ISSN 0429-9345

Manual on the production and use of live food for aquaculture

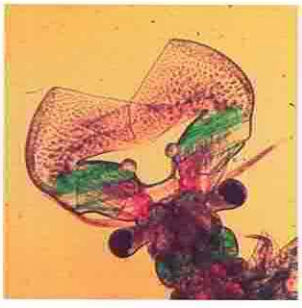
FAO
FISHERIES
TECHNICAL
PAPER

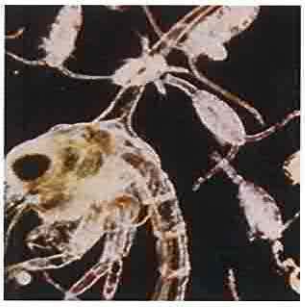
361











Food
and
Agriculture
Organization
of
the
United
Nations

Manual on the production and use of live food for aquaculture

FAO
FISHERIES
TECHNICAL
PAPER

361

Edited by
Patrick Lavens and Patrick Sorgeloos
Laboratory of Aquaculture and Artemia Reference Center
University of Ghent
Ghent, Belgium

Food
and
Agriculture
Organization
of
the
United
Nations

Rome, 1996

2.3.3. Isolating/obtaining and maintaining of cultures

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 culture, successive plating on agar media (See Worksheet 2.1), 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 sea water with 1 $g.l^{-1}$ bactopeptone. After sterilization, a drop of the culture to be tested is added and any residual bacteria will turn the bactopeptone solution turbid.

The collection of algal strains should be carefully protected against contamination during handling and poor temperature regulation. 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 (Fig. 2.4.).

2.3.4. Sources of contamination and water treatment

Contamination with bacteria, protozoa or another species of algae is a serious problem for monospecific/axenic cultures of micro-algae. The most common sources of contamination include the culture medium (sea water and nutrients), the air (from the air supply as well as the environment), the culture vessel, and the starter culture.

Seawater used for algal culture should be free of organisms that may compete with the unicellular algae, such as other species of phytoplankton, phytophagous zooplankton, or bacteria. Sterilization of the seawater by either physical (filtration, autoclaving, pasteurization, UV irradiation) or chemical methods (chlorination, acidification, ozonization) is therefore required. Autoclaving (15 to 45 min. at 120°C and 20 psi, depending on the volume) or pasteurization (80°C for 1-2 h) is mostly applied for sterilizing the culture medium in test tubes, erlenmeyers, and carboys. Volumes greater than 20 l are generally filtered at 1 µm and treated with acid (*e.g.* hydrochloric acid at pH 3, neutralization after 24 h with sodium carbonate) or chlorine (*e.g.* 1-2 $mg.l^{-1}$, incubation for 24 h without aeration, followed by aeration for 2-3 h to remove residual chlorine, addition of sodium thiosulfate to neutralize chlorine may be necessary if aeration fails to strip the chlorine). Water treatment is not required when using underground salt water obtained through bore holes. This water is generally free of living organisms and may contain sufficient mineral salts to support algal culture without further enrichment. In some cases well water contains high levels of ammonia and ferrous salts, the latter precipitating after oxidation in air.

Figure 2.4. Temperature controlled room for maintenance of algal stock cultures in a bivalve hatchery: stock cultures in test tubes (left) and inoculation hood (right).

A common source of contamination is the condensation in the airlines which harbor ciliates. For this reason, airlines should be kept dry and both the air and the carbon dioxide should be filtered through an in-line filter of 0.3 or 0.5 µm before entering the culture. For larger volumes of air, filter units can be constructed using cotton and activated charcoal (Fig.2.5.).

