

THE DEVELOPMENT OF AN ALGAL BIOASSAY PROCEDURE
USING STIGEOCLONIUM SUBSECUNDUM AND THE DEMONSTRATION
OF THE EFFECT OF INTERMITTENT CHLORINATION ON AN
ATTACHED FILAMENTOUS ALGA

by

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INTRODUCTION

The development of algal bioassay techniques in the United States was a result of the increasing concern of biologists and scientists in general to the alteration of natural waters by the introduction of substances which were toxic or stimulants to algal growth. The advantage of using a living organism as a bioassay organism lies in the fact that the organism is exposed to the total interactions of the various substances in the water. Isolation and characterization of the individual chemical substances in the water would be costly, time consuming, and the combined synergistic or antagonistic effects would still be unknown. The potential for algal growth or death in natural waters could be reflected by laboratory algal cultures using the particular water in question. In 1971, an algal bioassay procedure was developed and adapted as a standard procedure by the U. S. Environmental Protection Agency (U. S. Environmental Protection Agency, 1971). The bioassay utilized three algal species as indicator organisms for freshwater; a unicellular green alga (Selenastrum capricornutum, Chlorophyta: Oocystaceae), a colonial blue-green alga (Microcystis aeruginosa, Cyanophyta: Chroococcaceae), and a filamentous blue-green alga (Anabaena flos-aquae, Cyanophyta: Nostocaceae). These organisms, when grown in the standard algal medium, reflect the reaction of natural algal populations to the addition of either toxic or stimulatory substances (Maloney and Miller, 1975; Miller and Maloney, 1971; Miller, et al., 1974; and Payne, 1975). There are many shallow, lotic aquatic environments in the United States where the dominant algal biomass is

attached to a substrate. Whitford (1960) demonstrated that water, flowing over attached algae was physiologically more 'rich' in nutrients than the same water in static situations. Theoretically, this was caused by the constant replenishing of the water and nutrients next to the cell surface allowing a maximum diffusion gradient to exist between the organism and the environment. Attached algae in a stream or river also reflect to some extent the quality of water which flows over it (Cattaneo, 1975). It would seem, then, that an algal bioassay system utilizing algal cells attached to a substrate in a continuous flow system (to simulate shallow lotic environments) would be a complement to the bioassay system using suspended algal cells. Using this system, information could be obtained on the possible effects of stimulating or toxic substances on attached algae and periphyton in streams in the same fashion that the flask bioassay, using suspended cells, predicts the effects on lentic environments.

An algal bioassay system employing algal cells attached to a removable substrate would allow light microscopic observation of the cells both during and after the introduction of a specific toxicant. This is especially important for sublethal effects, which might not be manifested by the analysis of growth alone. The most important aspect of this system is that the algae would be characterized in terms of biomass per area rather than cells per volume. This is important, as the amount of algae present could have a definite effect on the toxicity of a specific substance (Steemann-Nielsen, et al., 1969).

The interest in the environmental impact of chlorine stems from

its use as an antifouling agent in the cooling systems of electric generating plants and as a disinfectant in the treatment of sewage. Both operations discharge a chlorinated effluent into a receiving stream, lake, or coastal water. Studies have been conducted to determine the effects of chlorine (both free and residual) on phytoplankton and phytoplankton productivity. Brook and Baker (1972) used the light and dark bottle oxygen technique to demonstrate that the chlorination of cooling water depressed rates of photosynthesis from 50% to 90% in freshwater phytoplankton present in the condenser discharge and the discharge canal of a steam electric generating plant. Using the C-14 technique for measuring primary productivity, Carpenter, et al. (1972) found 98% reductions in carbon fixation by estuarine phytoplankton entrained in the cooling water of a steam electric generating plant. In a similar situation, Hamilton, et al. (1970) also found reductions in photosynthetic rates (using the C-14 technique) in effluent cooling water. However, he did not find any reduction of primary productivity where the discharge canal from the power plant entered the bay. In addition, Hirayama and Hirano (1970) in laboratory experiments conducted with the marine phytoplankters Chlamydomonas sp. and Skeletonema costatum, concluded that power plants discharging chlorinated cooling water into the open sea would not cause great damage to marine phytoplankton in the immediate vicinity of the discharge.

Three papers by Kott (Kott, et al., 1966; Kott and Edlis, 1969; and Kott, 1969) explored the use of bromine and chlorine as algicides for planktonic algae. The conclusions of all three papers were the same; bromine was much more toxic to algae than chlorine, and a

combination of both halogens was more toxic than either halogen alone.

