Ser. Cient. INACH 34: 93-97, 1986

Techniques and equipment for culturing antarctic benthic marine algae, and for preparing specimens for electron microscopy

Técnicas y equipo para el cultivo de algas marinas bentónicas antárticas, y para preparar muestras para miscrocopía electrónica

MARGARET N. CLAYTON[1] and CHRISTIAN WIENCKE[2]

INTRODUCTION

"What we urgently need is an Antarctic biological station with modern laboratory facilities where algae can be studied alive in cultivation and be subject to cytological and physiological research, and where work can go on round the year". (Skottsberg 1964, p. 150).

More than 20 years later, there have been only a few attempts to cultivate Antartic species of benthic marine algae (Moe and Silva 1981, Moe and Henry 1982, Müller 1984), and only one species, *Ascoseira mirabilis*, has been successfully grown in culture (Moe and Henry 1982). Müller (1984) discussed some of the difficulties encountered in this work. In particular, he stressed the need for a land-based laboratory, access to the shore, and microscopic equipment to enable the investigator to recognise and select areas of reproductive tissue.

We are not aware of any previous attempts to fix benthic marine algae in Antarctica in preparation for electron microscopic studies. The procedures involved become relatively complicated in field laboratories because it is necessary to use a number of chemicals in precisely weighed quantities. Moreover, accurate balances, fume cupboards, and pH meters are normally not available.

METHODS AND OBSERVATIONS

Collection of specimens

It is most important that specimens of algae that are to be used for establishing cultures or for preservation of subsequent microscopic studies, should be perfectly fresh and healthy. Ideally, the algae should be collected from where they grow. Specimens washed up on the shore as drift may be used, but they should be examined carefully first as they are more likely to have been damaged. Because the great majority of Antarctic marine algae grow subtidally, the best specimens are collected by divers, preferably divers who are phycologist and familiar with the

[1] Botany Department, Monash University, Clayton 3168, Victoria, Australia.
[2] Biologische Anstalt Helgoland, D-2192, Helgoland. FRG. (Present address: Alfred-Wegener-Institut, Sektion Biologie II. D-2850 Bremerhaven, FRG).

appearance of mature, fertile plants. In our experience at Marsh Base, access to boats was a major constraint, and consequently most of our studies were made on drift algae. Once they are exposed to the atmosphere, subtidal algae deteriorate very rapidly. When collected, the specimens must be kept moist and *cold*. Some of the more delicate species are better also immersed in seawater. Specimens of *Desmarestia* spp., in particular *Desmarestia ligulata*, should always be carried separately and in seawater as the thalli deteriorate very rapidly. All the species of benthic marine algae we studied suffered immediate and severe damage when they were exposed to contact with rain or snow, and such specimens could not be used in our studies.

When specimens are to be used for culture, smaller pieces of thallus may be kept in an optimum condition if they are excised in the field, and placed in small tubes or sealable polythene bags. If this is to be done it is important to first ascertain how to recognise the appearance of the required (usually reproductive) parts of the thallus.

Equipment recommended (excluding diving equipment): buckets, polythene bags (various sizes; they should not leak; sealable ones are useful), plastic tubes, insulated carrying bag, waterproof boots, rubber gloves.

Culturing techniques

The methods we used to set up and maintain cultures of benthic marine algae in Antarctica (January and February 1986) were essentially adaptations of well known techniques (Stein, 1973). However, we found that, in this unique environment, many special precautions were necessary.

The first essential prerequisite is a land based laboratory where it is possible to carry out microscopic studies. It is extremely important that the air temperature in the laboratory is not significantly different from the outside. Even short exposure to elevated temperatures may be lethal for the algae. At Marsh Base we were able to set up one of the INACH laboratories for microscopy and also to use it as a culture room. Air temperatures in the laboratory varied between 3° and 9°. In order to maintain the cultures at a temperature closer to that of the coastal seawater (0°-2°), they were placed on ice (snow) in insulated polystyrene boxes (Figure 1). They were kept next to a window where they received natural daylight, but no direct sunlight. It was necessary to replace the snow every two days. No volatile chemicals were used in the same laboratory. This is a vital precaution. Substances such as glutaraldehyde and formaldehyde were kept in another room.