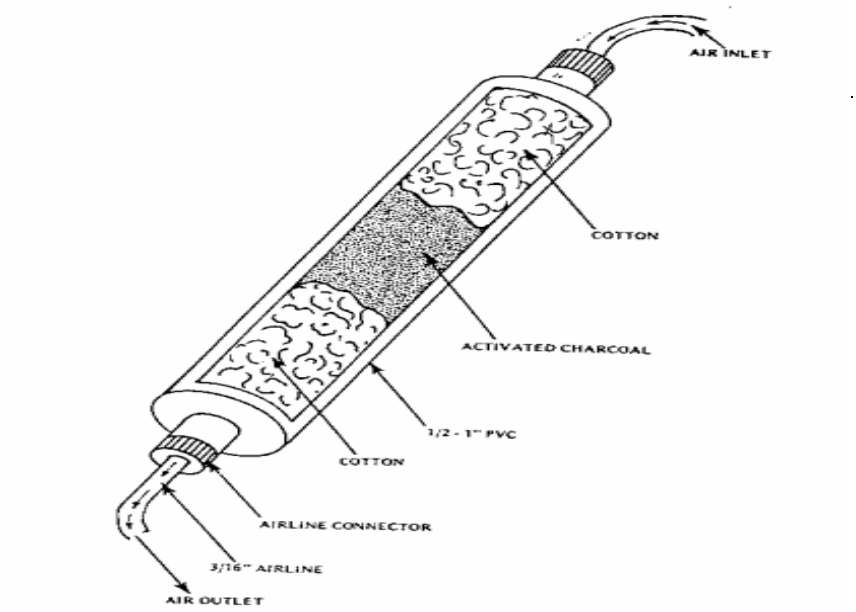


Figure 2.5. Aeration filter (Fox, 1983)

The preparation of the small culture vessels is a vital step in the upscaling of the algal cultures:

- wash with detergent
- rinse in hot water
- clean with 30% muriatic acid
- rinse again with hot water
- dry before use.

Alternatively, tubes, flasks and carboys can be sterilized by autoclaving and disposable culture vessels such as polyethylene bags can be used.

2.3.5. Algal culture techniques

Algae can be produced using a wide variety of methods, ranging from closely-controlled laboratory methods to less predictable methods in outdoor tanks. The terminology used to describe the type of algal culture include:

- **Indoor/Outdoor**. Indoor culture allows control over illumination, temperature, nutrient level, contamination with predators and competing algae, whereas outdoor algal systems make it very difficult to grow specific algal cultures for extended periods.

- **Open/Closed**. Open cultures such as uncovered ponds and tanks (indoors or outdoors) are more readily contaminated than closed culture vessels such as tubes, flasks, carboys, bags, etc.

- **Axenic (=sterile)/Xenic**. Axenic cultures are free of any foreign organisms such as bacteria and require a strict sterilization of all glassware, culture media and vessels to avoid contamination. The latter makes it impractical for commercial operations.

- **Batch, Continuous, and Semi-Continuous**. These are the three basic types of phytoplankton culture which will be described in the following sections.

Table 2.6. summarizes the major advantages and disadvantages of the various algal culture techniques.

Table 2.6. Advantages and disadvantages of various algal culture techniques (modified from Anonymous, 1991).

Culture type	Advantages	Disadvantages
Indoors	A high degree of control (predictable)	Expensive
Outdoors	Cheaper	Little control (less predictable)
Closed	Contamination less likely	Expensive
Open	Cheaper	Contamination more likely
Axenic	Predictable, less prone to crashes	Expensive, difficult
Non-axenic	Cheaper, less difficult	More prone to crashes
Continuous	Efficient, provides a consistent supply of high-quality cells, automation, highest rate of production over extended periods	Difficult, usually only possible to culture small quantities, complex, equipment expenses may be high
Semi-continuous	Easier, somewhat efficient	Sporadic quality, less reliable
Batch	Easiest, most reliable	Least efficient, quality may be inconsistent

2.3.5.1. Batch culture

The batch culture consists of a single inoculation of cells into a container of fertilized seawater followed by a growing period of several days and finally harvesting when the algal population reaches its maximum or near-maximum density. In practice, algae are transferred to larger culture volumes prior to reaching the stationary phase and the larger culture volumes are then brought to a maximum density and harvested. The following consecutive stages might be utilized: test tubes, 2 l flasks, 5 and 20 l carboys, 160 l cylinders, 500 l indoor tanks, 5,000 l to 25,000 l outdoor tanks (Figs.2.6., 2.7).

Table 2.7. Inoculation schedule for the continuous production of micro-algae using the batch technique. Every week a serial is initiated with 4 or 7 test tubes, depending on whether a new culture is required for harvesting every 2 days or daily.

