Introduction to Culturing of Marine Microalgae

1. Introduction to Marine Microalgae

The algae (singular alga) are generally considered simple plant-like organisms, both morphologically and anatomically. They are classified in the Protist Kingdom together with slime molds and protozoa and are found in waters of varying salinity, including both fresh water and marine environments. They vary in size from the minute (1-5 µm) to the very large species such as kelp, known to grow to 50 m or more in length. Although quite simple in form, the algae are an extremely diverse group. This diversity has occurred over 2 to 3 billion years. It is the result of the changes in cell differentiation and variations in planes of cell division and the differences are mainly morphological, not anatomical. Reproduction in the algae is primarily asexual, although sexual reproduction also occurs.

Algal classification systems can vary. Algal divisions have been established using several criteria including; pigmentation differences, type of energy storage products, cell wall composition and the presence or absence of flagella (as well as the number and placement of these flagella, when present).

The Principal Classes of Marine Phytoplankton include:

Bacillariophyceae	(diatoms)
Chlorophyceae	(green algae)
Chrysophyceae	(golden algae)
Cryptophyceae	(cryptophytes)
Cyanophyceae	(blue-green algae)
Dinophyceae	(dinoflagellates)
Haptophyceae	(haptophytes)

2. Culture Maintenance Conditions

Although it is desirable to maintain algal cultures using optimal growth conditions, it is not always practical as these vary greatly between different microalgae. This is especially true when keeping a collection of several different strains or species.

Culture medium

A newly isolated strain can be difficult to maintain, because no long-term culture strategy has yet been tried. If the optimum culture medium is not already known, you will need to quickly find a medium that suits the alga involved. A reasonably reliable way of doing this is to simply put the isolates into several culture media and wait to see which one they grow in. It is wise to maintain any new algae in these media (as long as they are showing signs of growth) for several months, if possible, as cultures will often grow quite happily for one or two subcultures before dying. Knowledge about the growth medium used for closely related algae is often valuable. If maintaining the original morphology of the cells is desirable it is recommended that a culture medium using a soil water extract is used, e.g. Erd-Schreiber.

Light

Over-illumination is a widespread mistake in the maintenance of cultures. Problems include; localised heating of algae, photo-oxidative stress and bleaching (loss of pigmentation) effects. Light and dark periods are required for the maintenance of most algae and some may be killed by continuous light. In most culture collections the light : dark regimes vary between 12 : 12 to 16 : 8 hour light : dark. The regime used will depend on whether the cultures are kept in a room where natural light is available or not and, if so, the time of year. Direct sunlight should always be avoided.

As the Plymouth Culture Collection is housed in a constant temperature (15°C) room with a large north facing window, lighting is used for 12hrs during autumn and winter and 8 hours during spring and summer. The use of cool white fluorescent tubes is recommended and these should be placed at a distance from the shelves, so as not to cause any overheating of the algae.

Temperature

Ideally, temperature should reflect the conditions from which the algae were isolated. Most Atlantic coast marine algae grow well between 15°C and 25°C. Any temperature above 30°C is usually fatal for these algae. Marine algae from the Pacific coast prefer temperatures between 5°C and 15°C, with lethal temperatures being as low as 20°C. Temperature stability should be kept to ± 2°C, where possible. Most larger service culture collections maintain almost all strains at between 15° and 20°C. Incubation temperatures higher than 20°C should be combined with increased light intensities to prevent photo-inhibition or damage.

Culture Vessels

Algal cultures can be maintained in a variety of containers including; glass or plastic flasks, tubes or Petri dishes. Reusable glassware tends to provide better growth, but can take up a lot of room and is more labour-intensive, as it has to be washed and sterilised. Disposable plastic is less labour-intensive but can work our more expensive in the long term. Whichever is used, the lid or top must fit well enough to prevent contamination whilst allowing gas exchange. Culture vessels should allow uniform illumination. It must be stressed that all culture vessels should be meticulously cleaned and sterilised.

3. Sterilisation and Sterile Technique

Category	Sterilisation Method	Effective Method	Applications	Limitations
Heat	Flame	Direct heat with fire (Bunsen burner)	Surface sterilisation (test tube openings, transfer loops etc)	Non heat-resistant materials (e.g. most plastics)
Heat	Autoclaving	15lb/sq in (1.06 Bar) pressure, 121°C – time varies according to quantities (20 minutes small volume – 60 minutes large volume)	General use: liquids and agar, glass, metal and some plastic vessels and equipment	Non heat-resistant materials: pH change; metal contamination
Heat	Dry Heat	220°C for 4 hours	Dry goods: glass and metal vessels and equipment	Non heat-resistant materials; liquids
Filtration	Filtration	0.2µm pore size filter	Liquids with heat – labile components	Small volumes, high-viscosity liquids, viruses not eliminated
Electromagnetic waves	Microwave	Liquids: 10 minutes at 800W. Dry goods: 15 minutes at 800W with water in separate container	Liquids: small volume of media. Dry goods: glassware and vessels	Small liquid volumes; dry goods with water require elimination of water

4. Isolation of Algae

Sample Collection

For coastal and oceanic sites plankton is usually concentrated by gentle towing with a plankton-net, e.g. 10 minutes with a 200µm and a 600µm mesh net. Some species may also be collected from rock pools or the shoreline. Gentle handling and time are important factors as damaged and dying cells will not produce viable cultures.

