2. MICRO-ALGAE

2.1. Introduction

Phytoplankton comprises the base of the food chain in the marine environment. Therefore, micro-algae are indispensable in the commercial rearing of various species of marine animals as a food source for all growth stages of bivalve molluscs, larval stages of some crustacean species, and very early growth stages of some fish species. 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 (Fig. 2.1.). Besides, for rearing marine fish larvae according to the "green water technique" algae are used directly in the larval tanks, where they are believed to play a role in stabilizing the water quality, nutrition of the larvae, and microbial control.

![Figure 2.1. The central role of micro-algae in mariculture (Brown et al., 1989).](image)

All algal species are not equally successful in supporting the growth and survival of a particular filter-feeding animal. Suitable algal species have been selected on the basis of their mass-culture potential, cell size, digestibility, and overall food value for the feeding animal. Various techniques have been developed to grow these food species on a large scale, ranging from less controlled extensive to monospecific intensive cultures. However, the controlled production of micro-algae is a complex and expensive procedure. A possible alternative to on-site algal culture is the collection of algae from the natural environment where, under certain conditions, they may be extremely abundant. Furthermore, in order to overcome or reduce the problems and limitations associated with algal cultures, various
investigators have attempted to replace algae using artificial diets either as a supplement or as the main food source. These various aspects of the production, use and substitution of micro-algae in aquaculture will be treated within the limits of this chapter.

2.2. Major classes and genera of cultured algal species

Today, more than 40 different species of micro-algae, isolated in different parts of the world, are cultured as pure strains in intensive systems. Table 2.1. lists the eight major classes and 32 genera of cultured algae currently used to feed different groups of commercially important aquatic organisms. The list includes species of diatoms, flagellated and chlorococcalean green algae, and filamentous blue-green algae, ranging in size from a few micrometer to more than 100 µm. 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*, *Monochrysis lutheri* and the chlorococcalean *Chlorella* spp. (Fig. 2.2.).

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Figure 2.2. Some types of marine algae used as food in aquaculture (a) *Tetraselmis* spp. (b) *Dunalieilla* spp. (c) *Chaetoceros* spp. (Laing, 1991).
Table 2.1. Major classes and genera of micro-algae cultured in aquaculture (modified from De Pauw and Persoone, 1988).

<table>
<thead>
<tr>
<th>Class</th>
<th>Genus</th>
<th>Examples of application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillariophyceae</td>
<td>Skeletonema</td>
<td>PL, BL, BP</td>
</tr>
<tr>
<td></td>
<td>Thalassiosira</td>
<td>PL, BL, BP</td>
</tr>
<tr>
<td></td>
<td>Phaeodactylum</td>
<td>PL, BL, BP, ML, BS</td>
</tr>
<tr>
<td></td>
<td>Chaetoceros</td>
<td>PL, BL, BP, BS</td>
</tr>
<tr>
<td></td>
<td>Cylindrotheca</td>
<td>PL</td>
</tr>
<tr>
<td></td>
<td>Bellerochea</td>
<td>BP</td>
</tr>
<tr>
<td></td>
<td>Actinocyclus</td>
<td>BP</td>
</tr>
<tr>
<td></td>
<td>Nitzchia</td>
<td>BS</td>
</tr>
<tr>
<td></td>
<td>Cyclotella</td>
<td>BS</td>
</tr>
<tr>
<td>Haptophyceae</td>
<td>Isochrysis</td>
<td>PL, BL, BP, ML, BS</td>
</tr>
<tr>
<td></td>
<td>Pseudoisochrysis</td>
<td>BL, BP, ML</td>
</tr>
<tr>
<td></td>
<td>dicrateria</td>
<td>BP</td>
</tr>
<tr>
<td>Chrysophyceae</td>
<td>Monochrysis (Pavlova)</td>
<td>BL, BP, BS, MR</td>
</tr>
<tr>
<td>Prasinophyceae</td>
<td>Tetraselmis (Platymonas)</td>
<td>PL, BL, BP, AL, BS, MR</td>
</tr>
<tr>
<td></td>
<td>Pyramimonas</td>
<td>BL, BP</td>
</tr>
<tr>
<td></td>
<td>Micromonas</td>
<td>BP</td>
</tr>
<tr>
<td>Cryptophyceae</td>
<td>Chroomonas</td>
<td>BP</td>
</tr>
<tr>
<td></td>
<td>Cryptomonas</td>
<td>BP</td>
</tr>
<tr>
<td></td>
<td>Rhodomonas</td>
<td>BL, BP</td>
</tr>
<tr>
<td>Cryptophyceae</td>
<td>Chlamydomonas</td>
<td>BL, BP, FZ, MR, BS</td>
</tr>
<tr>
<td></td>
<td>Chlorococcum</td>
<td>BP</td>
</tr>
<tr>
<td>Xanthophyceae</td>
<td>Olisthodiscus</td>
<td>BP</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>Carteria</td>
<td>BP</td>
</tr>
<tr>
<td></td>
<td>Dunaliella</td>
<td>BP, BS, MR</td>
</tr>
<tr>
<td>Cyanophyceae</td>
<td>Spirulina</td>
<td>PL, BP, BS, MR</td>
</tr>
</tbody>
</table>

PL, penaeid shrimp larvae; BL, bivalve mollusc larvae; ML, freshwater prawn larvae; BP, bivalve mollusc postlarvae; AL, abalone larvae; MR, marine rotifers (Brachionus); BS, brine shrimp (Artemia); SC, saltwater copepods; FZ, freshwater zooplankton
2.3.  Algal production

2.3.1.  Physical and chemical conditions

The most important parameters regulating algal growth are nutrient quantity and quality, light, pH, turbulence, salinity and temperature. The most optimal parameters as well as the tolerated ranges are species specific and a broad generalization for the most important parameters is given in Table 2.2. Also, the various factors may be interdependent and a parameter that is optimal for one set of conditions is not necessarily optimal for another.

2.3.1.1.  Culture medium/nutrients

Concentrations of cells in phytoplankton cultures are generally higher than those found in nature. Algal cultures must therefore be enriched with nutrients to make up for the deficiencies in the seawater. Macronutrients include nitrate, phosphate (in an approximate ratio of 6:1), and silicate.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Range</th>
<th>Optima</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>16-27</td>
<td>18-24</td>
</tr>
<tr>
<td>Salinity (g.l⁻¹)</td>
<td>12-40</td>
<td>20-24</td>
</tr>
<tr>
<td>Light intensity (lux)</td>
<td>1,000-10,000</td>
<td>2,500-5,000</td>
</tr>
<tr>
<td>(depends on volume and density)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photoperiod (light:dark, hours)</td>
<td>16:8 (minimum)</td>
<td>24:0 (maximum)</td>
</tr>
<tr>
<td>pH</td>
<td>7-9</td>
<td>8.2-8.7</td>
</tr>
</tbody>
</table>

Silicate is specifically used for the growth of diatoms which utilize this compound for production of an external shell. Micronutrients consist of various trace metals and the vitamins thiamin (B₁), cyanocobalamin (B₁₂) and sometimes biotin. Two enrichment media that have been used extensively and are suitable for the growth of most algae are the Walne medium (Table 2.3.) and the Guillard's F/2 medium (Table 2.4.). Various specific recipes for algal culture media are described by Vonshak (1986). Commercially available nutrient solutions may reduce preparation labour. The complexity and cost of the above culture media often excludes their use for large-scale culture operations. Alternative enrichment media that are suitable for mass production of micro-algae in large-scale extensive systems contain only the most essential nutrients and are composed of agriculture-grade rather than laboratory-grade fertilizers (Table 2.5.).
Table 2.3. Composition and preparation of Walne medium (modified from Laing, 1991).

