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A NOTE ON THE ISOLATION OF SMALL MARINE ALGAE AND FLAGELLATES FOR PURE CULTURES

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Increasing interest in the comparative biochemistry of marine algae and protozoa is creating a demand for experimental material in the form of pure, i.e. bacteria-free, cultures. For nutritional and biochemical studies pure cultures are, of course, obligatory; but the number of marine strains available for this sort of work is lamentably small. Indeed, none of the so-called μ-flagellates has hitherto been grown in pure culture as far as I know, although there exist pure strains of some of the larger and hardier forms.

Hardier species of motile or non-motile algae can be plated out by normal bacteriological techniques, provided that the material is growing sufficiently vigorously for there to be cells without attached bacteria. Marine examples of this class are species of *Chlorella*, *Chlamydomonas*, *Brachiomonas*, *Platymonas*, *Nannochloris*, *Phaeodactylum*, etc., many of which can be maintained on agar. For plating, I have found that test-tube slants (1% agar) are preferable to Petri dishes, since drying out of the medium is then delayed; but slants require more skill for the actual isolation than do plates.

The use of antibiotics seems, except with filamentous or gelatinous algae, to be applicable only to species which can be handled by simpler methods. This has recently been demonstrated by Spencer (1952).

Allen & Nelson's (1910) time-honoured 'dilution' method has been used with great success by Gross (1937), Parke (1949) and Butcher (1952) for obtaining unialgal cultures of many interesting marine species; but it is doubtful if pure cultures could ever be obtained by serial dilutions, since bacteria usually occur in far greater numbers than algae.

A method of purifying flagellates which is widely used among freshwater workers is the 'pipetting' or 'washing' method as developed by Pringsheim (1946). I want to show that, with very little modification, Pringsheim's technique can be used with success for some μ-flagellates.

The washing method has two advantages over all other methods; one is of principle, that the organism to be isolated is selected by the worker and not by the medium; the other is a practical one, that the ratio of bacteria to algae is reduced by astronomical proportions at each manipulation, which is not the case with serial dilutions. Like the plating method it depends for its success on the cells being free from attached bacteria in the first place. Motile forms

are mostly clean and therefore present no problem. With non-motile or mucilagenous species it is necessary to employ vigorously growing populations. Then, even such gelatinous algae as the freshwater *Paulschulzia pseudovolvox* may be purified by washing, and indeed, I have also washed the diatom *Phaeodactylum tricornutum* successfully.

Pringsheim's technique has been described in some detail in his book (1946) and in various articles (1950, 1951). The apparatus consists of a binocular dissecting microscope with a cardboard breath-guard; a store of sterilized Petri dishes, each with a watch-glass inside; micro-pipettes; and an appropriate variety of sterile culture media in test-tubes. Micro-pipettes are made simply by: (i) plugging a 9 in. length of $\frac{3}{8}$ in. glass tubing at both ends with cotton-wool; (ii) drawing this out in the centre to make two long-nosed pipettes with a 1 mm bore at the narrow end, these to be sterilized and stored sterile; (iii) immediately before use, further drawing out the thin part of the pipette to a bore of about 0·1 mm in a very narrow flame, and breaking the capillary tube cleanly at a convenient length by a sharp longitudinal tug with flamed forceps, an operation to be repeated before every manipulation to ensure sterility; and (iv) attaching a piece of rubber tubing, glass-stoppered at the free end, which serves to eject liquid from the pipette, and also has the necessary strength to overcome the strong capillary force in the tube.

Into the first of a series of six of the watch-glass combinations, each containing about 3 ml. of sterile culture medium, is placed a drop of material containing the desired organism. One normally starts with the highest powers of the binocular for identification, but once the look of the organism and its characteristic movement have been learned it is possible to go to the lower powers for isolation. Single cells are selected and drawn into the pipette by capillarity and transferred to the second glass of the series. For small organisms it is convenient to watch them being ejected into the new medium, as this may save a little time in searching. In any case they should be allowed to swim around for a while before they are picked up again, so that they become washed of the old medium. This treatment is repeated, until after the final washing they are transferred, one to each culture tube.

This method, as it stands is applicable down to about 15μ; but for smaller cells it becomes too tedious. I have used it in purifying cultures of *Oxyrrhis*, *Chlamydomonas*, *Brachiomonas*, *Platymonas*, *Syracosphaera*, etc.