Days	New culture available for harvest every 2 days				Harvest required daily						
1	t	t	t	t	t	t	t	t	t	t	t
2	t	t	t	t	t	t	t	t	t	t	t
3	t	t	t	t	t	t	t	t	t	t	t
4	t	t	t	t	t	t	t	t	t	t	t
5	t	t	t	t	t	t	t	t	t	t	t
6	t	t	t	t	t	t	t	t	t	t	t
7	t	t	t	t	t	t	t	t	t	t	t
8	e	e	e	e	e	e	e	e	e	e	e
9	e	e	e	e	e	e	e	e	e	e	e
10	e	e	e	e	e	e	e	e	e	e	e
11	e	e	e	e	e	e	e	e	e	e	e
12	E	e	e	e	E	e	e	e	e	e	e
13	E	e	e	e	E	E	e	e	e	e	e
14	E	E	e	e	E	E	E	e	e	e	e
15	E	E	e	e	E	E	E	E	e	e	e
16	f	E	E	e	f	E	E	E	E	e	e
17	f	E	E	e	f	f	E	E	E	E	e
18	f	f	E	E	f	f	f	E	E	E	E
19	f	f	E	E	f	f	f	f	E	E	E
20	F	f	f	E	F	f	f	f	f	E	E
21	F	f	f	E	F	F	f	f	f	f	E
22	F	F	f	f	F	F	F	f	f	f	f
23	F	F	f	f	F	F	F	F	f	f	f
24	L	F	F	f	L	F	F	F	F	f	f
25	L	F	F	f	L	L	F	F	F	F	f
26	*	L	F	F	*	L	L	F	F	F	F
27		L	F	F		*	L	L	F	F	F
28		*	L	F			*	L	L	F	F
29			L	F				*	L	L	F
30			*	L					*	L	L
31				L						*	L
32				*							*

t = 20 ml test tube
e = 250 ml erlenmeyer flask
E = 2 l erlenmeyer flask
f = 30 l fiberglass tank
F = 300 l fiberglass tank
L = use for larval feeding or to inoculate large volume (> 1.5 t) outdoor tanks
* = termination of 300 l fiberglass tank

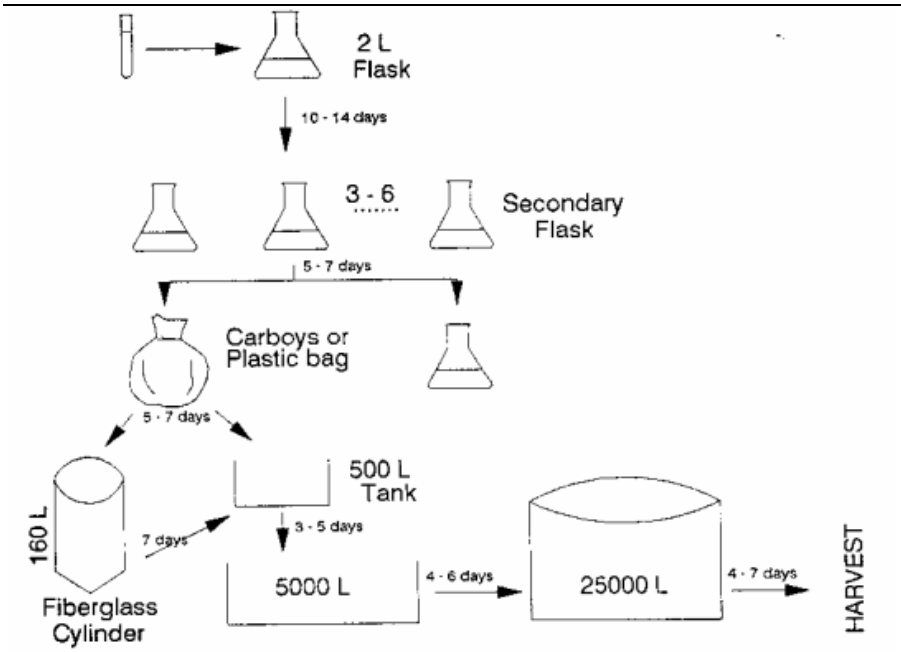


FIGURE 5. Progression of algal production at The Oceanic Institute.