Various methods were used to establish cultures depending on the species and whether the specimens were fertile. Initially, small pieces of thallus were selected, cut out with a razor blade, and carefully cleaned using filtered seawater. Cultures may be initiated with either vegetative or reproductive tissue. If specimens are fertile, the cleanest cultures are obtained using inocula of spores or gametes, rather than tissue fragments which are more likely to be contaminated with epiphytic diatoms and microorganisms. The former course was followed whenever possible. With Rodophyta, clean tissue fragments were placed in Petri dishes containing culture medium until the spores were released (1-10 days). The spores were then transferred to culture tubes. With many species of Phaeophyta the following technique was successful. Several small pieces of fertile tissue were placed on a Petri dish, each in a separate drop of sterile culture medium. The mass release of zooids began, either immediately or after periods of up to 1 hour. They were observed microscopically, and transferred to culture tubes using sterile pipettes. Drops of zooid suspension were also placed on sterile coverglasses. After the zooids had settled, the coverglasses were placed in culture medium in Petri dishes, which were then sealed with Parafilm.

Ascoseira mirabilis (Phaeophyta) presented a special problem. Although we collected many fertile specimens, zooids coul be obtained only by first cutting the fronds under seawater using a

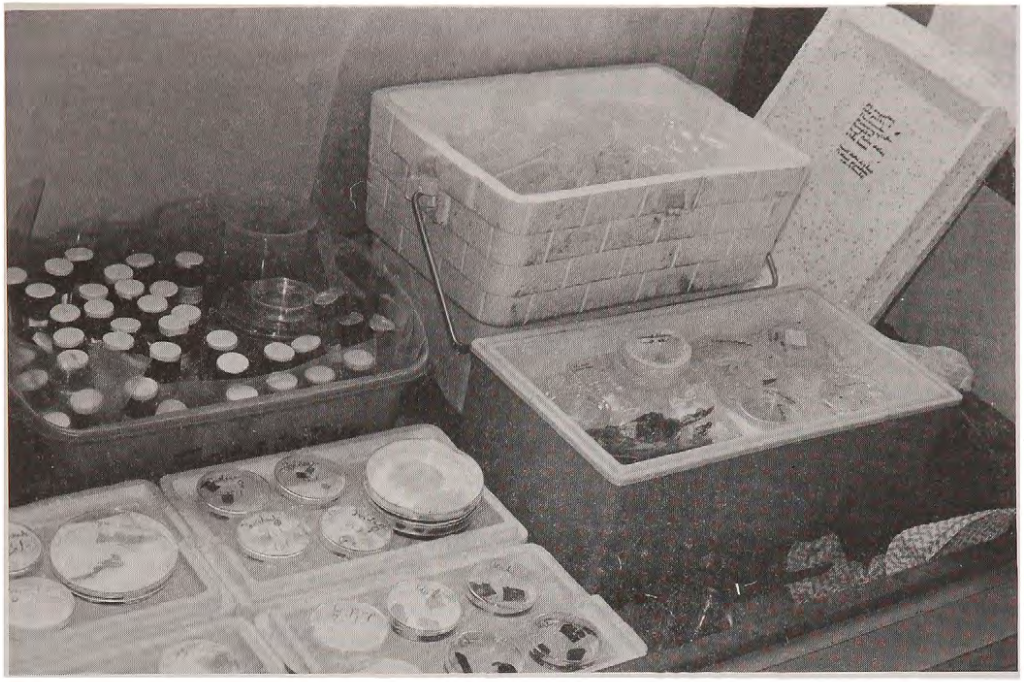

Fig. 1. Cultures of marine benthic algae at Marsh Base.

razor blade. The contents of the conceptacles swelled up in contact with water, and the zooids were then released.

