Short Communication

A Simple Technique for Establishing Axenic Cultures of Phytoflagellates

ICHIRO IMAI* and MINEO YAMAGUCHI

Nansei National Fisheries Research Institute, Ohno-cho, Saeki-gun, Hiroshima 739-04, Japan

(Received December 13, 1993—Accepted February 24, 1994)

Key words: phytoflagellates, red tide, axenic culture, shellfish poisoning

Many incidents of noxious red tides with fish mortalities and shellfish poisonings have occurred recurrently in Japanese coastal waters. Causative organisms of noxious red tides and shellfish poisonings are mainly phytoflagellates such as raphidophycean flagellates and dinoflagellates. To investigate the physiology and ecology of these organisms, it is essential to obtain the axenic culture¹⁾. A standard method of purifying flagellates is the 'micropipetting' or 'washing' method^{1–3)}, which is commonly employed for obtaining unialgal cultures. However, this technique is much labor-intensive and time-consuming when the axenic clonal cultures are directly obtained from natural samples, because great care is consistently needed during the course of aseptic procedures. In the present communication, we describe a simple technique to establish the axenic cultures of phytoflagellates from unialgal cultures utilizing their vertically migratory and phototactic behavior.

For the experiments establishing the axenic cultures, we used unialgal cultures of the following 13 species of marine phytoflagellates: *Chattonella antiqua*, *Fibrocapsa japonica*, and *Heterosigma akashiwo*, Raphidophyceae; *Alexandrium tamarense*, *Cochlodinium* sp., *Gymnodinium mikimotoi*

(*G. nagasakiense*), *Gyrodinium instriatum*, *Heterocapsa triquetra*, *Prorocentrum micans*, *P. minimum*, *P. triestinum*, and *Scrippsiella trochoidea*, Dinophyceae; *Eutreptiella* sp., Euglenophyceae. These flagellates were maintained in the modified SWM-3 culture medium^{4,5)} at a temperature of 22°C under a light intensity of about 50 $\mu\text{mol}/\text{m}^2/\text{sec}$ with a 14L : 10D photo-cycle.

The procedure for establishing the axenic cultures is shown in Fig. 1. The sterile culture medium of modified SWM-3 was filled in sterilized glass tubes (200 mm length and 10 mm inner diameter) with a silicon tube and a silicon cap at the lower end (Fig. 1, A). The unialgal culture (0.2–1.0 ml) of each species well grown in the above medium (late logarithmic or early stationary phase) was sucked into a syringe and gently injected into the culture medium through the silicon cap (Fig. 1, B). It is necessary to fold the silicon tube tightly so as to keep the flagellates at the bottom end. After the injection, an equal volume of the culture medium was sucked into the syringe, and then the syringe was pulled out. The glass tubes were stood and left in a clean bench for 15–20 min at a temperature of about 22°C and with a light intensity of 20–30 $\mu\text{mol}/\text{m}^2/\text{sec}$ from the ceiling (Fig. 1, C). The time of 15–20 min is considered to be enough for these flagellates to swim up to the surface of glass tubes 200 mm in length since the

* Corresponding author

Fig. 1. Procedure of the method for establishing axenic cultures of phytoflagellates utilizing vertically migratory and phototactic behavior.

- (A) Filling up sterile SWM-3 culture medium in glass tubes.
- (B) Injection of contaminated, unialgal cultures of phytoflagellates.
- (C) Leaving the glass tubes for 15–20 min with light from the ceiling for phytoflagellates to swim up from the bottom to the surface of the medium in glass tubes.
- (D) Inoculation of phytoflagellate cells at the surface of medium in glass tubes into sterile SWM-3 culture medium in flasks.

swimming velocities of marine phytoflagellates have been reported to be 1–2 m/hr^{6,7}). The phytoflagellates swam up to the surface of the culture medium in the glass tubes, and then these flagellates were aseptically pipetted (ca. 0.5 ml) into 25-ml sterile SWM-3 culture media contained in 50-ml flasks (Fig. 1, D). These flasks were incubated under the same conditions as the maintenance of the flagellates. After the growth of these species, the sterility test was made by culture method using a liquid peptone medium for heterotrophic bacteria containing 0.5 g Trypticase peptone and 50 mg yeast extract in one liter of seawater^{8,9}), into which (10 ml) a portion (0.5 ml) of phytoflagellate cultures was inoculated to examine the presence (or absence) of bacteria in the established cultures. When it was judged as axenic by the above method, further confirmation of the purity of established cultures was performed

by the direct observations with DAPI (4′6-diamidino-2-phenylindole) staining and epifluorescence microscopy^{10,11}).