Figure 2.6. Production scheme for batch culture of algae (Lee and Tamaru, 1993).

According to the algal concentration, the volume of the inoculum which generally corresponds with the volume of the preceding stage in the upscaling process, amounts to 2-10% of the final culture volume. An inoculation schedule for the continuous production according to the batch technique is presented in Table 2.7. Where small amounts of algae are required, one of the simplest types of indoor culture employs 10 to 20 l glass or plastic carboys (Fig. 2.8.), which may be kept on shelves backlit with fluorescent tubes (Fig. 2.9.).

Batch culture systems are widely applied because of their simplicity and flexibility, allowing to change species and to remedy defects in the system rapidly. Although often considered as the most reliable method, batch culture is not necessarily the most efficient method. Batch cultures are harvested just prior to the initiation of the stationary phase and must thus always be maintained for a substantial period of time past the maximum specific growth rate. Also, the quality of the harvested cells may be less predictable than that in continuous systems and for example vary with the timing of the harvest (time of the day, exact growth phase).

Another disadvantage is the need to prevent contamination during the initial inoculation and early growth period. Because the density of the desired phytoplankton is low and the concentration of nutrients is high, any contaminant with a faster growth rate is capable of outgrowing the culture. Batch cultures also require a lot of labour to harvest, clean, sterilize, refill, and inoculate the containers.

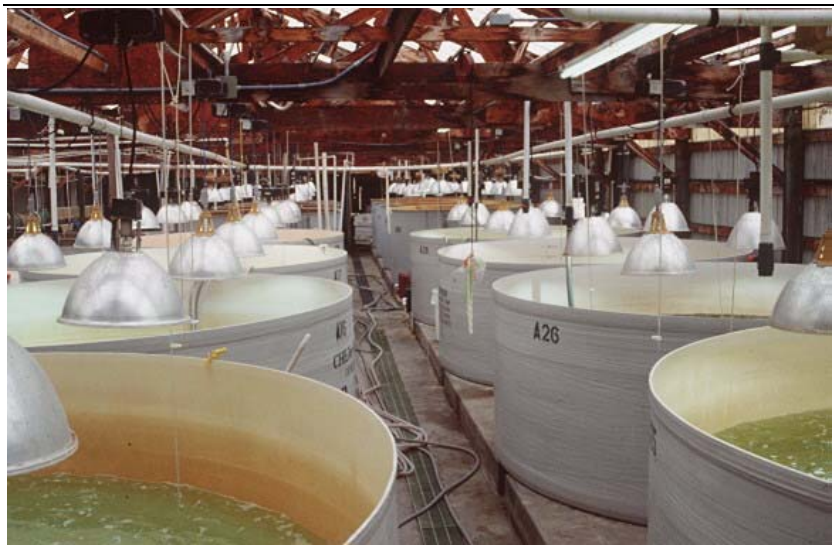


Figure 2.7.a. Batch culture systems for the mass production of micro-algae in 20,000 l tanks .

Figure 2.7.b. Batch culture systems for the mass production of micro-algae in 150 l cylinders.

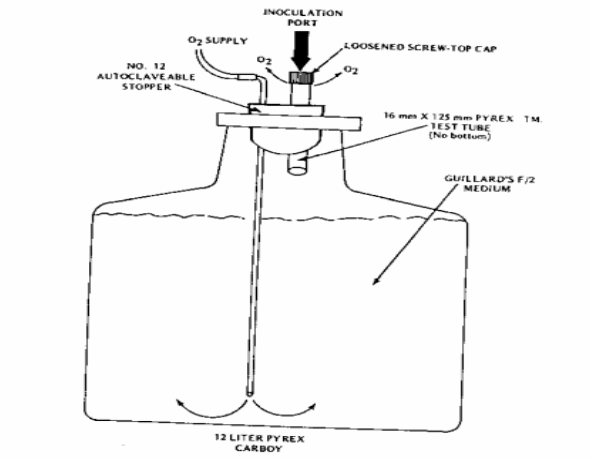


Figure 2.8. Carboy culture apparatus (Fox, 1983).

