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CONTINUOUS FLOW CULTURE OF BENTHIC DIATOMS AND ITS APPLICATION TO BIOASSAY[1,2]

C. Kwei Lin

Great Lakes Research Division, The University of Michigan
Ann Arbor, Michigan, 48109

ABSTRACT

The benthic diatom, Navicula seminulum *var.* hustedtii *Patr. was isolated from the field and studied in laboratory cultures. Experiments were conducted to determine the suitability of Millipore membranes and solidified agar substrate for diatom colonization under continuous flow conditions. Diatom colonization showed large variation and low reproducibility on Millipore membranes of different materials and pore sizes. Solidified agar substrate supported stable and reproducible colonization and is nutritionally neutral, translucent, homogeneous and easy to sample. The diatom colonization process on agar substrate involved four growth phases: i) pioneer; ii) exponential; iii) steady state; and, iv) vanishing. The culture system was also used in bioassay, testing the toxic effect of copper on the growth of benthic diatoms. The proposed method provides a useful means for studying autecology of benthic diatoms as well as for bioassay work.*

Key index words: artificial substrate; benthic diatom; bioassay; continuous culture; copper toxicity; colonization; Navicula; *substrate, artificial*

Benthic diatoms form an important component of primary producers in shallow portions of flowing and standing waters. Numerous field investigations have been made on production, species composition and seasonal succession of benthic diatom communities on natural and artificial substrates (10). Studies on production and metabolism of benthic diatoms in relation to environmental conditions have also been carried out in semi-controlled laboratory systems (13,17). The process and kinetics of diatom colonization on substrates, however, are not well understood.

Diatom community structure, which sometimes exhibits great sensitivity to environmental perturbation, has been recommended as a useful indicator for water quality (7,16,21). It is well recognized that the bioassay approach, using a single species sensitive to pollution, offers an efficient and effective means of evaluating water quality. Many other aquatic organisms, including fish, zooplankton and phytoplankton, have been adopted as standard laboratory test organisms. For example, the green alga *Selenastrum capricornutum* Printz, a freshwater phytoplankter, is widely used for nutrient bioassay (6). However, suitable indicative species and uniform methods have yet to be developed for periphytic systems. Preliminary application of toxicity bioassay using a single species of diatom in static and flow-through culture systems was developed by Patrick (15,16). In the flow-through culture, Patrick used Millipore membranes as artificial substrates for diatom colonization; earlier, a membrane substrate was also used for filamentous bluegreen algae by Watts and Harvey (20).

The present work describes diatom colonization on Millipore membranes of different materials and pore sizes. The colonization process on improved substrate and the potential bioassay method using solidified agar are also described.

METHODS AND MATERIALS

Isolation and culture. Initial culture of *Navicula seminulum* var. *hustedtii* Patr. was isolated from a stream in Chester County, Pennsylvania. Organisms growing on rocks were scraped into a beaker which contained modified Chu 14 medium (15), and were blended with a Waring blender at ca. 17,000 rpm 1–2 min to separate diatom colonies into a single-cell suspension. Microscope examination revealed that few cells were fractured by the blending process. With an inoculating loop, the diatom suspension was streaked onto petri dishes containing 2% agar enriched with culture medium. The plates were incubated at 18 C and 3,000 lx for 2 wk, during which the colonized diatoms on the plates were reisolated to fresh plates. A culture of single-species diatoms was obtained for 2–3 subcultures on agar plates. Actively growing cells were subsequently transferred to a liquid medium and incubated at the same temperature and light regime with stirring.

Continuous flow culture. The basic components of the culture system (Fig. 1) include: i) culture medium pump (Technicon proportioning pump model II, Tarrytown, New York) capable of delivering the medium in multi-channels with variable pumping rates; ii) culture flasks (125 ml filter flask) connected by T-shaped glass tubes for each treatment; and, iii) medium reservoir consisting of a 20 l pyrex carboy with a vent tube and medium connector at the top. The culture medium was introduced from the medium reservoir into each flask ca. 10 mm above the substrate through a Tygon tube at a rate of 6 $ml \cdot h^{-1}$. The effluent was discharged into a drain collector through the flask side arm.

