen, label the new flask with the algal species inoculated and the date of transfer.
- (h) Repeat procedure for all flasks within the booth. Once completed, turn off burner and open booth.
- (i) Remove all new flasks and place in the algal incubator or a well-lit area in the algae culture facility.
- (j) The remaining inoculum in the transfer flasks can be used to inoculate larger cultures such as 4 l flasks or carboys. (from: Bourne, Hodgson and Whyte, 1989)

Starter culture management

Procedures for the maintenance of starter cultures (inocula) are almost identical to those described above. These cultures are specifically grown to provide inocula to start larger volume cultures needed to produce food. A line of starter cultures is originally set-up from the stock culture of the required species. Starter cultures, like the stocks, can be grown in 500 ml boiling flasks in 250 ml of culture medium. Because they are needed to provide inocula it is necessary to grow them quickly. They are grown at 18 to 23 °C with an illumination of 4 750 to 5 250 lux. Starter cultures are generally aerated with an air/carbon dioxide (CO₂) mixture.

Starter cultures are grown for variable periods of time prior to use. In the case of diatom species, which have short generation times, this period is from 3 to 5 days. For the majority of flagellates it is 7 to 14 days. When ready for use a starter culture is sub-cultured using sterile techniques, as previously described. Twenty to 50 ml, (depending on species and the density of the culture), is transferred to a fresh 250 ml culture – to maintain the starter culture line. The remainder is used as an inoculum for larger cultures (up to 25 l in volume) to be grown for feeding or as an intermediate step in the process of large-scale culture, where they in turn act as the inocula for much larger cultures. Larger volume starter cultures may be needed to inoculate large-volume production cultures. For clarity, cultures of between 2 and 25 l volume will be referred to as intermediate-scale cultures. As an example, a 200 l production culture will initially begin with a 250 ml starter of the required species which is then transferred when it has grown to a larger volume 2 to 4 l starter. When a 200 l culture is about to be started, 200 to 400 ml of the 2 to 4 l starter culture is used to start a new 2 or 4 l starter culture and the remainder to start the 200 l production culture.

With larger volume starters it is advantageous to increase the level of illumination and to aerate the culture with an air/carbon dioxide mixture. It is advisable to dilute the medium to grow diatom species to a salinity of 20 to 25 PSU (practical salinity units, equivalent to parts per thousand) to obtain the best possible growth rates. Most flagellate species are best grown at about 30 PSU.

Intermediate-scale culture

Most laboratories and hatcheries requiring small volumes of algae for food use spherical glass flasks, plastic buckets or glass or clear plastic carboys of up to 25 l volume. These are generally operated as batch culture systems or semi-continuously. Batch culture involves the inoculation of the culture medium with the required species. The culture is then grown rapidly until a further increase in cell density is inhibited by the failure of the light to adequately penetrate the culture, The culture is then completely harvested, the container washed and

sterilized and started again with a new culture. The semi-continuous method involves starting the cultures in the same way but instead of completely harvesting them when they have grown; they are partially harvested before the light limiting stage is reached. The harvested volume is then replaced with freshly prepared culture medium and the process repeated 2 or 3 days later. In this way the life of a culture is extended. With some of the hardier species, e.g. *Tetraselmis suecica*, cultures will last for 3 months or more with harvests of 25 to 50% of the culture volume 3 times each week. Batch culture is generally used for delicate species and the rapidly growing diatoms. Semi-continuous culture is mainly used with hardier species of flagellates.

Growth phases of cultures

Harvesting takes place in semi-continuous culture during the exponential phase of growth. Batch harvests are made generally at the peak of exponential growth as the cultures enter the stationary phase. In this case the species cultured is the large, green flagellate, *Tetraselmis*. At inoculation from the starter culture, the starting cell density in the culture is 25 to 50 cells per ml (cells per microlitre). After inoculation these cells grow and divide increasingly rapidly as they acclimatize to the culture conditions. This acclimatization period, which lasts for 2 to 3 days, is called the lag phase. Once adapted to the conditions, the rate of cell division accelerates and increase in the number of cells in the culture is logarithmic. This period lasts for 4 to 6 days and is called the exponential growth phase. Cell division rate then slows as light penetration through the culture and/or nutrients become limiting. The culture then enters the stationary phase, which can last for many days in the case of flagellates or only for a short time for diatoms. Cultures of flagellates remain in this phase by the recycling of nutrients from dead and decaying cells, but in the case of diatoms, which may produce self-inhibiting metabolites, which attract bacterial growth, the culture collapses.

Details of intermediate-scale culture operation

The complexity of the culture operation depends on the requirement for algae and the cost constraints within which the system needs to operate. In the simplest form the culture system may be just a scaled-up version of the starter cultures, using 2 l to 25 l flat-bottomed, glass flasks or carboys. These are part filled with the culture medium – in this case sterile, nutrient-enriched seawater – and then they are inoculated with the required species and aerated with a mixture of 2% CO₂ carried in compressed air. The carbon dioxide is from a bottled gas source with gas pressure and flow regulation. This is to provide the carbon source for photosynthesis and to control pH within the range 7.5 to 8.2. The air/CO₂ mixture is filtered through a 0.2 µm porosity cartridge or membrane filter to remove the majority of air-borne contaminants and competing microorganisms. The culture medium is prepared from filtered or sterilized seawater.