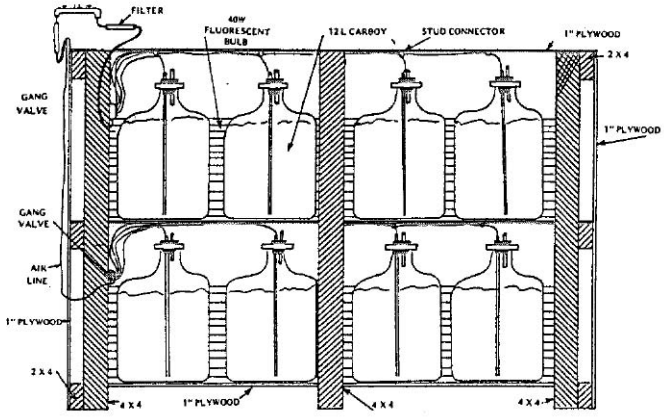


Figure 2.9. Carboy culture shelf (Fox, 1983).

2.3.5.2. Continuous culture

The continuous culture method, *i.e.* a culture in which a supply of fertilized seawater is continuously pumped into a growth chamber and the excess culture is simultaneously washed out, permits the maintenance of cultures very close to the maximum growth rate. Two categories of continuous cultures can be distinguished:

- turbidostat culture, in which the algal concentration is kept at a preset level by diluting the culture with fresh medium by means of an automatic system.

- chemostat culture, in which a flow of fresh medium is introduced into the culture at a steady, predetermined rate. The latter adds a limiting vital nutrient (*e.g.* nitrate) at a fixed rate and in this way the growth rate and not the cell density is kept constant.

Laing (1991) described the construction and operation of a 40 l continuous system suitable for the culture of flagellates, *e.g. Tetraselmis suecica* and *Isochrysis galbana* (Fig. 2.10.). The culture vessels consist of internally-illuminated polyethylene tubing supported by a metal framework (Fig. 2.11.). This turbidostat system produces 30-40 l per day at cell densities giving optimal yield for each flagellate species (Table 2.8.). A chemostat system that is relatively easy and cheap to construct is utilized by Seasalter Shellfish Co. Ltd, UK (Fig. 2.12.). The latter employ vertical 400 l capacity polyethylene bags supported by a frame to grow *Pavlova lutheri*, *Isochrysis galbana*, *Tetraselmis suecica*, *Phaeodactylum tricornutum*, *Dunaliella tertiolecta*, *Skeletonema costatum*. One drawback of the system is the large diameter of the bags (60 cm) which results in self-shading and hence relatively low algal densities.

The disadvantages of the continuous system are its relatively high cost and complexity. The requirements for constant illumination and temperature mostly restrict continuous systems to indoors and this is only feasible for relatively small production scales. However, continuous cultures have the advantage of producing algae of more predictable quality. Furthermore, they are amenable to technological control and automation, which in turn increases the reliability of the system and reduces the need for labor.

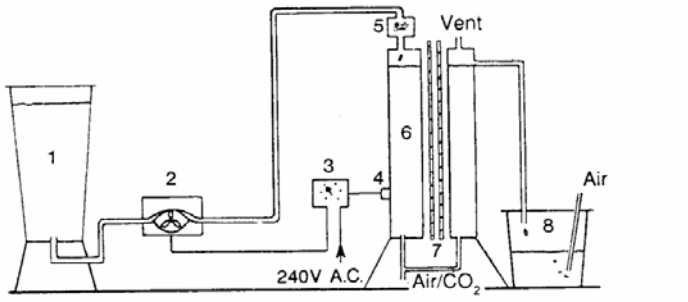


Figure 2.10. Diagram of a continuous culture apparatus (not drawn to scale): (1) enriched seawater medium reservoir (200 l); (2) peristaltic pump; (3) resistance sensing relay (50- 5000 ohm); (4) light-dependent resistor (ORP 12); (5) cartridge filter (0.45 μm); (6) culture vessel (40 l); (7) six 80 W fluorescent tubes (Laing, 1991).