Many power plants and sewage treatment plants discharge into shallow streams in rivers. In these situations, the predominant algal biomass is not planktonic, but rather benthic or attached to the substrate. The literature concerning the effect of chlorine on attached algae is rare. Markowski (1960) reported the growth of Enteromorpha intestinalis and E. ahneriana attached to concrete slabs located at the outfall of a cooling water effluent from a coastal steam electric generating plant. Because the author's aim was simply to observe the colonization of concrete slabs placed at the inlet and outfall of the power plant, no attempt was made to determine what effects, if any, the chlorinated effluent had on the attached algae. Betzer and Kott (1969) observed the effect of chlorine on the filamentous, attached alga Cladophora. However, the aim of their study was to compare the algicidal effect of halogens and copper sulfate on this alga. Although bioassays were performed, they were static bioassays in which filaments of 100 cells each were placed in beakers containing the water from which the alga was taken. Solutions of sodium hypochlorite were added to produce concentrations of chlorine in a range of from 1 to 20 ppm free chlorine. At the end of a specific contact time, the algal filaments were removed, rinsed, examined under a light microscope, and placed in another beaker containing the water of its original habitat. Various changes in color, cell wall damage, and viability were recorded by the authors with increasing chlorine concentrations and contact time. Using this method, the authors determined that 10 ppm chlorine with a

contact time of 2 hr was sufficient to kill all algal cells. The Cladophora cells used for the static bioassay came from flowing water, but there was no mention by the authors of the possible influence of the flowing water to the toxicity of chlorine to the attached alga.

One purpose of this research was to design and build an algal bioassay system which utilized an attached, filamentous alga. This bioassay system was to be constructed out of readily available material so as to make its use by other laboratories as easy as possible. A second purpose was to use the algal bioassay system to demonstrate the toxic effects of intermittent chlorination on an attached alga, such as might occur in a stream receiving discharged cooling water from an electric power plant. The technique used in this study was unprecedented.

METHODS AND MATERIALS

The Bioassay Organism

The alga used in this study, Stigeoclonium subsecundum Kutz., was selected as the organism of choice because of its ability to grow well on a variety of inorganic media as well as the ability to produce great numbers of zoospores which could attach to glass slides and grow into filaments. S. subsecundum offered three different cell types for study; basal or holdfast cells, erect filaments, and zoospores. Basal cells are somewhat protected from exposure to toxicants as they have a portion of the cell surface against the substrate. Cells of erect filaments would be surrounded by water on all sides, and a transition would exist between cells close to the glass and those further away leading to the terminal cells. Zoospores (flagellated unicells that escape from the filaments) are planktonic and zoospore production was also used to assess sublethal effects of chlorine on the alga.

S. subsecundum was isolated from a riffle in the Roanoke River at Riverside, Virginia (latitude 37° 14' 15" N., longitude 80° 10' 30" W.). Cladophora filaments from the riffle were placed in 250 ml flasks containing 100 ml of ASM-1 medium (Gorham, et al., 1964) and within 4 days, the Cladophora cells had died and the Stigeoclonium cells became the dominant alga in the cultures. The cultures were blended in a Waring Blender (21,000 rpm) and fresh ASM-1 medium added to produce a dilute suspension of cells. Drops of this suspension were spread on plates of ASM-1 solidified with agar (1.5%). After 3 weeks, isolated filaments of Stigeoclonium were observed growing on the agar surface. The

filaments were examined under a light microscope to insure that other alga contaminants were not present, after which the filaments were picked from the agar surface with a sterile wire and placed in liquid ASM-1 medium. The cultures resulting from the transfers were again checked after two weeks by microscopic examination to insure that the cultures were unialgal.

Preliminary studies were conducted prior to the toxicity testing to provide 1) a method for culturing and attaching the algal cells to glass slides, 2) a method for increasing the algal biomass on the glass slides, and 3) the mathematical determination of the relation between pigment extract and biomass as mg dry weight.

Culturing and Attachment of the Algal Cells to Glass Slides

A 'culture cycle' was necessary for the production of a continuous supply of cells which were as close to the same physiological state as possible. The culture cycle began with 100 ml cultures of Stigeoclonium subsecundum in 250 ml flasks on a shaker table moving at 100 oscillations/min at 20°C in a 12:12 light-dark photoperiod with a light intensity of 500 ft-c. Cool white fluorescent light was used throughout this study. After 2 weeks, cultures were poured into a 1.0 liter graduated cylinder and concentrated by settling overnight in a refrigerator at 4°C. The supernatant, usually between 700 and 800 ml, was removed by aspiration. The concentrated cell suspension was poured into a Waring Blender and sterile medium was added to bring the volume of the cell suspension to 500 ml. After blending 5 min (21,000 rpm), aliquots of the suspension were used to inoculate 100 ml of sterile medium in 250 ml flasks.