Cultures of Rhodophyta, Phaeophyta and Chlorophyta were also established using vegetative tissue. Fragments of actively growing tissue or entire very young individuals were used.

It is important that parallel microscopic observations are carried out on the specimens from which cultures are established, and on the reproductive cells themselves. This is necessary in order to determine the nature of the reproductive structures (gametangia, sporangia, cystocarps, etc.). In *Ascoseira mirabilis* such observations enabled us to demonstrate that the zooids, previously thought to be spores (Moe and Henry, 1982), were isogametes. Fertilisation was observed in this species for the first time.

Herbarium specimens were prepared from all species that were cultured. It is essential that voucher specimens are available for checking and future reference. In many instances, tissue samples from specimens used in setting up cultures were preserved for subsequent examination by light and electron microscopy. This kind of coordinated study is particularly valuable as it yields much related information about the same species.

Transport of cultures

This is a major problem. It is essential that *efficient*, insulated containers are available, together with an adequate supply of freeze packs, in order to maintain low temperatures during periods when the cultures cannot be refrigerated.

Equipment recommended: compound microscope with mirror and lamp, dissecting microscope, thermometer, insulated containers, presterilized culture vessels e.g. 5 or 10 ml polypropylene tubes, Petri dishes, presterilized culture medium with and without germanium

dioxide to control diatoms, filtered seawater (beaker, funnel, filter paper), dissecting instruments, razor blades, 70% ethanol for sterilizing instruments, sterile coverglasses, sterile pipettes, Parafilm, adhesive labels, waterproof marking pen, camera with adapter for microscope.

Survival and subsequent growth of algal cultures

After leaving Antarctica our return journeys lasted 24 days, during which time the cultures were without light, and we endeavoured to keep them at a temperature between 0° and 4°. This was not always possible and for one period of several hours the temperature rose to 7° or 8°. Back in our home laboratories the cultures were placed in illuminated culture rooms or cabinets at 1° or 2°. The algae began to develop slowly, the first, microscopic signs of growth being observed after periods which varied from two to five weeks in different species.

The following species were known, by mid-April 1986, to have been successfully cultured. RHODOPHYTA: *Iridae obovata* Kützing, *Leptosomia simplex* (A. and I. Gepp) Kylin; PHAEOPHYTA: *Ascoseira mirabilis* Skottsberg, *Desmarestia menziesii* J. Agardh, *Elachista antarctica* Skottsberg, *Himantothallus grandifolius* (A. and E.S. Gepp) Zinova, *Phaeurus antarcticus* Skottsberg, *Utriculidium durvillaei* (Bory) Skottsberg; CHLOROPHYTA: *Enteromorpha bulbosa* (Suhr) Montagne, *Prasiola crispa* subsp. *antarctica* (Kützing) Knebel f. *antarctica*, *Ulothrix* sp., *Urospora penicilliformis* (Roth) Areschoug.

Preparation for light and electron microscopy

The preservation schedule for algal material follows the general recipes given by Glauert (1974). It is clear that, under existing conditions, embedding and all further steps have to be carried out in the home laboratory; only fixation and part of dehydration can be completed in Antarctica.

In our work with Brown, Red, and Green algae, several methods were used. The following schedule represents a standard procedure which may be varied depending on the kind of material to be processed. Fixation: 1.5% formaldehyde, 1.5% glutaraldehyde, 50mM sodium cacodylate in seawater (pH 7.2) for 5-24 h. Washing: 5 changes of 50mM sodium cacodylate in seawater (pH 7.2). Postfixation: 1% osmium tetroxide in seawater for 2-12 h. Wash in 5 changes of distilled water or dehydrate immediately up to 70% ethanol in 10% steps.