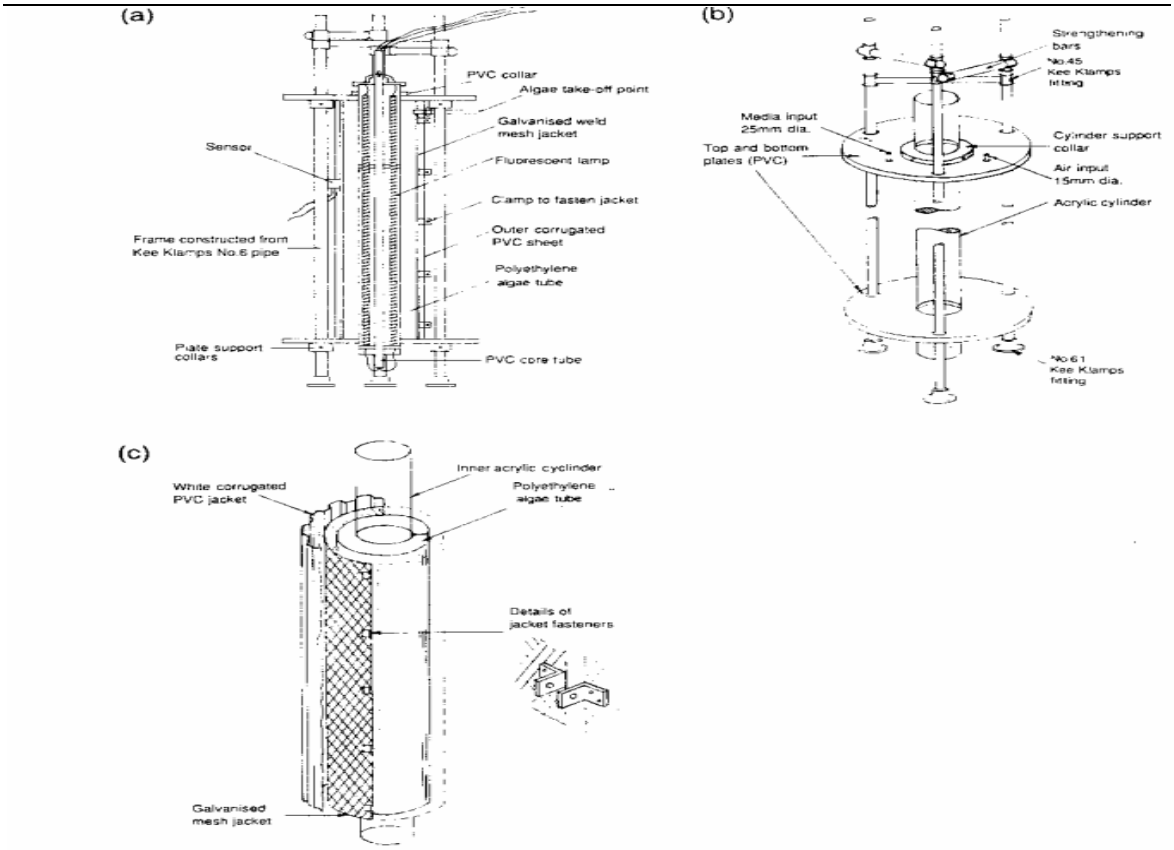


Figure 2.11. Schematic diagram of a 40 l continuous culture vessel (Laing, 1991).

Figure 2.12. Continuous culture of micro-algae in plastic bags. Detail (right) shows inflow of pasteurized fertilized seawater and outflow of culture.

Table 2.8. Continuous culture methods for various types of algae in 40 l internally-illuminated vessels (suitable for flagellates only) (modified from Laing, 1991),

Algae	Culture density for highest yield (cells per µl)	Usual life of culture (weeks)
Tetraselmis suecica	2 000	3-6
Chroomonas salina	3 000	2-3
Dunaliella tertiolecta	4 000	3-4
Isochrysis galbana *Monochrysis lutheri* *Pseudoisochrysis paradoxa*	20 000	2-3

2.3.5.3. Semi-continuous culture

The semi-continuous technique prolongs the use of large tank cultures by partial periodic harvesting followed immediately by topping up to the original volume and supplementing with nutrients to achieve the original level of enrichment. The culture is grown up again, partially harvested, etc. Semi-continuous cultures may be indoors or outdoors, but usually their duration is unpredictable. Competitors, predators and/or contaminants and metabolites eventually build up, rendering the culture unsuitable for further use. Since the culture is not harvested completely, the semi-continuous method yields more algae than the batch method for a given tank size.