Enough cell suspension was used to produce an absorbance of 0.1 in each flask. These flasks were then returned to the shaker table and the culture cycle continued. The remainder of the blended cell suspension (or portion thereof) was used for the attachment of the algal cells to the glass slides or 'stigeoclonimeters' (Fig. 2). The stigeoclonimeters were constructed from four 5 mm glass rods sealed to a 1 x 3 in. (2.54 x 7.62 cm) glass microscope slide for a top and one half of a glass microscope slide for the bottom. The stigeoclonimeter was held together by Dow-Corning Silicone Rubber Sealer (Dow-Corning Corp.). The design of the stigeoclonimeter allowed them to be placed on 5 mm glass rods attached to the inside of the battery jar test chamber (The construction of which will be described later).

As previously stated, separate experiments were performed to find the best set of conditions for the attachment of the algal cells to the stigeoclonimeters and to make the attachment as uniform as possible. Algal suspensions used for this study ranged from an absorbance of 0.2 to 1.2 at 412 nm with light intensities of 150 and 500 ft-c. The length of time the algal suspension was in contact with the stigeoclonimeters was also varied from 7 to 12 days. Both one and two week old cultures from the culture cycle were used for the experiments. These experiments produced the following procedure which was used to attach the algal cells to the stigeoclonimeter surface.

- a. Fifty stigeoclonimeters were placed in a 17.25 x 11.375 x 2.25 in. (43.8 x 28.9 x 5.7 cm) aluminum roasting pan. One liter of ASM-1 medium was added, the pan was covered with aluminum foil

and autoclaved for 10 min on the day prior to blending the concentrated algal suspension at 0800 hr.

- b. Sterile medium was added to the blended algal suspension to produce an absorbance of 0.85. One liter of this suspension was then added to the sterilized stigeocloniometers and algal medium in the aluminum pan.
- c. After the algal suspension was poured into the pan and mixed, a plastic transparent covering (Stretch-n-Seal, Colgate-Palmolive Co.) was placed over the top of the pan and the edges sealed around the pan lip with masking tape.
- d. The pan was covered with aluminum foil and placed in an environmental control chamber at 20°C.
- e. The following morning at 0800 hr., the aluminum foil was removed and the algae began a 12:12 light-dark photoperiod at 150 ft-c.
- f. The algal suspension in the pan was mixed once daily at 1200 hr by lifting and lowering one side of the pan several times.
- g. After 9 days, 500 ml of the algal suspension in the pan was withdrawn and replaced with 500 ml of sterile ASM-1 medium.
- h. On day 13, stigeocloniometers were removed from the pan and excess, unattached cells were removed by placing the stigeocloniometer surface under the water in the artificial stream and moving it rapidly back and forth. A small brush was used to clean algae from the upper surface of the piece of glass which formed the bottom of the stigeocloniometer. They were then placed in an artificial stream for specific periods

of time to increase the algal biomass and permit the algae to grow in the same water in which the toxicity tests would be conducted.

Figure 1 is a diagrammatic sketch of the procedure from flask culture (1) to toxicity testing of the attached algae in the battery jar test chambers (6).

The Medium for Growth and Toxicity Testing

While ASM-1 was the algal medium used for the culture cycle and attachment phase, flowing dechlorinated tap water was the medium used to increase the algal biomass on the stigeocloniometers in the artificial stream (described later) and as the medium in which the toxicity tests were conducted. The dechlorinated tap water was produced by running tap water through charcoal columns which removed all but 0.01 to 0.05 ppm residual chlorine. This small residual was removed by first bubbling air through a sodium thiosulfate solution (715 g/l), then passing that air through the water containing the small chlorine residual. The chlorine residual was completely removed in this manner with no evidence of thiosulfate contamination of the water. Vigorous bubbling worked best and an air flow of 4000 cc/min was used.

Blacksburg tap water is soft (Table 1) and is taken from the New River. Phosphate (in the form of sodium tripolyphosphate) is added at the water treatment plant for corrosion control. It is not known whether or not S. subsecundum can utilize the polyphosphate, but the polyphosphate does hydrolyze to the ortho form, which can be utilized by the alga.