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Quantities</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution A (at 1 ml per liter of culture)</strong></td>
<td></td>
</tr>
<tr>
<td>Ferric chloride (FeCl₃)</td>
<td>0.8 g(¹)</td>
</tr>
<tr>
<td>Manganous chloride (MnCl₂·4H₂O)</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Boric acid (H₃BO₃)</td>
<td>33.6 g</td>
</tr>
<tr>
<td>EDTA(²), di-sodium salt</td>
<td>45.0 g</td>
</tr>
<tr>
<td>Sodium di-hydrogen orthophosphate (NaH₂PO₄·2H₂O)</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Sodium nitrate (NaNO₃)</td>
<td>100.0 g</td>
</tr>
<tr>
<td>Solution B</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Make up to 1 litre with fresh water (c)</td>
<td>Heat to dissolve</td>
</tr>
<tr>
<td><strong>Solution B</strong></td>
<td></td>
</tr>
<tr>
<td>Zinc chloride (ZnCl₂)</td>
<td>2.1 g</td>
</tr>
<tr>
<td>Cobaltous chloride (CoCl₂·6H₂O)</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Ammonium molybdate (NH₄)₆Mo₇O₂₄·4H₂O)</td>
<td>0.9 g</td>
</tr>
<tr>
<td>Cupric sulphate (CuSO₄·5H₂O)</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Concentrated HCl</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Make up to 100 ml fresh water (c)</td>
<td>Heat to dissolve</td>
</tr>
<tr>
<td><strong>Solution C (at 0.1 ml per liter of culture)</strong></td>
<td></td>
</tr>
<tr>
<td>Vitamin B₁</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Solution E</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>Make up to 200 ml with fresh water (c)</td>
<td></td>
</tr>
<tr>
<td><strong>Solution D (for culture of diatoms-used in addition to solutions A and C, at 2 ml per liter of culture)</strong></td>
<td></td>
</tr>
<tr>
<td>Sodium metasilicate (Na₂SiO₃·5H₂O)</td>
<td>40.0 g</td>
</tr>
<tr>
<td>Make up to 1 litre with fresh water (c)</td>
<td>Shake to dissolve</td>
</tr>
<tr>
<td><strong>Solution E</strong></td>
<td></td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.1 g</td>
</tr>
<tr>
<td>make up to 250 ml with fresh water (c)</td>
<td></td>
</tr>
<tr>
<td><strong>Solution F (for culture of Chroomonas salina - used in addition to solutions A and C, at 1 ml per liter of culture)</strong></td>
<td></td>
</tr>
<tr>
<td>Sodium nitrate (NaNO₃)</td>
<td>200.0 g</td>
</tr>
<tr>
<td>make up to 1 litre with fresh water (c)</td>
<td></td>
</tr>
</tbody>
</table>

(¹) Use 2.0 g for culture of *Chaetoceros calcitrans* in filtered sea water; (²) Ethylene diamine tetra acetic acid; (c) Use distilled water if possible.
Table 2.4. Composition and preparation of Guillard's F/2 medium (modified from Smith et al., 1993a).

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Final concentration (mg/ℓ seawater)(^a)</th>
<th>Stock solution preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO(_3)</td>
<td>75</td>
<td><strong>Nitrate/Phosphate Solution</strong> Working Stock: add 75 g NaNO(_3) + 5 g NaH(_2)PO(_4) to 1 liter distilled water (DW)</td>
</tr>
<tr>
<td>NaH(_2)PO(_4).H(_2)O</td>
<td>5</td>
<td><strong>Silicate Solution</strong> Working Stock: add 30 g Na(_2)SiO(_3) to 1 liter DW</td>
</tr>
<tr>
<td>Na(_2)SiO(_3).9H(_2)O</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Na(<em>2)C(</em>{10})H(_14)O(_8)N(_2).H(_2)O (Na(_2)EDTA)</td>
<td>4.36</td>
<td><strong>Trace Metal/EDTA Solution</strong> Primary stocks: make 5 separate 1-liter stocks of (g/ℓ DW) 10.0 g CoCl(_2), 9.8 g CuSO(_4), 180 g MnCl(_2), 6.3 g Na(_2)MoO(_4), 22.0 g ZnSO(_4)</td>
</tr>
<tr>
<td>CoCl(_2).6H(_2)O</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CuSO(_4).5H(_2)O</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>FeCl(_3).6H(_2)O</td>
<td>3.15</td>
<td>Working stock: add 1 ml of each primary stock solution + 4.35 g Na(_2)C(_10)H(_14)O(_8)N(_2) + 3.15 g FeCl(_3) to 1 liter DW</td>
</tr>
<tr>
<td>MnCl(_2).4H(_2)O</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Na(_2)MoO(_4).2H(_2)O</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>ZnSO(_4).7H(_2)O</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.1</td>
<td><strong>Vitamin Solution</strong> Primary stock: add 20 g thiamin HCl + 0.1 g biotin + 0.1 g B(_{12}) to 1 liter DW</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>B(_{12})</td>
<td>0.0005</td>
<td>Working stock: add 5 ml primary stock to 1 liter DW</td>
</tr>
</tbody>
</table>
Table 2.5. Various combinations of fertilizers that can be used for mass culture of marine algae (modified from Palanisamy et al., 1991).

<table>
<thead>
<tr>
<th>Fertilizers</th>
<th>Concentration (mg.l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>150</td>
</tr>
<tr>
<td>Urea</td>
<td>7.5</td>
</tr>
<tr>
<td>Calcium superphosphate</td>
<td>25</td>
</tr>
<tr>
<td>Clewat 32</td>
<td>-</td>
</tr>
<tr>
<td>N:P 16/20 fertilizer</td>
<td>-</td>
</tr>
<tr>
<td>N:P:K 16-20-20</td>
<td>-</td>
</tr>
<tr>
<td>N:P:K 14-14-14</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3.1.2. Light

As with all plants, micro-algae photosynthesize, i.e. they assimilate inorganic carbon for conversion into organic matter. Light is the source of energy which drives this reaction and in this regard intensity, spectral quality and photoperiod need to be considered. Light intensity plays an important role, but the requirements vary greatly with the culture depth and the density of the algal culture: at higher depths and cell concentrations the light intensity must be increased to penetrate through the culture (e.g. 1,000 lux is suitable for erlenmeyer flasks, 5,000-10,000 is required for larger volumes). Light may be natural or supplied by fluorescent tubes. Too high light intensity (e.g. direct sun light, small container close to artificial light) may result in photo-inhibition. Also, overheating due to both natural and artificial illumination should be avoided. Fluorescent tubes emitting either in the blue or the red light spectrum should be preferred as these are the most active portions of the light spectrum for photosynthesis. The duration of artificial illumination should be minimum 18 h of light per day, although cultivated phytoplankton develop normally under constant illumination.