To determine substrate suitability for continuous culture of benthic diatoms, several Millipore membranes and solidified agar were tested. The membrane materials included glass fiber filter (borosilicate), Duralon (nylon), Celotate (cellulose acetate), and MF (mixed cellulose esters). The effect of pore size on

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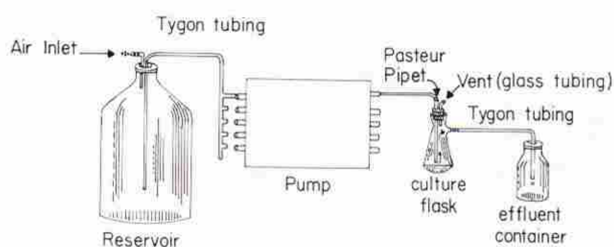

FIG. 1. Diagram of basic units in continuous flow culture system.

colonization was determined using MF membranes of pore size 0.22, 0.45, 1.2 and 5.0 μm. To remove possible impurities, the membranes were washed and soaked in distilled water overnight before inoculating with diatom cells. After rinsing with distilled water, each membrane was inoculated with 1.2 ml of diatom stock culture, containing 6×10^3 cells in homogenized suspension, by gentle vacuum pressure at ca. 5 lb/in^2. By calculation, this inoculation gave an initial density of ca. 360 cells·cm^{-2}. The membrane was transferred into the bottom of a culture flask which was connected to the pump and the algae were cultured in the flow-through medium for periods of 9–17 days.

At the end of each experiment, membranes were taken out and the diatom cells removed by scraping with a spatula and cells were counted with a hemacytometer (8). The total number of detached cells collected in the collecting bottle was also counted. The number of cells growing attached and detached from membranes was expressed by unit area (1 cm^2)/day.

Agar substrate was formed by plating 8 ml sterilized hot agar (Difco Noble) at the bottom of a 125 ml filter flask. A firm, homogeneous and translucent substrate 5 mm thick formed upon solidification. To each culture flask, ca. 2 ml of unicellular suspension of stock culture containing 10^4 cells was introduced onto the agar surface. The flask was swirled gently to spread the suspension evenly over the substrate. After 2 days of standing incubation, flasks were connected to the pump and the continuous flow culture begun. Three hardnesses of agar substrate (1, 2, 3% w/w agar concentration) were tested for colonization rates.

To determine the diatom growth on an agar surface during the experimental period, subsamples were taken by inserting a 5 ml cut-off sterile plastic pipet into the agar substrate. Numbers of diatom cells growing on the sampled agar disc were counted using a hemacytometer, after liquifying and removing the agar by dissolving it in 60% nitric acid. The acid, which did not cause noticeable separation of diatom valves, was removed by repeated washing and centrifuging. The total population that colonized on the entire agar surface in each flask was calculated by multiplying the mean cell number of the samples and the ratio to the bottom area of the entire flask. Rates of population increase between sampling dates were calculated by the following formula (8):

$$k = \ln(Nt_2/Nt_1) \cdot (1.443/t),$$

where k is the mean number of cell divisions·day^{-1}; Nt_2 and Nt_1 are mean cell numbers per flask at times t_2 and t_1 respectively; and t is the time interval (days).

The colonization process in continuous cultures of *N. seminulum* var. *hustedtii* on agar was observed in detail for 4 wk. This culture system was also used in bioassay experiments testing the toxic effect of copper on diatom growth. Three concentrations of copper triethanolamine complex (algicide manufactured by Applied Biochemists, Inc., Mequon, Wisconsin) were filled in separate medium reservoirs and administered into the continuous flow system for 14 days after which the algal yield was determined by both cell numbers and chlorophyll *a* (1).

TABLE 1. *Cell numbers attached to and detached from membrane materials and agar surfaces. (Numbers based on cells·cm^{-2}·day^{-1}.)*

Substrate variation	Cells attached			Cells detached		
	Mean	1 SD	Confidence interval (95%)	Mean	1 SD	Confidence interval (95%)
Millipore membrane material $\times 10^3$ cells						
Glass fiber	9.61	3.59	32.24	2.18	0.09	3.22
Duralon	22.43	4.10	36.82	1.47	1.31	11.80
Celotate	2.88	1.35	12.12	—	—	—
MF	21.79	6.79	60.97	3.40	1.22	10.93
Pore sizes (MF) $\times 10^3$ cells						
0.22 μm	17.30	0.58	5.15	16.68	4.96	44.32
0.45	2.92	2.64	23.52	11.74	6.71	60.31
1.2	4.08	7.58	45.93	—	—	—
5	16.48	7.58	68.01	16.68	2.02	18.12
Agar (% hardness) $\times 10^4$ cells						
1	27.72	1.38	12.35	0.044	0.003	0.035
2	24.72	1.65	12.50	0.032	0.002	0.016
3	23.00	0.28	2.44	0.015	0.001	0.014