Increasing Algal Biomass on the Stigeocloniometers

A separate study was conducted to identify the best possible way to increase the amount of algal biomass on the stigeocloniometer surface. Initially a flat, fiberglass pan 24 x 36 x 1.25 in. (60.96 x 91.44 x 3.18 cm) with dechlorinated tap water flowing through it was tried. The stigeocloniometers were placed in the middle of the pan side by side to form two rows each of 25 stigeocloniometers parallel to the long axis of the pan. Because of problems with this procedure (reviewed in results and discussion) an artificial stream was built (Fig. 3) in order to increase the algal biomass on the stigeocloniometers in a more uniform manner.

Dechlorinated tap water entered through the end of the stream and was dispersed through holes in a rigid plastic tube (A in Fig. 3) which ran the width (6 in. or 15.25 cm) of the stream. The stigeocloniometers were placed in the artificial stream at C, against the barrier (B in Fig. 3). The long axis of the stigeocloniometers were parallel to the barrier, so that two rows of 25 stigeocloniometers were formed. The top of the barrier above C was identical to the thickness of the stigeocloniometer (7 mm). The depth of the water over the stigeocloniometers was 5 mm with a flow of 650 cc/min. Light intensity at the surface was 500 ft-c with a 12:12 light-dark photoperiod. After a specific time period in the artificial stream, and hence a specific biomass increase, the stigeocloniometers with their attached algae were placed in the battery jar test chambers (Figs. 4 & 5) for the toxicity testing.

Test Chambers and the Continuous Flow System

Used in the Toxicity Studies

Battery jars 12 in. (30.48 cm) tall and 6 in. (15.24 cm) in dia. were used as test chambers. Figures 4 and 5 show the construction of the test chambers utilized in the continuous flow system. The vertical glass rods shown in Fig. 4 were omitted from Fig. 5 to facilitate clarity of the diagram. Clear Dow-Dorning Silicone Rubber Sealer (Dow-Corning Corp.) was used exclusively in the construction of the continuous flow test chambers and will be referred to as rubber sealer. Seven 5 mm glass rods each 11.5 in. (29.21 cm) long were attached to the inside of the battery jars at the bottom by rubber sealer (B in Fig. 4). Prior to attaching the glass rods, two stigeocloniometers were placed on each glass rod (the stigeocloniometers were used as guides) and the rod was then placed inside the battery jar while the battery jar rested on its side. It was intended that the edges of the stigeocloniometers should be in contact with one another so as to form one continuous surface of 42 in^2 (271 cm^2). As soon as this was accomplished, the bottom of the glass rods were cemented in place. Glass rods (7 mm dia.) and 0.25-.05 in. (0.635-1.27 cm) long were attached to the side of the 5 mm glass rods with rubber sealer near the bottom to act as 'stops' (F in Fig. 4) keeping the total stigeocloniometer surface equidistant between the surface of the water and the bottom of the battery jar.

The tops of the battery jar test chambers were circular pieces of 0.5 in. (1.27 cm) plywood (C in Fig. 4), 6.5 in. (16.51 cm) of dia. with

a 2.375 in. (6 cm) dia. center hole (E in Fig. 4). Three additional one inch (2.54 cm) dia. holes were placed in the top; two of these opposite each other with the third equidistant between the first two (D in Fig. 4). The top was secured by a 20.4 in. (51.8 cm) piece of plastic tubing 0.25 in. (0.635 cm) outside dia. (G in Fig. 4) attached around the underside of the top with rubber sealer. A thin coat of rubber sealer was applied to all wood surfaces prior to use. The central hole in the wooden top was occupied by a no. 13 rubber stopper (F in Fig. 5) with a 0.5 in. (1.27 cm) dia. hole through its center. A 0.875 in. (2.2 cm) dia. bearing (G in Fig. 5) was mounted over the top of this hole and held in place by rubber sealer. On the bottom of the rubber stopper, a circular piece of plexiglass (not shown) with a 7 mm hole in the center acted as a guide for the shaft of the paddle (a 7 mm glass rod) that extended up through the rubber stopper and the bearing. The difference in diameter between 7 mm glass rod and the inside diameter of the bearing was made up for by wrapping a piece of plastic electrical tape around the glass rod at that point.

The paddle itself (J in Fig. 5) was composed of four 7.75 x 0.375 in. (19.7 x 0.953 cm) plexiglass blades 0.318 cm thick. The bottom of the paddle was 0.125 in. (0.318 cm) above the bottom inside the battery jar with 0.875 in. (2.2 cm) plexiglass circles located on the shaft at both ends of the paddle blades. The shaft of the paddle (I in Fig. 5) was connected to the shaft of the motor by rubber tubing.