2.3.1.3. pH

The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2-8.7. Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH. The latter is accomplished by aerating the culture (see below). In the case of high-density algal culture, the addition of carbon dioxide allows to correct for increased pH, which may reach limiting values of up to pH 9 during algal growth.
2.3.1.4. Aeration/mixing

Mixing is necessary to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification (e.g. in outdoor cultures) and to improve gas exchange between the culture medium and the air. The latter is of primary importance as the air contains the carbon source for photosynthesis in the form of carbon dioxide. For very dense cultures, the CO₂ originating from the air (containing 0.03% CO₂) bubbled through the culture is limiting the algal growth and pure carbon dioxide may be supplemented to the air supply (e.g. at a rate of 1% of the volume of air). CO₂ addition furthermore buffers the water against pH changes as a result of the CO₂/HCO₃⁻ balance. Depending on the scale of the culture system, mixing is achieved by stirring daily by hand (test tubes, erlenmeyers), aerating (bags, tanks), or using paddle wheels and jetpumps (ponds). However, it should be noted that not all algal species can tolerate vigorous mixing.

2.3.1.5. Temperature

The optimal temperature for phytoplankton cultures is generally between 20 and 24°C, although this may vary with the composition of the culture medium, the species and strain cultured. Most commonly cultured species of micro-algae tolerate temperatures between 16 and 27°C. Temperatures lower than 16°C will slow down growth, whereas those higher than 35°C are lethal for a number of species. If necessary, algal cultures can be cooled by a flow of cold water over the surface of the culture vessel or by controlling the air temperature with refrigerated air-conditioning units.

2.3.1.6. Salinity

Marine phytoplankton are extremely tolerant to changes in salinity. Most species grow best at a salinity that is slightly lower than that of their native habitat, which is obtained by diluting sea water with tap water. Salinities of 20-24 g.l⁻¹ have been found to be optimal.

2.3.2. Growth dynamics

The growth of an axenic culture of micro-algae is characterized by five phases (Fig. 2.3.):

- lag or induction phase

This phase, during which little increase in cell density occurs, is relatively long when an algal culture is transferred from a plate to liquid culture. Cultures inoculated with exponentially growing algae have short lag phases, which can seriously reduce the time required for upscaling. The lag in growth is attributed to the physiological adaptation of the cell metabolism to growth, such as the increase of the levels of enzymes and metabolites involved in cell division and carbon fixation.
During the second phase, the cell density increases as a function of time $t$ according to a logarithmic function:

$$C_t = C_0 e^{mt}$$

with $C_t$ and $C_0$ being the cell concentrations at time $t$ and 0, respectively, and $m =$ specific growth rate. The specific growth rate is mainly dependent on algal species, light intensity and temperature.

Cell division slows down when nutrients, light, pH, carbon dioxide or other physical and chemical factors begin to limit growth.

In the fourth stage the limiting factor and the growth rate are balanced, which results in a relatively constant cell density.

During the final stage, water quality deteriorates and nutrients are depleted to a level incapable of sustaining growth. Cell density decreases rapidly and the culture eventually collapses.

In practice, culture crashes can be caused by a variety of reasons, including the depletion of a nutrient, oxygen deficiency, overheating, pH disturbance, or contamination. The key to the success of algal production is maintaining all cultures in the exponential phase of growth. Moreover, the nutritional value of the produced algae is inferior once the culture is beyond phase 3 due to reduced digestibility, deficient composition, and possible production of toxic metabolites.
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.
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.).
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).

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

#### 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.

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

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

<table>
<thead>
<tr>
<th>Algae</th>
<th>Culture density for highest yield (cells per µl)</th>
<th>Usual life of culture (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tetraselmis suecica</em></td>
<td>2 000</td>
<td>3-6</td>
</tr>
<tr>
<td><em>Chroomonas salina</em></td>
<td>3 000</td>
<td>2-3</td>
</tr>
<tr>
<td><em>Dunaliella tertiolecta</em></td>
<td>4 000</td>
<td>3-4</td>
</tr>
<tr>
<td><em>Isochrysis galbana</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monochrysis lutheri</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudoisochrysis paradoxa</em></td>
<td>20 000</td>
<td>2-3</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Commercial name of chamber</th>
<th>Chamber vol (ml)</th>
<th>Depth (mm)</th>
<th>Objective used for magnification</th>
<th>Cell size (mm)</th>
<th>Cell conc counted easily</th>
</tr>
</thead>
<tbody>
<tr>
<td>Redgwick Rafter</td>
<td>1.0</td>
<td>1.0</td>
<td>2.5-10</td>
<td>50-100</td>
<td>30-10^4</td>
</tr>
<tr>
<td>Palmer Malony</td>
<td>0.1</td>
<td>0.4</td>
<td>10-15</td>
<td>5-150</td>
<td>10^2-10^5</td>
</tr>
<tr>
<td>Speirs Levy</td>
<td>4.10^3</td>
<td>0.2</td>
<td>10-20</td>
<td>5-75</td>
<td>10^4-10^7</td>
</tr>
<tr>
<td>Improved Neaubouer</td>
<td>2.10^4</td>
<td>0.1</td>
<td>20-40 (phase)</td>
<td>2-30</td>
<td>10^4-10^7</td>
</tr>
<tr>
<td>Petroff Houser</td>
<td>2.10^5</td>
<td>0.02</td>
<td>40-100</td>
<td>0.5-5</td>
<td>10^4-10^8</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Algal species</th>
<th>Dry weight (pg cell⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Isochrysis galbana</em></td>
<td>8.0, 16.1, 20.1, 23.5, 30.5</td>
</tr>
<tr>
<td><em>Isochrysis</em> sp. (clone, T-ISO)</td>
<td>14.1, 17.3, 29.7</td>
</tr>
<tr>
<td><em>Skeletonema costatum</em></td>
<td>52.2</td>
</tr>
<tr>
<td><em>Thalassiosira pseudonana</em></td>
<td>13.2, 17.8, 28.4</td>
</tr>
<tr>
<td><em>Chaetoceros neogracle</em> (C. gracilis)</td>
<td>23.8, 30.6, 74.8</td>
</tr>
<tr>
<td><em>Tetraselmis suecica</em></td>
<td>66, 168, 194-244, 247, 292</td>
</tr>
</tbody>
</table>

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.
2.3.10. Algal production cost

Estimates of the algal production cost range from US$ 4 to 300 per kg dry biomass (Table 2.11.). Algal production in outdoor ponds is relatively cheap, but is only suitable for a few, fast-growing species and is characterized by a poor batch-to-batch consistency and unpredictable culture crashes due to contaminations and/or fluctuating climatological conditions. Indoor algal production offers a better control of the culture conditions and the algal species being grown, but is more expensive than outdoor culture due to space, energy, and skilled labour requirements.