RESULTS AND DISCUSSION

Effects of Millipore membranes on colonization. The size of populations that attached to the substrate of the four pore sizes varied considerably and inconsistently, with wider confidence intervals (95%) in the larger pore size membranes (Table 1). Membranes of 0.22 and 5 μm pores tended to support more growth than those of 0.45 and 1.2 μm. The numbers of diatom cells attached to the membranes also varied considerably among several materials. Colonization was greater on nylon and cellulose ester membranes than those of cellulose acetate and borosilicate (Table 1). Marked variations in attached cell numbers, as indicated by the broad confidence limits, also occurred between the duplicates of all materials tested. Although the confidence limits might be narrowed by increasing the number of replicates, the main factor causing the inconsistency was from the dislodging of inoculum and subsequent growth, as indicated by the number of detached cells. Variation in duration and pressure applied in the vacuum inoculation process did not enhance initial diatom attachment. Diatom cells physically "fastened" by vacuum to the substrate did not show growth within 1 wk after inoculation, indicating that the prolonged lag phase was probably due to the physical damage from the pressure applied in filtration. Filtering of natural phytoplankton samples with vacuum pressure has been reported to increase leakage of cellular content and removal of extracellular products (2). Many diatoms produce polysaccharides as extracellular products (9,12) and removal of extracellular compounds may affect the diatom's initial ability to attach. Secretion of a cementing substance is believed to be instrumental in attachment (22) and flocculation (18) for aquatic bacteria.

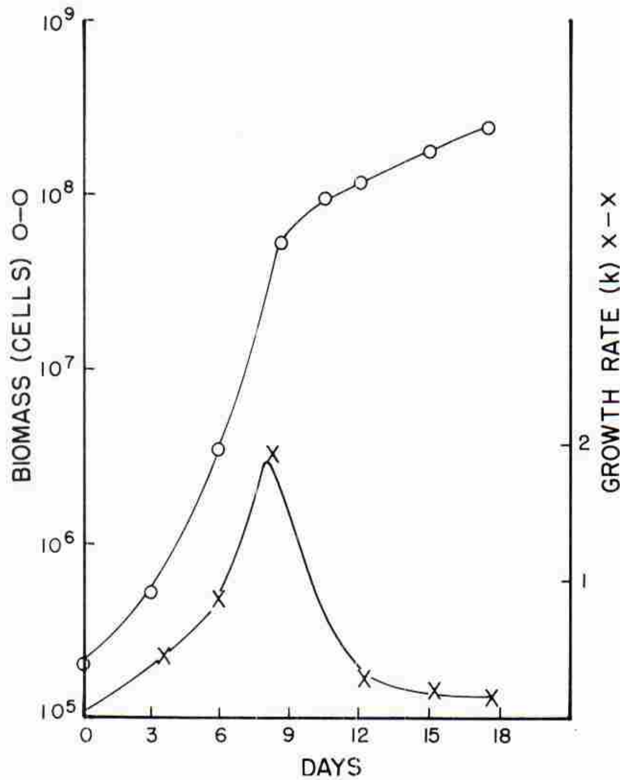

FIG. 2. Development of population size and change of growth rate of *Navicula seminulum* var. *hustedtii* in continuous flow culture.