Water entered the battery jar through and was regulated by a stopcock (C in Fig. 5). Constant head pressure was maintained by a

mixing chamber (not shown) with an overflow located about 2.5 ft (76 cm) above the battery jars. The dechlorinated tap water was distributed to the test chambers from the bottom of the mixing chamber by 'Y' tubing connectors and rubber tubing. To avoid extensive modification of the battery jars, a siphon outlet system was made to maintain a constant level of water in the battery jar. The effluent was made of 6 mm inside dia. glass tubing (D in Fig. 5) with the mouth of the effluent tube bent into the current created by the paddle. After coming through a no. 4 rubber stopper in the top of the battery jar, the 6 mm glass tube was connected to a 4 mm inside dia. bent glass tube (K in Fig. 5) which enabled the water to siphon out of the battery jar into a plastic funnel (E in Fig. 5) which was connected to a drain. The reduction in size of the effluent tubing was necessary to prevent small bubbles from becoming trapped and forming an air-lock. The end of the 4 mm tube was placed through a no. 10 rubber stopper (L in Fig. 5) which rested in the plastic funnel. The funnel was supported by a ring stand (not shown) such that the level of the water in the battery jar could be adjusted by the vertical position of the funnel.

The motors used to turn the paddles in the battery jars were variable speed 115 volt, AC/DC, 60 cycle electric motors which were connected to a 0.75 amp variac. During this study, the speed of the paddle was 550 rpm, which gave a current speed at the outer edge of the paddle of 0.75 m/sec.

The Chlorine Solution

The rotation of the paddle created a vortex at the water surface which provided an excellent point for the introduction of the chlorine solution as mixing occurred almost instantaneously. This was confirmed by dye tests. A 5 mm hole in the no. 13 rubber stopper (H in Fig. 5) permitted the addition of the chlorine solution into the vortex. The chemistry of chlorine and hypochlorite salts in water was recently reviewed by Palin (1974). A variable speed pump was used for the introduction of one liter of a free chlorine solution. A stock calcium hypochlorite solution was prepared for each test by dissolving 500 mg of the granular salt in two liters of distilled water. The pH of the resulting solution was adjusted with concentrated HCl to 7.4 and the solution was immediately poured into amber bottles and stored at 4°C until needed. Various portions of this stock solution were used to make up the solution which was introduced into the test chamber. Because the system was continuous flow with water constantly entering and leaving, the introduction of chlorine for 30 min caused an increase in chlorine concentration in the test chamber to a maximum concentration at the end of 30 min. The maximum concentration attained in the test chamber depended on the concentration of chlorine in the one liter bottle connected to the peristaltic pump. For example, when diluted to one liter, 12 ml of the stock solution gave a concentration of 10 ppm free chlorine. When this solution was introduced into the 3700 ml volume of water in the test chamber over a period of 30 min, a peak concentration of 0.5 ppm total chlorine was produced. The concentration

of chlorine in the test chamber was then gradually reduced to zero due to the continuous flow of water through the system. Thus a curve of chlorine concentration (Fig. 6) was produced in the test chamber to simulate a 'pulse' of chlorine moving down a stream as measured at one point. For a peak concentration of 0.25 ppm total chlorine, 8 ml of the stock solution was diluted to one liter producing a concentration of 3.0 ppm free chlorine in the one liter bottle. When the solution of free chlorine was added to the test chamber, the small amount of ammonium ion in the dechlorinated water was partially converted to chloramine. This was always less than 10% of the total chlorine and thus the distinction between free and residual chlorine was not made and the concentrations were reported as total chlorine. Determination of the total chlorine was by amperometric titration.

The chlorine solution was added to one of the three test chambers at 6 hr intervals for one week. The peristaltic pump was controlled by an automatic timer for the early morning chlorination. At the time of the first chlorination, one of the two remaining battery jars was removed from the continuous flow system and the amount of algae on 12 stigeocloniometers was determined. The remaining test chamber served as the control.

The amount of algae attached to the stigeocloniometers was varied while holding the chlorine concentration constant. Each test was the reaction of a specific amount of attached algae to a specific concentration of chlorine. Subsequent tests then explored the reactions of different amounts of algae to the same chlorine dose.