An international survey among the operators of bivalve hatcheries showed that only facilities capable of producing mass quantities of specific micro-algae are able to attain production costs below US$ 100 per kg of dry weight (Fig. 2.13.).

<table>
<thead>
<tr>
<th>Production cost (US$.kg⁻¹ dry weight)</th>
<th>Remarks</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td><em>Tetraselmis suecica</em> 200 l batch culture</td>
<td>calculated from Helm <em>et al.</em> (1979)</td>
</tr>
<tr>
<td>167</td>
<td>various diatoms continuous flow cultures (240 m³)³</td>
<td>calculated from Walsh <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>4-20</td>
<td>outdoor culture indoor culture</td>
<td>De Pauw and Persoone (1988)</td>
</tr>
<tr>
<td>160-200</td>
<td>summer-winter production continuous flow cultures in bags (8 m³) and tanks (150 m³)³</td>
<td>Dravers (pers. comm. 1990)</td>
</tr>
<tr>
<td>23-115</td>
<td>tank culture (450 m³)³</td>
<td>Donaldson (1991)</td>
</tr>
<tr>
<td>50 - 400</td>
<td>international survey among bivalve hatchery operators in 1991</td>
<td>Coutteau and Sorgeloos (1992)</td>
</tr>
</tbody>
</table>

³ total volume available for algal production
Nutritional value of micro-algae

The nutritional value of any algal species for a particular organism depends on its cell size, digestibility, production of toxic compounds, and biochemical composition. The gross composition of 16 species of micro-algae is compared in Table 2.12. Although there are marked differences in the compositions of the micro-algal classes and species, protein is always the major organic constituent, followed usually by lipid and then by carbohydrate. Expressed as percentage of dry weight, the range for the level of protein, lipid, and carbohydrate are 12-35%, 7.2-23%, and 4.6-23%, respectively.

The content of highly unsaturated fatty acids (HUFA), in particular eicosapentaenoic acid (20:5n-3, EPA), arachidonic acid (20:4n-6, ARA), and docosahexaenoic acid (22:6n-3, DHA), is of major importance in the evaluation of the nutritional composition of an algal species to be used as food for marine organisms. The fatty acid composition of 10 species of micro-algae grown under defined conditions and harvested during the log phase is presented in Fig. 2.14. Significant concentrations of EPA are present in the diatom species (*Chaetoceros calcitrans*, *C. gracilis*, *S. costatum*, *T. pseudonana*) and the prymnesiophyte *Platymonas lutheri*, whereas high concentrations of DHA are found in the prymnesiophytes (*P. lutheri*, *Isochrysis* sp.) and *Chroomonas salina*.

Micro-algae can also be considered as a rich source of ascorbic acid (0.11-1.62% of dry weight, Fig. 2.15.).

The nutritional value of micro-algae can vary considerably according to the culture conditions. For example the effect of the composition of the culture medium on the proximate composition of various species of micro-algae is demonstrated in Table 2.13.

---

2.4. Nutritional value of micro-algae

The nutritional value of any algal species for a particular organism depends on its cell size, digestibility, production of toxic compounds, and biochemical composition. The gross composition of 16 species of micro-algae is compared in Table 2.12. Although there are marked differences in the compositions of the micro-algal classes and species, protein is always the major organic constituent, followed usually by lipid and then by carbohydrate. Expressed as percentage of dry weight, the range for the level of protein, lipid, and carbohydrate are 12-35%, 7.2-23%, and 4.6-23%, respectively.

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The nutritional value of micro-algae can vary considerably according to the culture conditions. For example the effect of the composition of the culture medium on the proximate composition of various species of micro-algae is demonstrated in Table 2.13.
Table 2.12. Concentrations of chlorophyl a, protein, carbohydrate and lipid in 16 species of micro-algae commonly used in aquaculture (modified from Brown, 1991).

<table>
<thead>
<tr>
<th>Algal class</th>
<th>Species</th>
<th>Dry weight (pg.cell⁻¹)</th>
<th>Chl a</th>
<th>Protein</th>
<th>Carbo-hydrate</th>
<th>Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillariophyceae</td>
<td>Chaetoceros calcitrans</td>
<td>11.3</td>
<td>0.34</td>
<td>3.8</td>
<td>0.68</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Chaetoceros gracilis</td>
<td>74.8</td>
<td>0.78</td>
<td>9.0</td>
<td>2.0</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>Nitzchia closterium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Phaeodactylum tricornutum</td>
<td>76.7</td>
<td>0.41</td>
<td>23.0</td>
<td>6.4</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>Skeletonema costatum</td>
<td>52.2</td>
<td>0.63</td>
<td>13.1</td>
<td>2.4</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Thalassiosira pseudonana</td>
<td>28.4</td>
<td>0.27</td>
<td>9.7</td>
<td>2.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>Dunaliella tertiolecta</td>
<td>99.9</td>
<td>1.73</td>
<td>20.0</td>
<td>12.2</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>Nannochloris atomus</td>
<td>21.4</td>
<td>0.080</td>
<td>6.4</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Cryptophyceae</td>
<td>Chroomonas salina</td>
<td>122.5</td>
<td>0.98</td>
<td>35.5</td>
<td>11.0</td>
<td>14.5</td>
</tr>
<tr>
<td>Eustigmatophyceae</td>
<td>Nannochloropsis oculata</td>
<td>6.1</td>
<td>0.054</td>
<td>2.1</td>
<td>0.48</td>
<td>1.1</td>
</tr>
<tr>
<td>Prasinophyceae</td>
<td>Tetraselmis chui</td>
<td>269.0</td>
<td>3.83</td>
<td>83.4</td>
<td>32.5</td>
<td>45.7</td>
</tr>
<tr>
<td></td>
<td>Tetraselmis suecica</td>
<td>168.2</td>
<td>1.63</td>
<td>52.1</td>
<td>20.2</td>
<td>16.8</td>
</tr>
<tr>
<td>Prymnesiophyceae</td>
<td>Isochrysis galbana</td>
<td>30.5</td>
<td>0.30</td>
<td>8.8</td>
<td>3.9</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Isochrysis aff. Galbana (T-iso)</td>
<td>29.7</td>
<td>0.29</td>
<td>6.8</td>
<td>1.8</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>Pavlova lutheri</td>
<td>102.3</td>
<td>0.86</td>
<td>29.7</td>
<td>9.1</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>Pavlova salina</td>
<td>93.1</td>
<td>0.34</td>
<td>24.2</td>
<td>6.9</td>
<td>11.2</td>
</tr>
</tbody>
</table>