It is possible to make use of phototactic responses for the purification of some μ-flagellates. This avoids most of the difficulties arising from their small size. For example, an organism of 5μ can be washed with less trouble with the aid of phototaxis than one of 50μ handled in the normal way; but of course success depends on one's being able to induce phototaxis. Luckily, many species seem to exhibit a very strong negative taxis when they are transferred from the wild to a culture medium. All that is needed is to put a drop of the material gently at the window side of the watch-glass and wait 5 min (or

perhaps 10) when the flagellates will have congregated at the side away from the light, leaving most of the unwanted bacteria on the other side. From here the flagellates are transferred *en masse* with a rather large-bore micro-pipette to the window side of the next watch-glass, and so on until the last. Then under medium power of the binocular the flagellates, mere specks, are picked up singly with a fine-bore micro-pipette and placed in culture tubes. The last operation is very rapid: fifty isolations can be made in half an hour or so. The proportion of contaminated to clean cultures obtained with six washes is about 1:10.

I do not know what the size limit of this method is; but provided an organism is visible, if only as a speck, it is possible to pick it up. The essentials are that there is a preponderance of the desired flagellate in one's material to begin with, and secondly that it is motile and phototactic.

My successes during last summer with this method include two members of the Chrysophyceae, *Monochrysis lutheri* and *Prymnesium parvum*; one of the Cryptophyceae, *Hemiselmis* sp., and several Chlorophyceae, all below 10μ. As an example of the efficiency of the procedure, *Monochrysis lutheri* (5–7μ) was found at a cell concentration of 16,000,000 per ml. in a pool of salinity 25‰. One day was devoted to preparing the media and isolating the organism. Forty-eight isolations were put into twelve different combinations of medium; and out of this forty-eight, ten flagellate cultures and three bacterial cultures resulted. All the successful ones contained glucose and liver extract. *Hemiselmis* (6μ), on the other hand, was more difficult. The natural material was at a cell concentration of about 30,000,000 per ml. from a pool of low salinity. I made three attempts on the same scale as for *Monochrysis*. In the first I ended up with a large number of pure cultures of a minute heterothallic *Chlamydomonas*; not until the third attempt did I obtain any growth of *Hemiselmis*. Then only three cultures resulted of which two were bacteria-free.

In general there is no difficulty in ridding a motile organism of bacteria; it is getting it to grow by itself which often presents problems. Synthetic media are inappropriate for preliminary studies, even for the few species which are known to grow in such media. It is often necessary and always safer to use natural extracts, natural sea water, soil extract, etc. Moreover, since the aim is bacteria-free cultures there is nothing to prevent the use of yeast, beef and liver extracts as well (Pringsheim, 1946).

The flagellate is probably phototrophic, but there is a strong possibility that it has one or more heterotrophic requirements in addition; these are best met by including extracts of organic materials, liver and yeast being the most efficient.

I find it convenient to prepare all stock media with an artificial sea water, sterilize them, then mix them with equal proportions of separately autoclaved natural sea water. In this way many combinations of yeast, beef, soil extracts,

peptones, etc., and other nutrients having various pH's can be quickly prepared, while at the same time ensuring a high proportion of natural sea water. Unautoclaved, but Seitz-filtered, media can also be included. The idea is to use a large range of media and isolate a large number of cells on the chance that one of the combinations will be suitable.

One word about sterility testing. As a routine, four tests are made: (i) fresh-water liquid, (ii) fresh-water agar, (iii) salt-water liquid, and (iv) salt-water agar. The medium contains yeast, beef, and soil extracts, glucose and acetate. In addition, all cultures are maintained, as far as possible, in organic media so that chance contamination becomes visible.

All the species which have been mentioned here are supra-littoral or neritic forms, and it is probable that they are easier to handle than the pelagic ones. Nevertheless, the μ-flagellates among them are delicate: they need natural sea water for their growth and show the usual tendency to burst at the least provocation. It is possible that pelagic species could be handled in the same way as the littoral forms. I have no doubt that, wherever feasible, physical manipulation is the surest and quickest means of obtaining pure cultures, and in my view it is also the easiest. The object of this note has been to record that its range of application has been extended to include some organisms as small as 5μ.

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