Biomass and Pigment Extract Determinations

As previously stated, a separate study was conducted to determine the mathematical relationship between algal biomass and pigment extract. This was necessary so that dry weight determinations at the beginning and end of a toxicity test could be made without the use of an oven. Early in this study, it was found that 90% acetone would not completely remove the pigments of S. subsecundum cells. In order to determine the biomass of small amounts of algae which might be left after repeated chlorination, a total pigment extract was needed and a 95% methanol extract satisfied this requirement. The wavelength of maximum absorbance of the pigment extract was found to be 412 nm. It was realized that by reading the pigment extract at 412 nm, carotenes and xanthophylls as well as chlorophyll a and b were being measured. However, because the peak at 412 nm was over twice the height of the peak of 665 nm, the 412 nm wavelength was chosen to have the maximum sensitivity to small amounts of pigment in the extract.

All absorbance readings in this and the other preliminary studies as well as the toxicity studies were taken at 412 nm with a 1 cm light path on a Coleman 124 dual-beam spectrophotometer.

The relation between the absorbance of the pigment extract and the biomass was established by taking cells which had been scraped off the stigeocloniometers and pooling them, washing the suspension with a sodium bicarbonate solution (45 ppm) and using equal aliquots of many cell suspensions for dry weights and pigment extracts. The sodium bicarbonate solution was used to reduce plasmolysis during the washings

procedure. Algal suspensions for dry weight were placed in an oven 8 hr at 75°C. Algal suspensions for pigment extracts were filtered with Gelman type A/E glass fiber filters. The filter was put in a screw capped test tube with 10 ml of 95% methanol, and placed in a freezer. Preliminary experiments demonstrated that total pigment extracts could be obtained after 24 hr in the freezer. Extracts were then centrifuged at 2400 rpm and the absorbance at 412 nm determined. Thus, two sets of figures were generated which related a range of weights and pigment extracts for cells grown under specific conditions. This data was used to calculate regression formulas relating algal biomass to the pigment extract absorbance. The only exception to this procedure was for the cells grown in the battery jar test chambers. At the end of one week in the battery jar, the algal biomass on each stigeocloniometer had become so large that the pigment extract was much too dense to read (e.g. greater than 2.0 absorbance units). Therefore, a 1:10 dilution was made of the pigment extract to allow it to be read on the spectrophotometer. Then, the relation between dry weight and a 1:10 dilution was made as described above. Regression formulas were calculated for algae on the stigeocloniometers after 12 days in the aluminum pan, after 24, 48, 60, 72, 96, and 120 hr in the artificial stream, and after one week in the battery jar.

To confirm the regression formulas concerning algal growth in the artificial stream, 20 stigeocloniometers were removed for each specific time period (24, 48, 60, 72, 96, and 120 hr). The algae attached to each of 10 stigeocloniometers was scraped into ten tared aluminum pans

for direct dry weight determinations after 8 hr at 75°C. Pigment extracts were taken of the algae on each of the remaining 10 stigeocloniometers by scraping the algal biomass onto the glass fiber filters and placing them in 10 ml of 95% methanol as previously described. Algal cells attached to 20 stigeocloniometers after the 12 day attachment period were used to check the 'zero hours in stream' regression formula. Fourteen stigeocloniometers (seven for direct dry weight and seven for pigment extract) were used to confirm the regression formula for algal cells growing in the battery jar for one week. Using the specific regression formula in question, algal biomass values were computed from the pigment extracts and the average compared to the average of the direct dry weight measurements. If the difference between the two values for biomass was greater than 0.5 mg, the regression was run again on new data and then checked again. This process continued until the difference between the average biomass values calculated from pigment extracts and measured directly was less than 0.5 mg.

Zoospore Counts

Zoospores leaving the algal mat in the battery jar were collected and concentrated by placing the effluent line from the plastic funnel into a Foerst plankton centrifuge (Foerst Mechanical Specialties Co., Chicago). The effluent line from the centrifuge was placed in a two liter volumetric flask. The flask was allowed to fill until it reached a volume of 1995 ml at which time the effluent line from the battery jar was removed from the centrifuge. The volume of the concentrate in

the centrifuge was 5 ml to which was added an equal volume of algal preservative (6-3-1; formalin, ethanol, acetic acid). The resulting 10 ml was poured into a centrifuge tube with the aid of a rubber policeman and centrifuged at 2400 rpm to concentrate the zoospores into a volume of 2.0 ml. Counting of the zoospores was accomplished by a Hemocytometer and the average number of zoospores per 0.1 mm^3 was determined by eight separate counts.