Percentage of dry weight

<table>
<thead>
<tr>
<th>Algal class</th>
<th>Species</th>
<th>Weight of constituent (pg.cell⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillariophyceae</td>
<td>Chaetoceros calcitrans</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>Chaetoceros gracilis</td>
<td>74.8</td>
</tr>
<tr>
<td></td>
<td>Nitzchia closterium</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Phaeodactylum tricornutum</td>
<td>76.7</td>
</tr>
<tr>
<td></td>
<td>Skeletonema costatum</td>
<td>52.2</td>
</tr>
<tr>
<td></td>
<td>Thalassiosira pseudonana</td>
<td>28.4</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>Dunaliella tertiolecta</td>
<td>99.9</td>
</tr>
<tr>
<td></td>
<td>Nannochloris atomus</td>
<td>21.4</td>
</tr>
<tr>
<td>Cryptophyceae</td>
<td>Chroomonas salina</td>
<td>122.5</td>
</tr>
<tr>
<td>Eustigmatophyceae</td>
<td>Nannochloropsis oculata</td>
<td>6.1</td>
</tr>
</tbody>
</table>
Table 2.12.(contd.) Concentrations of chlorophyll a, protein, carbohydrate and lipid in 16 species of micro-algae commonly used in aquaculture (modified from Brown, 1991).

<table>
<thead>
<tr>
<th>Algal class</th>
<th>Species</th>
<th>Dry weight (pg.cell⁻¹)</th>
<th>Chl a</th>
<th>Protein</th>
<th>Carbohydrate</th>
<th>Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prasinophyceae</td>
<td><em>Tetraselmis chui</em></td>
<td>269.0</td>
<td>1.42</td>
<td>31</td>
<td>12.1</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td><em>Tetraselmis suecica</em></td>
<td>168.2</td>
<td>0.97</td>
<td>31</td>
<td>12.0</td>
<td>10</td>
</tr>
<tr>
<td>Prymnesio phyceae</td>
<td><em>Isochrysis galbana</em></td>
<td>30.5</td>
<td>0.98</td>
<td>29</td>
<td>12.9</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td><em>Isochrysis aff. Galbana</em> (T-iso)</td>
<td>29.7</td>
<td>0.98</td>
<td>23</td>
<td>6.0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td><em>Pavlova lutheri</em></td>
<td>102.3</td>
<td>0.84</td>
<td>29</td>
<td>9.0</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td><em>Pavlova salina</em></td>
<td>93.1</td>
<td>0.98</td>
<td>26</td>
<td>7.4</td>
<td>12</td>
</tr>
</tbody>
</table>

The protein content per cell, which is considered as one of the most important factors determining the nutritional value of micro-algae as feed in aquaculture, was found to be more susceptible to medium-induced variation than the other cellular constituents.

Moreover, the growth of animals fed a mixture of several algal species is often superior to that obtained when feeding only one algal species. A particular alga may lack a nutrient, while another alga may contain that nutrient and lack a different one. In this way, a mixture of both algal species supplies the animals with an adequate amount of both nutrients. An extensive review of the nutritional aspects of micro-algae used in mariculture of bivalve molluscs, crustaceans, and fish is presented in Brown *et al.* (1989).
Table 2.13. Cellular density (10^6 cells.ml⁻¹) and proximate composition (pg.cell⁻¹) of four marine micro-algae grown in different culture media (Algal-1 is a commercial nutrient) (modified from Herrero et al., 1991)

<table>
<thead>
<tr>
<th></th>
<th>Cellular density</th>
<th>Protein</th>
<th>Carbohydrates</th>
<th>Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T. suecica</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walne</td>
<td>2.29</td>
<td>13.31</td>
<td>6.20</td>
<td>7.04</td>
</tr>
<tr>
<td>ES</td>
<td>2.58</td>
<td>16.98</td>
<td>6.93</td>
<td>7.22</td>
</tr>
<tr>
<td>F/2</td>
<td>2.38</td>
<td>21.75</td>
<td>8.37</td>
<td>7.92</td>
</tr>
<tr>
<td>Algal-1</td>
<td>4.11</td>
<td>32.22</td>
<td>8.83</td>
<td>8.65</td>
</tr>
<tr>
<td><strong>D. tertiolecta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walne</td>
<td>4.04</td>
<td>13.37</td>
<td>13.22</td>
<td>22.28</td>
</tr>
<tr>
<td>ES</td>
<td>4.24</td>
<td>14.88</td>
<td>15.73</td>
<td>23.94</td>
</tr>
<tr>
<td>F/2</td>
<td>4.97</td>
<td>13.26</td>
<td>17.91</td>
<td>23.67</td>
</tr>
<tr>
<td>Algal-1</td>
<td>8.45</td>
<td>18.82</td>
<td>11.08</td>
<td>18.18</td>
</tr>
<tr>
<td><strong>I. galbana</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walne</td>
<td>10.11</td>
<td>5.17</td>
<td>4.28</td>
<td>25.95</td>
</tr>
<tr>
<td>ES</td>
<td>12.09</td>
<td>7.23</td>
<td>5.21</td>
<td>28.38</td>
</tr>
<tr>
<td>F/2</td>
<td>10.81</td>
<td>8.13</td>
<td>5.59</td>
<td>26.82</td>
</tr>
<tr>
<td>Algal-1</td>
<td>16.15</td>
<td>9.57</td>
<td>4.28</td>
<td>20.68</td>
</tr>
<tr>
<td><strong>P. tricornutum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walne</td>
<td>19.01</td>
<td>2.65</td>
<td>6.42</td>
<td>6.51</td>
</tr>
<tr>
<td>ES</td>
<td>16.23</td>
<td>5.21</td>
<td>9.20</td>
<td>6.45</td>
</tr>
<tr>
<td>F/2</td>
<td>24.65</td>
<td>3.34</td>
<td>6.90</td>
<td>5.52</td>
</tr>
<tr>
<td>Algal-1</td>
<td>39.04</td>
<td>4.20</td>
<td>5.98</td>
<td>5.79</td>
</tr>
</tbody>
</table>
Figure 2.14. Fatty acid composition of 10 species of micro-algae. Relative amounts of (a) C16- and C18-polyunsaturated fatty acids (PUFA); (b) 20:5n-3 and 22:6n-3; (c) (n-3) and (n-6) PUFA. Species abbreviations are: C. CAL: Chaetoceros calcitrans; C.GRA: C. gracilis; SKEL: Skeletonema costatum; THAL: Thalassiosira pseudonana; ISO: Isochrysis sp. (Tahitian); PAV: Pavlova lutheri; DUN: Dunaliella tertiolecta; NAN: Nannochloris atomus; TET: Tetraselmis suecica; CHRO: Chroomonas salina (Volkman et al., 1989).
2.5. Use of micro-algae in aquaculture

Micro-algae are an essential food source in the rearing of all stages of marine bivalve molluscs (clams, oysters, scallops), the larval stages of some marine gastropods (abalone, conch), larvae of several marine fish species and penaeid shrimp, and zooplankton.