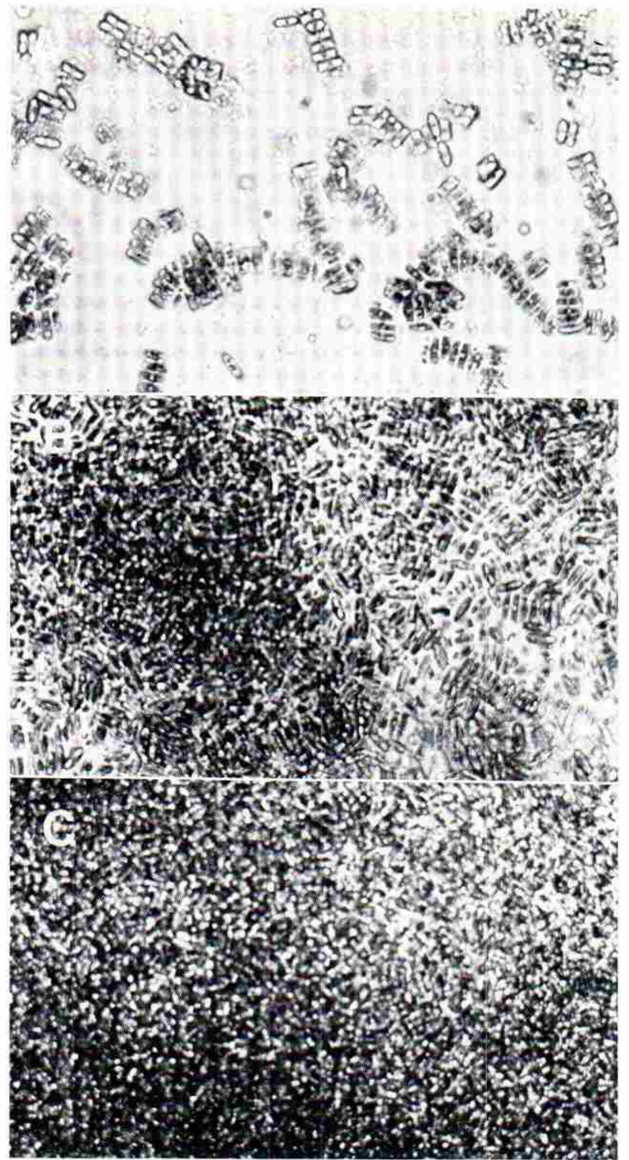

FIG. 3. Microphotographs of *Navicula seminulum* var. *hustedtii* showing different stages of colonization on agar substrate in continuous culture. × 300. A. Initial stage forming single-layer horizontal chains of cell. B. End of exponential growth with mono- or bi-layer cell coverage. C. Linear growth phase with multilayer cell coverage.

Effect of agar hardness. Diatom populations that colonized on wetted agar surfaces of all hardnesses were much greater and less variable than those on membranes (Table 1). The cell numbers detached from each flask in the flow-through system, ranging from 1.8×10^4 to 2.4×10^4, only constituted <0.5% of the attached populations. A slightly greater population growth occurred on 1% than on 3% agar, and indicates the substrate is more likely to exchange nutrient between liquid and solid phases than a harder surface. Microscope examination of algal growth on agar plates of three hardnesses revealed that diatom cells were able to bore into the softer agar quite readily. In nature, a similar vertical movement of epipelic algae has been observed, particularly among diatoms that dwell on mud surfaces (5,19). Soft agar would provide a convenient substrate for investigating vertical movement of epipelic species.

Colonization process. The population development on a uniform surface involved several growth phases (Fig. 2). During the first 5 day period of flow-through culture, the population increased slowly from an initial doubling rate of 0.2 to a rate of 0.7. The brief lag phase during the pioneer stage was probably due to the recovery from preparation of inoculum as well as acclimation to the fresh growth medium. A similar lag phase was observed in the colonization of mixed species by Kevern et al. (11) in their estimation of benthic productivity on plexiglass plates used as substrates in laboratory streams.

In the initial colonizing state, diatom colonies established themselves at dispersed points on the wetted surface at the end of the 5 day period (Fig. 3A). With few exceptions the cells divided in one dimension and formed long chains of daughter cells that attached themselves on girdle sides to the substrate with abundant translucent gelatinous material.

During the process of developing single-layer colonies, algal cells received continuous nutrient supplies and adequate illumination; the population grew

TABLE 2. *Effect of copper triethanolamine under continuous flow system on 1% agar substrate.*

	Copper concentration—ppm			
	Control (0)	0.5	1.0	3.0
cells/flask $\times 10^8$	3.65±0.21	2.05±0.35	0.65±0.21	0.33±0.13
chlorophyll *a* μg	290.88±0.40	126.41±8.05	34.05±0.26	0.17±0.07
cells/effluent $\times 10^4$	4.20	5.50	0.10	0.13
mean number of divisions	15	14	12	11

exponentially with a maximum growth rate of 1.98 between days 6 and 8. At the end of this period the available substrate surface was almost entirely covered with a layer of cells, totalling ca. 5×10^7 cells. Under this restricted spatial arrangement the crowded cells colonized in a vertical dimension forming new layers of cells on top of the previous growth (Fig. 3B). The cells attached to each other on either valve or girdle surfaces. With increasing numbers of cell layers, the growth rate of the entire population shifted from exponential to linear, falling to 0.5 by the 10th day and to 0.15 by the 12th day. This low level of population growth, characterizing a steady state, was sustained until the end of the experimental period (17 days). The multilayer of diatom coverage (Fig. 3C) formed during this phase attenuated the light transmittance at the bottom of the flask to 2–3% of the incident level. Also the cell size became progressively smaller with successive division. The time period required by benthic diatoms to complete the colonization of a given area of substrate is apparently dependent on the species involved as well as environmental conditions (4,14).