Photographic Methods

After each test, two stigeocloniometers were removed from both the control and chlorinated test chambers and placed in separate 100 ml beakers containing dechlorinated tap water. Algal cells were then scraped from the stigeocloniometer surface with a razor blade, placed on a glass microscope slide, and observations made with a Leitz ortholux microscope equipped with a Nikon camera and light meter. The film used was Kodak photomicrography color film 2483 from which both color slides and black and white prints were made.

RESULTS & DISCUSSION

Algal Cell Attachment to Stigeocloniometers

Low light intensity (150 ft-c) produced more attached algae than did 500 ft-c in all cases. The latter light intensity produced an algal scum on the surface of the medium which became small islands of hard, crustose algae. Of the suspensions of blended algal cells used in this study, the suspension having an absorbance of 0.85 seemed to be the best. The suspensions having an absorbance above this (1.0 and 1.2 absorbance units) did not produce greater amounts of attached algae over the same time period. Cell suspensions with an absorbance below 0.85 (0.1 and 0.4 absorbance units) did not maintain a uniform suspension above the stigeocloniometers and produced very little attached algae, given the same time period. The cover was also important and the stretch-n-seal covering worked better than either glass or plexiglass. A spectral scan from 380 to 760 nm was performed on the stretch-n-seal and it was found not to absorb light in the visible spectrum. The length of time the blended algal suspension was in contact with the stigeocloniometer surface also determined the amount of algae which became attached. It was found that after 12 days there was very little increase in the amount of attached algae on the stigeocloniometer surface. This was due to a decrease in the rate of zoospore production in the algal suspension as well as the increased growth of unattached filaments which shaded the attached algae. The age of the culture prior to blending was found to be very important. For example, blended week-old cultures diluted to an absorbance of 0.85 produced an

average biomass of 1.1 mg dry weight of algae attached to the stigeocloniometer surface. If blended two week old cultures were used, the average dry weight biomass was 1.9 mg after 12 days.

Increasing the Algal Biomass on the Stigeocloniometer Surface

Attempts were made to increase the growth of the algae attached to the stigeocloniometer surface in the aluminum pan by removing the algal suspension and replacing it with new ASM-1 medium. Growth of the attached algae was, however, very slow and uneven in this situation.

A shallow fiberglass pan containing flowing dechlorinated tap water did permit the increase of algal biomass on the stigeocloniometer at a faster rate, but it was found by dye studies that most of the water flowed around the stigeocloniometers rather than over them. This eventually caused the exposed edges of the stigeocloniometers to have visibly more algal growth than the rest of the surface. In addition, it was extremely hard to achieve a uniform flow of water through the pan so that both ends of the stigeocloniometer rows received equal current velocities.

The artificial stream (Fig. 3) did away with many of these problems. The barrier (B in Fig. 3) allowed the dechlorinated water to flow evenly over the surfaces of the stigeocloniometers. Dye studies showed that the water advanced along an even front down the length of the stream. Since the edges of the stigeocloniometers were either against the barrier, the sides of the stream, or against each other, the increased growth of algae along the edges was not observed.

Two stigeocloniometers without attached algae were always placed at the end of the two rows of stigeocloniometers so that the water flowed evenly away from the surface of the algal mat into the remaining length of the stream.

During the first 24 hr in the stream, there was found to be no change in the algal biomass. Average algal biomass values in the stream for 10 stigeocloniometers were 1.4, 1.8, 2.3, and 3.5 mg dry weight per stigeocloniometer surface for 48, 60, 72, and 96 hr, respectively, with an initial average biomass of 1.1 mg dry weight per stigeocloniometer surface.

Biomass and Pigment Determinations

The changing relationship between algal biomass and algal pigment was reflected by the changing regression formulas relating the two variables. Regression formulas which related the algal biomass on the surface of the stigeocloniometers to the pigment extract in 95% methanol were found to be as follows:

$$\begin{array}{ll}
 Y = 4.690715 (X) + 0.481226 & (0 \text{ hr in stream}) \\
 Y = 5.810427 (X) - 0.054085 & (24 \text{ hr in stream}) \\
 Y = 5.507812 (X) - 0.275946 & (48 \text{ hr in stream}) \\
 Y = 6.047324 (X) - 1.638078 & (60 \text{ hr in stream}) \\
 Y = 4.322594 (X) + 0.074709 & (72 \text{ hr in stream}) \\
 Y = 4.434328 (X) + 0.069908 & (96 \text{ hr in stream}) \\
 Y = 4.394702 (X) + 0.087794 & (120 \text{ hr in stream})
 \end{array}$$

where X equals absorbance of the pigment extract at 412 nm and Y equals mg dry weight. The correlation coefficients of the above regression

formulas were all greater than 0.95.