2.5.1. Bivalve molluscs

Intensive rearing of bivalves has so far relied on the production of live algae, which comprises on average 30% of the operating costs in a bivalve hatchery. The relative algal requirements of the various stages of the bivalve culture process depend on whether the operation aims at the mass-production of larvae for remote setting or growing millions of seed till planting size. In either case, the juveniles, representing the largest biomass in the hatchery and demanding the highest weight-specific rations, consume the largest volumes of algal culture. The algal species that were reported in an international survey among hatchery operators in 1991 are listed in Table 2.14. Eight algal species (Isochrysis sp., clone T-Iso; C. gracilis; C. calcitrans; T. suecica; T. pseudonana, clone 3H; P. lutheri; I. galbana; S. costatum) were widely used and represented over 90% of the volume of algal culture produced in 23 facilities.

Figure 2.15. Ascorbic acid in microalgae harvested from logarithmic (grey filling) and stationary phase (black filling) cultures, expressed as (a) cellular levels (fg.cell⁻¹), (b) % dry weight, (c) concentrations (fg. µm⁻³) (Brown and Miller, 1992).
Table 2.14. Algal species used in hatchery and nursery rearing of bivalve molluscs as reported in an international questionnaire. Species are ranked according to decreasing frequency of use (Coutteau and Sorgeloos, 1992).

<table>
<thead>
<tr>
<th>Algal species</th>
<th>frequency of use†</th>
<th>total daily production n‡</th>
<th>volume (m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isochrysis sp., clone T-Iso</td>
<td>31</td>
<td>18</td>
<td>23.8</td>
</tr>
<tr>
<td>Chaetoceros gracilis</td>
<td>23</td>
<td>11</td>
<td>14.1</td>
</tr>
<tr>
<td>Chaetoceros calcitrans</td>
<td>16</td>
<td>10</td>
<td>6.0</td>
</tr>
<tr>
<td>Tetraselmis suecica</td>
<td>15</td>
<td>10</td>
<td>39.1</td>
</tr>
<tr>
<td>Thalassiosira pseudonana, clone 3H</td>
<td>14</td>
<td>9</td>
<td>112.0</td>
</tr>
<tr>
<td>Pavlova lutheri</td>
<td>11</td>
<td>7</td>
<td>11.7</td>
</tr>
<tr>
<td>Isochrysis galbana</td>
<td>8</td>
<td>5</td>
<td>9.1</td>
</tr>
<tr>
<td>Skeletonema costatum</td>
<td>6</td>
<td>3</td>
<td>58.8</td>
</tr>
<tr>
<td>Chroomonas salina</td>
<td>5</td>
<td>3</td>
<td>0.76</td>
</tr>
<tr>
<td>Dunaliella tertiolecta</td>
<td>4</td>
<td>2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Figure 2.16. Requirements for cultured algae in hatchery and nursery culture of bivalve molluscs (Utting and Spencer, 1991).
Table 2.14. (contd.) Algal species used in hatchery and nursery rearing of bivalve molluscs as reported in an international questionnaire. Species are ranked according to decreasing frequency of use (Coutteau and Sorgeloos, 1992)

<table>
<thead>
<tr>
<th>Algal Species</th>
<th>Number of Hatcheries</th>
<th>Number of Forms</th>
<th>Daily Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chaetoceros simplex</td>
<td>3</td>
<td>3</td>
<td>1.76</td>
</tr>
<tr>
<td>Chaetoceros muelleri</td>
<td>3</td>
<td>2</td>
<td>5.0</td>
</tr>
<tr>
<td>Nannochloropsis sp.</td>
<td>3</td>
<td>2</td>
<td>0.20</td>
</tr>
<tr>
<td>Cyclotella sp.</td>
<td>2</td>
<td>1</td>
<td>0.36</td>
</tr>
<tr>
<td>Phaeodactylum tricomutum</td>
<td>2</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td>Tetraselmis chui</td>
<td>2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Pavlova salina</td>
<td>1</td>
<td>1</td>
<td>3.18</td>
</tr>
<tr>
<td>Dicruteria sp.</td>
<td>1</td>
<td>1</td>
<td>4.07</td>
</tr>
<tr>
<td>Tetraselmis levis</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Dunaliella perva</td>
<td>1</td>
<td>1</td>
<td>0.012</td>
</tr>
<tr>
<td>Thalassiosira weissflogii</td>
<td>1</td>
<td>1</td>
<td>0.12</td>
</tr>
<tr>
<td>Chlamydomonas sp.</td>
<td>1</td>
<td>1</td>
<td>0.52</td>
</tr>
<tr>
<td>Chlorella sp.</td>
<td>1</td>
<td>1</td>
<td>0.36</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>43</strong></td>
<td><strong>23</strong></td>
<td><strong>295</strong></td>
</tr>
</tbody>
</table>

†: number of hatcheries growing each algal species (from 43 completed forms)
‡: number of hatcheries providing data which allowed to calculate daily production per algal species (from 23 completed forms)

The larvae of most bivalve species have similar food preferences; suitable algal species including *C. calcitrans*, *T. pseudonana* (3H), *I. galbana*, and *T. suecica* (for larvae > 120 µm in length). Combinations of flagellates and diatoms provide a well balanced diet which will generally accelerate the rate of larval development to metamorphosis in comparison with unialgal diets. The quantity fed depends upon the larval density, but suitable cell concentrations (expressed as cells.µl⁻¹) are given by each of the following combinations:

- *I. galbana*; 50
- *C. calcitrans*; 250
- *I. galbana/C. calcitrans*; 25/125
- *I. galbana/C. calcitrans/T. suecica*; 33/83/3.3 (larvae > 120µm)
Because of the high cost of cultured algae, bivalve hatcheries prefer to move juveniles to outdoor nursery systems at a maximum size of 1-2 mm length. In this way, the duration of the juvenile phase in closely controlled hatchery conditions is relatively short for oysters at about 20 days but much longer for the slower growing clams at up to 60 days. Bivalve food rations are preferentially expressed as daily weight-specific rations, such as number of cells or percent dry weight of algae per live weight of bivalves. Seed growth is largely influenced by food ration and the optimal ration for maximum growth depends upon the species and culture conditions of the algae making up the diet, and the bivalve culture conditions. Under practical hatchery conditions, high food rations are often fed, which may be as high as 5-6% dry weight of algae per live weight of spat per day.