There are various options for culture water treatment:

- a) Either the seawater is filtered to remove bacteria using 0.22 or 0.45 µm membrane cartridge filters, or,
- b) It is batch or continuously pasteurized at 65 to 75°C or,
- c) It is autoclaved at 1.06 kg per cm² for 20 minutes (After autoclaving the medium must be allowed to stand for 2 days in a suitable container closed from the atmosphere). Or,
- d) It is chemically sterilized with sodium hypochlorite solution at 25 mg per l free-chlorine (by adding 0.5 ml of domestic bleach – 5% sodium hypochlorite – per l of filtered seawater).



- e) Before use, the residual free-chlorine is neutralized by adding an excess of sodium thiosulphate solution (50.0 mg per l) prepared in distilled water.

Note: Methods (a) and (c) are most commonly used for small-scale culture preparation; (b) and (d), after prior filtration to 1 or 2 μm particle size, for large-scale culture.

After the sterilizing treatment, nutrient additions are made. Note that diatoms require the addition of silica (Si) to the basic nutrients. The medium is then ready to dispense aseptically to the culture flasks, which are then ready to be inoculated. To obtain the maximum productivity of most species it may be necessary to dilute the seawater with pure (distilled) freshwater (or from an uncontaminated source) before filtration or autoclaving. Growth and cell division rates of *Chaetoceros calcitrans*, *Thalassiosira pseudonana* and *Skeletonema costatum* are optimal at a salinity of about 20 to 25 PSU. Productivity of many of the flagellates is optimal at 25 to 30 PSU.

Estimating algal density

Accurate estimates of cell density can be made using a haemocytometer.

Haemocytometers are thick glass slides with two chambers on the upper surface, each measuring 1.0 x 1.0 mm. A special cover slip is placed over these two chambers giving a depth of 0.1 mm making the total volume of each chamber 0.1 mm³. The base of each chamber is marked with a grid to aid in counting cells within the area. Prior to counting motile algal species, 1 or 2 drops of 4% formalin should be added to a 10 to 20 ml sample of the culture to be counted. With the cover slip in position, one or two drops of the algal sample are introduced by means of a Pasteur pipette to fill both chambers. Cell density is estimated as follows. The central grid of each chamber (outlined in the circle) is sub-divided into 25 squares, each measuring 0.2 x 0.2 mm. The numbers of cells in 10 randomly chosen 0.2 x 0.2 mm squares are counted and the average or mean is calculated. This gives the mean number of algal cells per 0.2mm x 0.2mm x 0.1mm, or 0.004 mm³.

Example:

- a. Counts of algal cells: 40 + 30 + 50 + 60 + 55 + 65 + 70 + 45 + 40 + 70 = 525

$$\text{Average} = 52.5 \text{ cells per } 0.004 \text{ mm}^3$$

- b. Multiply the average by 250 to give the average number of cells per mm³.
c. Since there are 1000 mm³ in 1 ml, multiply the value calculated in B by 1000.

In this example, the cell density would be $52.5 \times 250 \times 1000 = 13.1 \text{ m}$ (13.1×10^6) cells per ml.

Extensive outdoor culture

Commercial hatcheries need to produce large volumes of good quality, high-food-value algae daily to support economic-scale seed production. Outdoor tank culture makes use of natural light. Culture in rectangular or circular tanks with overhead illumination is used in shrimp hatcheries in India. This involves the fertilization of a large volume of seawater with the basic nutrients necessary for production, namely nitrogen, phosphorus and silica in one form or another. It is possible to induce monospecific blooms by prior fine (<2 μ particle retention) filtration of the impounded seawater and the introduction of an inoculum of the required species, as long as it is hardy and vigorous. However, it is difficult to maintain such blooms for long periods because they rapidly become contaminated with other microorganisms.

Principles of large-scale culture management

The objective in culture management is to obtain the greatest possible daily yield of algae so that the culture systems are operated cost effectively. This yield must be sustained for long periods of time to maintain the hatchery output of post larvae. Ineffective management of algal culture greatly influences the potential for production and ultimately the selling price of the seed.

Troubleshooting

Cultures will fail to grow, will become overly contaminated with competing micro-organisms or will crash even in the best-run hatcheries. Below are some pointers to check to determine the source of such failures.

1. Air supply: Is there adequate air entering the cultures? Are the cells sedimenting to the bottom of the culture vessel? This may happen when culturing certain diatoms, in which case the air flow rate should be increased. It should not happen in the case of commonly cultured flagellates. If it does, then the problem lies elsewhere.
2. Temperature: Check min/max thermometer. Were there any increases or decreases in the temperature of the algal culture facility over the past 24 hours? Most of the commonly cultured algal species cannot tolerate temperatures above 26°C for extended periods – or temperatures below 12 °C. Temperatures in the range 18 to 23 °C are ideal for indoor.
3. PH: Check CO₂ supply; Is the CO₂ cylinder empty? Check pH of the algal cultures using a pH probe. Is the pH too high (above 8.5)? Is the pH too low (below 7.5)? Adjust the CO₂ supply accordingly.
4. Nutrients: Check records for the last time the cultures received nutrients. This is particularly important for semi-continuous cultures.
5. Contamination. Are the walls of the culture container, particularly at the water/air interface, visibly foaming or fouled with what appears to be detritus? If so, the culture is at the end of its useful life and needs to be replaced. If this is a continuing problem in the early stages of the culture cycle with a particular species, then check the starter cultures for signs of contaminating organisms and replace them as necessary.