Equipment

An inverted microscope with a long-working distance condenser and good range of lenses (x4, x10, x20 and x40 magnification) to provide easy access for pipettes is essential. A mechanical stage designed for both multiwell plates and slides is very desirable. Borosilicate glass has been used traditionally but is now being replaced by plasticware. Advantages of plasticware are that it is pre-sterilised in ready-to-use packages and some multiwell plates can be coated with growth substances which may enhance growth of many algae.

Culture Medium

Many diatoms and dinoflagellates can be isolated into full strength medium. We usually isolate into Erd-Schreiber or Keller's medium. However, some species are quite sensitive so isolating into very dilute culture medium may be required. The strength of the medium can then be increased incrementally to improve growth of the culture.

Single-Cell Isolation

This is carried out using a micropipette. Traditionally a fine glass Pasteur pipette or a glass capillary tube has been used. We usually use a sterile extended fine tip plastic transfer pipette (Alpha Laboratories Ltd. Cat. No. LW4231). The aim of micropipette isolation is to pick up a cell from the sample and deposit it, without damage, into a sterile droplet of medium. We do this using a multitest slide. The sterile droplet containing the target cell, and probably other cells, is examined under the microscope. Using a clean pipette, the target cell is transferred to a second drop of medium. This is repeated until a single cell can be transferred into the culture vessel. We usually use sterile plastic 24-well tissue culture plates for initial culturing. The process needs to be carried out enough times to ensure clean isolation of a single cell but without causing cell damage by excessive handling. Daily microscopic examination should be carried out and once the culture is growing it can be transferred to a 100ml glass flask. Once the culture is established it can be grown in larger quantities.

Other methods of isolation include; the use of agar, dilution techniques, centrifugation and phototaxis (see Andersen, 2005, Algal Culturing Techniques, Elsevier Academic Press).

5. Sub-culturing

Aseptic technique should always be adopted and where possible a lamina flow hood used to reduce any possibility of contamination. For routine maintenance the goal is to sub-culture the alga at the end of its exponential growth phase. This depends on the sensitivity of the strain, with sensitive strains requiring a transfer cycle of 1-2 weeks and the most robust strains only requiring transfer every 2-3 months. At the Plymouth Collection, the majority of strains are sub-cultured every 4 weeks. Algae kept on agar slopes, however, can be transferred once every 6 months.

Transferring liquid cultures

1) Wear protective clothing and clean all surfaces with a laboratory disinfecting agent, e.g. Virkon. Wash hands.
2) Take a sterile culture vessel, write culture medium, culture name and/or number on it and add the current date.
3) Pour required amount of culture medium into culture vessel.
4) With a sterile pipette, transfer a small amount of liquid algal culture (generally 1-2mls per 250ml depending on cell numbers) into culture vessel containing the fresh medium.

Transferring agar cultures

1) Wear protective clothing and clean all surfaces with a laboratory disinfecting agent, e.g. Virkon. Wash hands.
2) Take a sterile culture vessel (test tube or Petri dish) containing agar medium, write culture name and/or number on it and add the current date.
3) Flame transfer loop in the blue part of the Bunsen burner flame until the metal glows red and let cool.
4) Lift lid of culture vessel containing agar culture, slowly insert the loop and pick up the cells without scratching the agar. Replace lid.
5) Open the new culture vessel and gently brush the surface of the agar (without scratching the surface). Replace lid.
6) After use, flame the end of the transfer loop to disinfect.

6. Counting Cells in Culture

There are several microscope counting devices available. The choice depends on several factors, including; culture density, size and shape of the cells, presence of extracellular material such as mucilage. We usually use a Sedgwick-Rafter Counting Cell or a Haemocytometer.

Sedgwick-Rafter Counting Cell

The chamber is rectangular (50x20mm), 1mm deep, with an area of 1000mm^2 and a volume of 1.0ml. It is best used for relatively large cells, 20-500µm with cell densities ranging from ~30 per ml up to about 10^5 per ml. The base is divided into 1mm squares. A cover glass traps liquid to the correct depth. Using an inverted microscope or low power compound microscope, cells in each cubic millimetre can be counted. The chamber and coverslip must be perfectly clean, usually by rinsing in ethanol or methanol. The chamber must be completely filled with no air bubbles present.

Haemocytometer

The haemocytometer has 2 chambers each with nine 1-mm squares with several layers of subdivision. The most common ruling is the 'Improved Neubauer' ruling which comprises, 3mm x 3mm divided into nine 1mm^2 areas, with a depth of 0.1mm. Another useful chamber is the Fuchs-Rosenthal, which also has a total ruled area of 9mm^2 but has a depth of 0.2mm, making it useful for bigger cells. The chamber and coverslip must be perfectly clean, usually done by rinsing in ethanol or methanol. The coverslip is firmly fixed to the chamber by cohesion as shown by the presence of diffraction rings. It is essential that the coverslip is symmetrically placed over the double rulings to prevent non-random cell distribution. The cell suspension is transferred to the chamber using a fine pipette. The chamber must be filled using a single application, without overflowing into the channels and must not contain bubbles. The cells must be allowed to settle prior to counting. For cells touching the centre line of the triple ruling it is important that only 2 sides of the square are included in the count and cells touching the centre line of the other 2 sides are excluded. To maintain an acceptable degree of error, at least 50 cells should be counted. To calculate cell density:

Cells per µl = number of cells counted x 1/area x 1/depth x dilution

For motile cells fixation is necessary, this is commonly done using Lugol's iodine fixative or formaldehyde based fixative. For Lugol's iodine, mix together, 10g potassium iodide, 5g crystalline iodine and 100ml distilled water. Once dissolved add 1 drop (~0.05ml) per 5ml of algal sample.