2.5.2. Penaeid shrimp

A typical algal feeding regime for penaeid larvae is given in Table 2.15. Algae are added during the non-feeding nauplius stage so that algae are available immediately upon molting into the protozoea stage. Algal species most often used are *Tetraselmis chui*, *Chaetoceros gracilis*, and *Skeletonema costatum*. As feeding preference changes from primarily herbivorous to carnivorous during the mysis stages, the quantity of algae is reduced. Nevertheless, a background level of algae is maintained as this may stabilize water quality. The "same-tank method", in which the algae are cultured in the same water as that of the larvae using sunlight and fertilizers, was originally developed in Japan for culturing larval *Penaeus japonicus* and is extensively described by Liao et al. (1993).

<table>
<thead>
<tr>
<th>Substage</th>
<th>Chaetoceros neogracile (C. gracilis)</th>
<th>Tetraselmis chuii</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₅ or N₆</td>
<td>60,000</td>
<td>0-15,000</td>
</tr>
<tr>
<td>P₁</td>
<td>100,000-120,000</td>
<td>30,000</td>
</tr>
<tr>
<td>P₂</td>
<td>120,000</td>
<td>35,000</td>
</tr>
<tr>
<td>P₃</td>
<td>120,000</td>
<td>35,000</td>
</tr>
<tr>
<td>M₁</td>
<td>100,000</td>
<td>30,000</td>
</tr>
<tr>
<td>M₂</td>
<td>75,000</td>
<td>20,000</td>
</tr>
<tr>
<td>M₃</td>
<td>50,000-75,000</td>
<td>20,000</td>
</tr>
<tr>
<td>PL₁ to PL₆</td>
<td>20,000-75,000</td>
<td>5,000-20,000</td>
</tr>
</tbody>
</table>

2.5.3. Marine fish

Apart from the requirement for micro-algae for culturing and/or enriching live prey organisms such as *Artemia* and rotifers (see Chapters 3. and 4.3.), algae are often used directly in the tanks for rearing marine fish larvae. This "green water technique" is part of the commonly applied techniques for rearing larvae of gilthead seabream *Sparus aurata* (50,000 cells ml⁻¹ of *Isochrysis* sp. + 400,000 cells.ml⁻¹ of *Chlorella* sp. per day), milkfish *Chanos chanos* (between 500 and 3,500 *Chlorella* cells.ml⁻¹ are added from hatching till day 21), Mahimahi *Coryphaena hippurus* (200,000 cells.ml⁻¹ of either *Chaetoceros gracilis*, *Tetraselmis chui*, or...
Chlorella sp.), halibut Hippoglossus hippoglossus (Tetraselmis sp.), and turbot Scophthalmus maximus (60,000 cells.ml⁻¹ of Tetraselmis sp. or 130,000 cells.ml⁻¹ of I. galbana).

The effects of the presence of micro-algae in the larval rearing tank are still not fully understood and include:

- stabilizing the water quality in static rearing systems (remove metabolic by-products, produce oxygen);
- a direct food source through active uptake by the larvae with the polysaccharides present in the algal cell walls possibly stimulating the non-specific immune system in the larvae;
- an indirect source of nutrients for fish larvae through the live feed (i.e. by maintaining the nutritional value of the live prey organisms in the tank);
- increasing feeding incidence by enhancing visual contrast and light dispersion, and
- microbial control by algal exudates in tank water and/or larval gut.

2.6. Replacement diets for live algae

The high costs associated with algal production, the risks for contamination, and temporal variations in the algal food value still pose problems for any aquaculture operation depending on the mass-cultures of unicellular algae. In order to overcome or reduce the problems and limitations associated with algal cultures, various investigators have attempted to replace algae by using artificial diets either as a supplement or as the main food source. Different approaches are being applied to reduce the need for on-site algal production, including the use of preserved algae, micro-encapsulated diets, and yeast-based feeds.

To date, the requirement for live algae in the mass-production of prey-organisms has been largely reduced. In this way, baker's yeast, marine yeasts and lipid-enriched yeast diets are now routinely used as a sole diet or in combination with the alga Chlorella for rearing the rotifer B. plicatilis (see Chapter 3). In addition, considerable progress has been made in the replacement of live algae in the larval rearing of commercially important shrimp species. Partial replacement of live algae using micro-encapsulated and yeast-based diets is now routine in hatcheries for penaeid shrimp. Complete substitution of live algae by a commercial micro-encapsulated diet has been accomplished recently for the production of various penaeid species using seawater filtered to 5 µm, eliminating the algae but not the bacteria, which apparently contribute important micronutrients (and possibly immunostimulants). In marine fish hatcheries, the tendency is to apply a "clear water technique" instead of a "green water technique". However, the omission of algae in the larval tanks, which requires optimization of feeding strategies and zootechnical aspects, still often results in less predictable culture performance. Despite extensive research efforts, the use of artificial diets in the culture of bivalve molluscs is still very limited. The advantages and disadvantages of each of the three classes of replacement diets for live algae are briefly discussed below.
2.6.1. Preserved algae

A possible alternative to on-site algal culture could be the distribution of preserved algae that are produced at relatively low cost in a large facility under optimal climatological conditions and using the most cost-effective production systems. Centrifugation of algae into a paste form and subsequent refrigeration until required is widely applied in North America by oyster hatcheries using remote setting techniques. However, the limited shelf-life and/or the high prices of the presently available algal pastes (US$ 200 or more per kg dry weight) have discouraged many growers from using them. Recently, the development of preservation techniques has extended the shelf-life of *Thalassiosira pseudonana* concentrates from about 10 days to more than one year, which makes it possible to utilize excess and off-season algal production. Outdoor pond production on a large scale has lead to the bulk availability of a limited number of "algal meals", such as spray-dried *Spirulina* and a spray-dried extract of *Dunaliella salina*. The latter may be used as a supplement to live algae to improve the growth of bivalve larvae.

In addition, recent techniques have been developed for the large scale production of marine micro-algae under heterotrophic growth conditions, by utilizing organic carbon instead of light as an energy source. Heterotrophic algal cultures can attain up to 1,000 times higher densities than photoautotrophic cultures and can be preserved by spray-drying. Projected costs of producing algae within industrial fermentors vary from US$ 5 to 25 per kg (Gladue, 1991). Unfortunately, heterotrophic mass-production techniques have only been realized for a few algal species, and most of the species that are known to be of high nutritional value (*e.g.* *Chaetoceros*, *Isochrysis*, *Skeletonema*, *Thalassiosira*, *Monochrysis*) are not capable of growing in the dark. Furthermore, heterotrophic conditions may result in a drastic change in the gross composition and reduced (n-3) HUFA content as compared to light-grown algae. Nevertheless, further developments in this rather new technology may improve the biochemical composition and the range of dried algae available in the future.

2.6.2. Micro-encapsulated diets

Through micro-encapsulation techniques dietary ingredients can be encapsulated within digestible capsules and delivered to suspension-feeders without losses of nutrients to the aqueous medium. Possible problems arising from the use of microparticulate feeds include settling, clumping and bacterial degradation of the particles, leaching of nutrients, and low digestibility of the cell wall material. In this regard, low susceptibility to bacterial attack and high digestibility for the filter-feeder may be conflicting requirements for a capsule wall.