The numbers of detached cells collected in the washout increased as the colonized population sizes increased, but never exceeded 0.5% of the colonized population. However, in prolonged culture (up to 25 days) the thick sheath of algal growth began to break away or slough off from the attachment surface. Sloughing is considered an important factor in benthic diatom succession in nature. Kevern et al. (11) observed that decay of underlying layers of a thick periphyton mat caused portions of the mat to float away. Such massive dislodging is probably the cause of abrupt and complete disappearance of the benthic flora observed in natural streams (3).

Preliminary application of continuous flow system. Copper triethanolamine has been used as an algicide to control nuisance growth in natural waters. In the presence of 3 ppm growth of *Navicula seminulum* var. *hustedtii* was reduced to ca. one-tenth that in the control under a continuous flow system on agar at the end of the 12 day assay period (Table 2). The toxic effect of copper on diatom growth, as measured by chlorophyll *a* production, was drastically increased at the highest concentration (3 ppm), but was not parallel to the change in cell numbers. At this copper concentration, chlorophyll *a* was bleached severely. A small proportion of cells (<0.1%) was found in the effluent in all treatments.

CONCLUSIONS

Colonization of benthic diatoms on Millipore membranes varies markedly among different pore sizes and materials. Large loss of inoculum from membrane substrate resulted in low reproducibility that reduced its applicability as an artificial substrate. The solidified agar substrate possesses the following properties: i) homogeneous firm surface structure and composition; ii) nutritionally neutral, non-toxic, hydrophilic and sterilizable material; iii) ability to receive inocula of single species in known quantities; and, iv) convenient methods for taking representative samples. If fully developed, this continuous flow culture system will provide a simple and inexpensive method for investigating the autecology of benthic diatoms, as well as for bioassay work.

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EFFECT OF BLUE-GREEN LIGHT ON PHOTOSYNTHETIC PIGMENTS AND CHLOROPLAST STRUCTURE IN THE MARINE DIATOM *STEPHANOPYXIS TURRIS*[1]

S. W. Jeffrey[2]

C.S.I.R.O. Division of Fisheries & Oceanography, Box 21, Cronulla, N.S.W. 2230, Australia

and

Maret Vesk

Electron Microscope Unit, Sydney University, N.S.W. 2006, Australia

ABSTRACT

Blue-green light increased the chlorophyll concentration and chloroplast number of cells of Stephanopyxis turris *(Grev.) Ralfs, compared to white light controls. Light fields for growth were 400 $\mu W \cdot cm^{-2}$ (12:12 LD cycles). Chlorophyll increased up to 100%/cell, but no change in the ratio of chlorophylls to major carotenoids occurred. The effect was, therefore, not that of complementary chromatic adaptation. At the same time, blue-green light enhanced the photosynthetic fixation of CO_2. At the ultrastructure level, an increase in, and rearrangement of, the thylakoid system occurred.*

Key index words: blue-green light; chloroplast pigments; chloroplast structure; diatom; light; pigments, chloroplast; Stephanopyxis

Light penetrating the euphotic zone of the ocean consists predominantly of low intensity blue-green radiation, with a maximum at ca. 480 nm (27,45). While biologically significant levels of light penetrate to a depth of about 200 m, all red light is eliminated below depths of 5–10 m. Unicellular marine phytoplankton are normally found in highest concentration between 25–150 m, and are rarely found in significant concentrations at the ocean surface exposed to the full visible spectrum of white light. The algae use a variety of photosynthetic pigments, carotenoids, biliproteins and accessory chlorophylls to trap this blue-green radiation efficiently. Light quality is known to regulate the relative concentrations of the biliproteins in some red and blue-green algae (5,6,13). It is not known to what extent this phenomenon (complementary chromatic adaptation) can also regulate the relative concentration of the blue-green absorbing carotenoids in diatoms, chrysomonads and dinoflagellates, the major phytoplankton. Halldal (14) suggests, from the constancy of spectral response of brown algae collected from various depths, that the pigment system of these algae

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[2] Address for reprint requests.

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