Table 1 presents a summary of results obtained with 13 species of marine phytoflagellates. The axenic cultures were established in the following seven species: *C. antiqua* and *H. akashiwo*, Rhodophyceae; *A. tamarense*, *Cochlodinium* sp., *G. instriatum*, and *S. trochoidea*, Dinophyceae; *Eutreptiella* sp., Euglenophyceae. In the cases of the remaining species, it is suspected that there is a close association between bacteria and these phytoflagellates, e.g. embedding of bacteria in mucilaginous materials on the surface of the flagellates.

The present method is simpler than that proposed by Paasche¹²) utilizing the phototaxis of phytoflagellates in a delicate pipette. Although this method was not always feasible for all

Table 1. Summary of results of establishing axenic cultures of marine phytoflagellates.

Species of phytoflagellates	n ^a	Incubation time (min)	Number of growth	Number of axenic culture
Raphidophyceae				
<i>Chattonella antiqua</i>	3	20	2	2
<i>Fibrocapsa japonica</i>	3	15	0	0
<i>Heterosigma akashiwo</i>	3	15	3	3
Dinophyceae				
<i>Alexandrium tamarense</i>	3	15	3	2
<i>Cochlodinium</i> sp.	3	15	3	3
<i>Gymnodinium mikimotoi</i> (<i>G. nagasakiense</i>)	3	20	3	0
<i>Gyrodinium instriatum</i>	3	15	3	1
<i>Heterocapsa triquetra</i>	3	20	3	0
<i>Prorocentrum micans</i>	3	20	3	0
<i>P. minimum</i>	3	20	3	0
<i>P. triestinum</i>	3	20	3	0
<i>Scrippsiella trochoidea</i>	3	15	2	2
Euglenophyceae				
<i>Eutreptiella</i> sp.	2	15	2	2

^a Number of glass tubes injected with unialgal culture

phytoflagellates examined, this simple technique is worth trying to establish the axenic cultures from unialgal cultures by virtue of the needlessness of microscopy for the scientists except the specialists of microalgae.

References

- 1) Pringsheim, E.G. 1946. Pure Culture of Algae. Cambridge University Press, Cambridge, 119p.
- 2) Droop, M.R. 1954. A note on the isolation of small marine algae and flagellates for pure cultures. J. mar. biol. Ass. U.K. **33**: 511-514.
- 3) Iwasaki, H. 1967. The isolation and Culture of Unicellular Algae. Japan Fisheries Resource Conservation Association, Tokyo, 55 p. (in Japanese)
- 4) Chen, L.C.M., T. Edelstein and J. McLachlan. 1969. *Bonnemaïsonia hamifera* Hariot in nature and in culture. J. Phycol. **5**: 211-220.
- 5) Itoh, K. and I. Imai. 1987. Rafido so (Raphidophyceae), in "A Guide for Studies of Red Tide Organisms", ed. by Japan Fisheries Resource Conservation Association, Shuwa, Tokyo, pp. 122-130. (in Japanese)
- 6) Eppley, R.W., O. Holm-Hansen and J.D.H. Strickland. 1968. Some observations on the vertical migration of dinoflagellates. J. Phycol. **4**: 333-340.
- 7) Yamochi, S. and T. Abe. 1984. Mechanisms to initiate a *Heterosigma akashiwo* red tide in Osaka Bay II. Diel vertical migration. Mar. Biol. **83**: 255-261.
- 8) Ishida, Y., K. Shibahara, H. Uchida and H. Kadota. 1980. Distribution of obligately oligotrophic bacteria in Lake Biwa. Bull. Jpn. Soc. Sci. Fish. **46**: 1151-1158.
- 9) Imai, I. 1987. Size distribution, number and biomass of bacteria in intertidal sediments and seawater of Ohmi Bay, Japan. Bull. Jpn. Soc. Microb. Ecol. **2**: 1-11.
- 10) Porter, K.G. and Y.S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25**: 943-948.
- 11) Imai, I. 1984. Size distribution and biomass of bacteria in Suo-Nada, western Seto Inland Sea. Bull. Nansei reg. Fish. Res. Lab. No. 17: 183-196. (in Japanese with English abstract)
- 12) Paasche, E. 1971. A simple method for establishing bacteria-free cultures of phototactic flagellates. J. Cons. int. Explor. Mer. **33**: 509-511.