2.3.6. Algal production in outdoor ponds

Large outdoor ponds either with a natural bottom or lined with cement, polyethylene or PVC sheets have been used successfully for algal production. The nutrient medium for outdoor cultures is based on that used indoors, but agricultural-grade fertilizers are used instead of laboratory-grade reagents (Table 2.5). However, fertilization of mass algal cultures in estuarine ponds and closed lagoons used for bivalve nurseries was not found to be desirable since fertilizers were expensive and it induced fluctuating algal blooms, consisting of production peaks followed by total algal crashes. By contrast, natural blooms are maintained at a reasonable cell density throughout the year and the ponds are flushed with oceanic water whenever necessary. Culture depths are typically 0.25-1 m. Cultures from indoor production may serve as inoculum for monospecific cultures. Alternatively, a phytoplankton bloom may be induced in seawater from which all zooplankton has been removed by sand filtration. Algal production in outdoor ponds is relatively inexpensive, but is only suitable for a few, fast-growing species due to problems with contamination by predators, parasites and "weed" species of algae. Furthermore, outdoor production is often characterized by a poor batch to batch consistency and unpredictable culture crashes caused by changes in weather, sunlight or water quality.

Mass algal cultures in outdoor ponds are commonly applied in Taiwanese shrimp hatcheries where *Skeletonema costatum* is produced successfully in rectangular outdoor concrete ponds of 10-40 tons of water volume and a water depth of 1.5-2 m.

2.3.7. Culture of sessile micro-algae

Farmers of abalone (*Haliotis* sp.) have developed special techniques to provide food for the juvenile stages which feed in nature by scraping coralline algae and slime off the surface of rocks using their radulae. In culture operations, sessile micro-algae are grown on plates of corrugated roofing plastic, which serve as a substrate for the settlement of abalone larvae. After metamorphosis, the spat graze on the micro-algae until they become large enough to feed on macro-algae. The most common species of micro-algae used on the feeder plates are pennate diatoms (*e.g. Nitzchia*, *Navicula*). The plates are inoculated by placing them in a current of sand filtered seawater. Depending on local conditions, the micro-algae cultures on the plates take between one and three weeks to grow to a density suitable for settling of the larvae. As the spat grow, their consumption rate increases and becomes greater than the natural production of the micro-algae. At this stage, the animals are too fragile to be transferred to another plate and algal growth may be enhanced by increasing illumination intensity and/or by the addition of fertilizer.

2.3.8. Quantifying algal biomass

There are several ways to evaluate the quantity of algal biomass present in cultures either by counting the number of cells or through determination of volume, optical density or weight.

Cells can be counted either with an electronic particle counter or directly under a microscope, using a haematocytometer. The Coulter® counter and similar instruments need appropriate calibration for each algal species to be counted. Detailed instructions on operation of electronic cell counting can be found in Sheldon and Parsons (1967). The presence of contaminating particles in the same size range as the algae and failure of cells to separate after cell division may be possible sources of erroneous counts. Counting with a microscope has the advantage of allowing control of the quality of the cultures. The major difficulty in microscopic counts is reproducibility, which is a function of the sampling, diluting, and filling of the counting chamber, as well as the choice of the right type of counting chamber and range of cell concentration. Counting chambers, recommended for various cell sizes and concentrations, are listed in Table 2.9. Worksheet 2.2. details on the operation of two types of counting chambers, namely Fuchs-Rosenthal and Bürker.

A relationship between optical density and cellular concentration can be established using a spectrometer. However, variations may occur due to the fact that the chlorophyll concentration in the algal cell varies according to the culture conditions and therefore affects this relationship. In this way, a culture under low lighting conditions will be comparatively more pigmented and will eventually result in higher readings for optical density.

Table 2.9. Cell counting chambers and their properties (modified from Vonshak, 1986).