2.6.3. Yeast-based diets

Because of their suitable particle size and high stability in the water column yeasts can easily be removed from suspension and ingested by filter-feeding organisms. Furthermore, as opposed to most of the other alternatives to live algae, yeasts can be mass-produced at a relatively low cost. The potential of yeasts as a food in aquaculture has been proven by their successful application in the rearing of rotifers and some species of penaeid shrimp. However, a limited nutritional value of yeasts was reported for various species of filter-feeders and attributed to their nutritionally deficient composition and/or undigestible cell wall. Despite this, the nutritional value and digestibility of yeast-based diets can be improved through the addition of limiting essential nutrients and the chemical treatment of the yeast
cell wall, respectively. In this way, about 50% of the algae can be substituted by yeast-based diets with minimal effects on the growth of juvenile hard clam, *Mercenaria mercenaria* (Coutteau et al., 1994).

### 2.7. Literature of interest


2.8 WORKSHEETS

WORKSHEET 2.1: ISOLATION OF PURE ALGAL STRAINS BY THE AGAR PLATING TECHNIQUE

The following agar plating technique can be used to isolate algal strains from raw seawater and for the maintenance of existing algal strains.

- prepare a 0.9% agar medium by weighing out 9 g of agar powder and placing it into a 2 l conical flask to which 1 l of sea water is added
- heat the flask on a Bunsen flame and let it boil twice, i.e. heat until it boils, let it cool and let it boil a second time.
- add nutrients (see Tables 2.3 & 2.4) before autoclaving
- cover the flask with aluminium foil
- autoclave at 125 °C for 30 minutes at 1 atm
- sterilise Petridishes by incubation for 30 minutes at 150°C
- agar plates are prepared aseptically by pouring the warm autoclaved agar into the sterile Petridishes near a Bunsen flame or in a laminar flow, cover up the Petridishes and leave them to cool for about 2 h
- streak the algal sample onto the agar surface with a sterile platinum loop (previously heated to red-hot and cooled)
- place the Petri dishes upside-down on an illuminated glass rack
- depending on the density of the inoculum, cell colonies can be observed to grow on the surface after 5 - 21 days
- select the best colonies and transfer them with a sterile platinum loop into a test tube filled with 5-10 ml of culture medium and shake it regularly during incubation on an illuminated glass rack.
- when a colour change is observed in the tube, check under the microscope the isolated algal strain
A variety of counting chambers (normally used for blood cell counts) can be used for cell counts (algae, yeast) (see Table 2.9.). Two types are most common: Fuchs-Rosenthal and Bürker (Fig. 2.17.). Both types have 2 rafters allowing for 2 subsamples to be examined. They have the following characteristics:

<table>
<thead>
<tr>
<th></th>
<th>Fuchs - Rosenthal</th>
<th>Bürker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth (in mm)</td>
<td>0.200</td>
<td>0.100</td>
</tr>
<tr>
<td>Surface of smallest</td>
<td>0.0625</td>
<td>0.0400</td>
</tr>
<tr>
<td>square (in mm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>minimal</td>
<td>cell</td>
<td>10³</td>
</tr>
<tr>
<td>concentration</td>
<td>(in cells.ml⁻¹)</td>
<td></td>
</tr>
</tbody>
</table>

- dilute sample if needed (use formalin 4% to fixate moving algal cells)
- clean slide and cover-glass with Kleenex-paper
- press cover glass onto the slide until the Newton diffraction rings appear
- fill both slides of the counting chamber under the cover-glass with a single smooth flow of suspension using a Pasteur pipet (avoid air bubbles)
- count cells in, respectively, 80 (Fuchs-Rosenthal) and 20 (Bürker) small squares under a microscope (objective 40 x). Count cells which touch the upper and left border but not those which touch the lower and right borders (see schematic diagram)
- the subsample on the other side of the chamber is counted in the same way
- calculation for Fuchs-Rosenthal numbers of cells.ml⁻¹ = \((n₁ + n₂)/(2x80) \times 80 \times 10³ \times d = (n₁ + n₂)/2 \times 10³ \times d\)
- calculation for Bürker numbers of cells.ml⁻¹ = \((n₁ + n₂)/(2x20) \times 250 \times 10³ \times d = (n₁ + n₂)/160 \times 10⁶ \times d\)
  with :  
  - \(n₁\) = number of cells counted in upper rafter
  - \(n₂\) = number of cells counted in lower rafter
  - \(d\) = dilution factor
- For greater accuracy make 3 duplicate counts (3 separate dilutions each counted in two rafters).
Figure 2.17. Counting directions (follow arrow) for Fuchs-Rosenthal chamber (upper diagram) and for Bürker (down left corner). For both, count the cells in the square and those which touch the top and left border (•). Do not count the ones touching the right and lower border (○) (see down right corner).
WORKSHEET 2.3. : CELLULAR DRY WEIGHT ESTIMATION OF MICRO-ALGAE.

Dry weight of algal cells can be determined by filtering and drying algae from aliquots of culture of known concentration

- determine accurately (3 duplicate counts, see Worksheet 2.2.) the concentration of the algal culture to be sampled for dry weight analysis
- filter an exact volume of culture on prepared glass-fiber filters (1 µm pore size) using a Büchner setup connected to a vacuum pump (triplicate). Wash the filter with a solution of ammonium formate (0.5 M) to remove salts
- follow the same procedure with control filters on which an equal volume of 0.22-µm filtered seawater is filtered (triplicate). The strength of the applied vacuum will determine the amount of salts retained on the control filters.
- dry the filters at 100 °C for 4 h to volatilize the ammonium formate
- weigh on an analytical balance
- calculate the dry weight per algal cell according to the formula:
  \[ \text{DW (g.cell}^{-1}) = \frac{\text{DW}_A - \text{DW}_C}{\text{N}.\text{V}} \]

  with  
  \( \text{DW}_A \) = average dry weight retained on algal filter (g)  
  \( \text{DW}_C \) = average dry weight retained on control filter (g)  
  \( \text{N} \) = algal concentration (cells.ml\(^{-1}\))  
  \( \text{V} \) = volume of algal culture and filtered seawater filtered on algal and control filter, respectively (ml)

In order to improve the correction for salt residues and the variation among samples, cellular dry weight can be determined from regression analysis of DW retained on the filter versus number of algal cells filtered (Fig. 2.18).
Figure 2.18. Dry weight analysis of algae by means of linear regression of dry weight retained on the filter versus number of algae cells filtered. Each data set represents the dry weight determination for algae obtained from one culture of, respectively *Isochrysis* sp. (T-iso) and *Chaetoceros neogracile* (Chg). Linear regression equators are: T-iso: $y = 1.83 \times 10^7 + 107.7 \quad (r^2 = 0.99)$; Chg: $y = 2.61 \times 10^6 + 130.1 \quad (r^2 = 0.95)$