As normally no fume cupboard will be available, all the fixations should be done outside in a well ventilated space. We were able to construct a temporary fixing cabinet using wooden boxes. This provided some protection for the specimens. Gloves without fingertips help to keep the hands warm. To facilitate the preparation of solutions in the absence of accurate balances, it is useful to have some of the chemical (e.g. cacodylate, paraformaldehyde) preweighed in small amounts. An important consideration is the solubility of the various substances at low temperatures. If caffeine is used in the fixative medium, it will be insoluble at concentrations higher than 0.5% when air temperatures are between 2° and 9°. For the preparation of formaldehyde solution, finely ground paraformaldehyde dissolves much more quickly than the coarser varieties. The addition of a few drops of 1M NaOH may also help. The adjustment of pH is relatively easy using pH paper, and we found that paper sensitive in the ranges 1-14 and 6-8 was useful. Fixation times at Antarctic temperatures are at least three times as long as at normal room temperature (20°). One early symptom of insufficient time in aldehyde fixative is seen if, when placed in osmium, the specimen is very slow to darken. When this happens, the fixation should be repeated with a new specimen using a longer time.

The procedure for microscopy may be terminated in Antarctica either in distilled water or in 50-70% ethanol (or acetone). When leaving the specimen in distilled water for any length of time,

the use of sterile distilled water is recommended to prevent the growth of microorganism. Extended periods in ethanol (or acetone) may result in the extraction of some ethanol-soluble substances, and should be avoided. If possible, specimens should be refrigerated until the dehydration procedures are completed, and they are embedded.

Labelling the specimens can be difficult in Antarctic temperatures. We found that adhesive labels did not stick wery well to the fixation vials which were also sometimes wet with condensation. Transparent tape may be used to further secure the labels, but we strongly recommend additional labels, both inside the vial with the specimen, and on the lid.

Equipment recommended: (Wherever possible polyethylene laboratory ware was used in order to reduce the weight and cost for air transport).

razor blades
forceps
file for osmium ampoule
pH paper
Pasteur pipettes
beakers, 400, 800 ml
filter funnel and paper
camping bunsen burner and fuel
glass vials 10 ml
polythene bags for waste
waterproof matches
Parafilm
dental wax for cutting specimen
preparation needle
container for osmium solution
pipettes 2, 5, 10 ml
measuring cylinders 50, 250 ml
Pileus ball
glass Erlenmeyer flask 250 ml
flask with stopper 500 ml
squeeze bottle, distilled water
paper tissues
aluminium foil
adhesive labels and transparent tape

Chemicals: osmium tetroxide in 0.1 g ampoules, 25% glutaraldehyde, paraformaldehyde, sodium cacodylate, NaOH 1M, HCl 1M, ethanol or acetone 10-70% , distilled sterilized water.

ACKNOWLEDGEMENTS

We are grateful for the organisational and financial support provided by the Instituto Antártico Chileno (INACH). The research was funded by a Marine Sciences and Technologies grant (Australia) and a grant from the Ian Potter Foundation to M. Clayton, and a grant from the Deutsche Forschungsgemeinschaft (DFG) to Klaus Lüning, Biologische Anstalt Helgoland (Hamburg).

REFERENCES

GLAUERT, A.M. 1974. [Ed.] Practical Methods in Electron Microscopy, Volume 3. North-Holland, Amsterdam, 353 pp.

MOE, R. and E.C. HENRY 1982. Reproduction and early development of *Ascoseira mirabilis* Skottsberg (Phaeophyta), with notes on Ascoseirales Pretrov. Phycologia 21, 55-56.

MOE, R. and P.C. SILVA 1981. Morphology and taxonomy of *Himantothallus* (including *Phaeoglossum* and *Phyllogigas*) an Antarctic member of the Desmarestiales. (Phaeophyceae). J. Phycol. 17, 15-29.

MÜLLER, D.G. 1984. Reproducción sexual de algas. Bol. Antártico Chileno 4, 19-20.

SKOTTSBERG, C.J.F. 1964. Antarctic phycology. In: R. Carrick, M.W. Holdgate and J. Prevost editors, "Biologie Antarctique" pp. 147-154, Hermann, Paris.

STEIN, J.R. (editor). 1973. Handbook of Phycological Methods. Culture Methods and Growth Measurements. Cambridge University Press, Cambridge.