Commercial name of chamber	Chamber vol (ml)	Depth (mm)	Objective used for mag-nification	Cell size (mm)	Cell conc counted easily
Redgwick Rafter	1.0	1.0	2.5-10	50-100	$30\text{-}10^4$
Palmer Malony	0.1	0.4	10-15	5-150	$10^2\text{-}10^5$
Speirs Levy	4.10^3	0.2	10-20	5-75	$10^4\text{-}10^7$
Improved Neau-bouer	2.10^4	0.1	20-40 (pha-se)	2-30	$10^4\text{-}10^7$
Petroff Houser	2.10^5	0.02	40-100	0.5-5	$10^4\text{-}10^8$

Cellular volume is measured by centrifuging samples and measuring the volume of the concentrated paste.

Measuring the dry weight of a culture is one of the most direct ways to estimate biomass production. For this, the cells of a representative sample of the culture are separated from the culture medium by either centrifugation or filtration on a glassfiber filter. The cells of marine algae are washed with isotonic ammonium formate (0.5 M) to remove salts without causing the cells to burst. Ammonium formate does not leave any residues as it decomposes to volatile compounds during the drying process (*e.g.* 2 h at 100°C). The results can be expressed as dry weight per volume or, when combined with a determination of the cell concentration, per algal cell (see Worksheet 2.3.).

For a particular algal species, dry weight per cell may vary greatly according to the strain and culture conditions. Published data on the dry weight content for species commonly used in mariculture are presented in Table 2.10. The density of harvested algal cultures generally ranges between 80 and 250 mg of dry weight per liter.

Table 2.10. Cellular dry weight reported in literature for algal species commonly used in mariculture.

Algal species	Dry weight (pg cell $^{-1}$)
Isochrysis galbana	8.0, 16.1, 20.1, 23.5, 30.5
Isochrysis sp. (clone, T-ISO)	14.1, 17.3, 29.7
Skeletonema costatum	52.2
Thalassiosira pseudonana	13.2, 17.8, 28.4
Chaetoceros neogracile (C. gracilis)	23.8, 30.6, 74.8
Tetraselmis suecica	66, 168, 194-244, 247, 292

2.3.9. Harvesting and preserving micro-algae

In most cases, it is unnecessary to separate micro-algae from the culture fluid. Excess and off-season production may, however, be concentrated and preserved. The various techniques employed to harvest micro-algae have been reviewed by Fox (1983) and Barnabé (1990). High-density algal cultures can be concentrated by either chemical flocculation or centrifugation. Products such as aluminum sulphate and ferric chloride cause cells to coagulate and precipitate to the bottom or float to the surface. Recovery of the algal biomass is then accomplished by, respectively, siphoning off the supernatant or skimming cells off the surface. Due to the increased particle size, coagulated algae are no longer suitable as food for filter-feeders. Centrifugation of large volumes of algal culture is usually performed using a cream separator; the flow rate being adjusted according to the algal species and the centrifugation rate of the separator. Cells are deposited on the walls of the centrifuge head as a thick algal paste, which is then resuspended in a limited volume of water. The resulting slurry may be stored for 1-2 weeks in the refrigerator or frozen. In the latter case, cryoprotective agents (glucose, dimethylsulfoxide) are added to maintain cell integrity during freezing. However, cell disruption and limited shelf-life remain the major disadvantages of long-term preserved algal biomass. Concentrated cultures of *Tetraselmis suecica* kept in darkness at 4°C maintain their viability, whereas the latter is completely lost upon freezing. Furthermore, cultures stored in hermetically sealed vials lose their viability more rapidly than those kept in cotton-plugged vials.

The success of any farming operation for fish and shellfish depends upon the availability of a ready supply of larvae or "seed" for on-growing to market size. The cultivation of fish and shellfish larvae under controlled hatchery conditions requires not only the development of specific culture techniques, but in most cases also the production and use of live food organisms as feed for the developing larvae. The present manual reviews and summarizes the latest developments concerning the production and use of the major live food organisms currently employed in larviculture worldwide. It describes the main production techniques as well as their application potential in terms of their nutritional and physical properties and feeding methods. The manual is divided into sections according to the major groups of live food organisms used in aquaculture, namely micro-algae, rotifers, *Artemia*, natural zooplankton, and copepods, nematodes and trochophores. The document has been prepared to help meet the needs of aquaculture workers of member countries for the synthesis of information in the field of aquaculture nutrition and feed development.

ISBN 92-5-103934-8 ISSN 0429-9345

9 789251 039342

M-44 W3732E/1/12.96/2000