The changes were due to the fact that the algal cells were taken from a static, nutrient rich, hard water (the ASM-1 algal medium in the aluminum pan) under low light intensity (150 ft-c) and placed in a flowing, low nutrient, soft water (the dechlorinated tap water in the artificial stream) under high light intensity (500 ft-c). Although the changes in the biomass/pigment relationship of the algal cells were not the topic of this research, it was interesting to note that the changes were characteristic of those previously reported in the literature. For example, the decrease in pigment relative to the algal biomass, as shown by the slope of the regression formulas from zero to 48 hr in stream, has been observed in Chlorella pyrenoidosa and C. vulgaris and is true for the green algae in general (Jorgensen, 1969). Light intensity is also related to changes in membrane potential and hence ion transport across the cell wall. Uptake of such ions as K^+ , Na^+ , Cl^- , NO_3^- , NO_2^- , and SO_4^{-2} have been found to be light dependent (Soeder and Stengel, 1974). Adaptation to the new light intensity was expected to take approximately 30 hr (Steemann-Nielsen, et al., 1962) and the increase in the slope of the regression line at 60 hr was unexpected. The 60 hr regression formula was computed because a great increase in growth was observed to occur after 48 hr in the stream. However, once the toxicity tests were underway, the 60 hr regression formula was not needed. All experiments in the literature which refer to light adaptation have used unicellular algae in suspension, never filamentous algal cells attached to a substrate in flowing water. Thus, the longer

time required for adaptation of the attached alga could reflect differences in physiology due to differences in habitat.

After 72 hr in the stream, the relation between biomass and pigment extract absorbance stabilized as shown by the slopes of the 72, 96, and 120 hr regression formulas. Therefore, to obtain biomass values from extracts of algae grown in the stream for 72 hr or longer, the 72 hr regression formula was used. The regression formula for the algal cells grown for one week in the battery jar test chamber was found to be

$$Y = 87.62076 (X) + 6.12928$$

with a correlation coefficient of 0.96.

The growth of the attached algae in the battery jars was much faster than in the artificial stream. During a one week period, the algal mat grew to a biomass of between 40 and 52 mg dry weight per stigeocloniometer surface depending upon the initial algal biomass present at the time the stigeocloniometers were placed in the battery jar. It was interesting that the smaller the initial biomass, the faster the growth of the algal mat. An initial average of 0.52 mg dry weight per stigeocloniometer surface grew to an average of 43.6 mg dry weight per stigeocloniometer surface, over 80 times the initial value. An initial average value of 2.9 mg dry weight grew to a final average value of 51.4 mg dry weight per stigeocloniometer or 18 times the original value.

Chlorine Toxicity and Minimal Resistant Biomass

During preliminary trials of the bioassay system, it was discovered that the effect of intermittent chlorination on the algal mat in the battery jar was dependent upon the initial algal biomass present on the stigeocloniometer surface. If a sufficient algal biomass was present, a peak of 0.5 ppm free chlorine every six hours for one week had no effect. If, however, an insufficient algal biomass was present, all the cells would be killed within the week. Thus, there seemed to be a minimal resistant biomass (MRB), i.e. a lower limit of the algal biomass which was able to survive intermittent chlorination and continue to grow.

In order to find the MRB for a chlorine dosage of 0.5 ppm every six hours for one week, slides were removed from the aluminum pan at the end of the attachment period, placed in the stream for 24 hr and then tested. When the cells on the stigeocloniometers were killed by the intermittent chlorination, the next test (using the same chlorine dosage) was made on cells which had been in the stream for 48 hr. The S. subsecundum cells on the surface of the stigeocloniometers which were placed in the artificial stream for 24 and 48 hr (corresponding to average biomass values of 1.17 and 1.51 mg dry weight) did not survive the 0.5 ppm intermittent chlorination. However, algal cells which had grown in the stream for 72 hr and attained an average biomass of 2.31 mg dry weight per stigeocloniometer surface did survive and grew despite the intermittent chlorination. The average biomass on the stigeocloniometer surface at the end of one week was 26.5 mg dry weight

with the control battery jar having an average of 51.7 mg dry weight per stigeocloniometer (Table 2). The next set of stigeocloniometers were allowed to grow in the stream for 96 hr before testing. This produced an average of 2.85 mg dry weight per stigeocloniometer at the beginning of the test. This biomass survived and after one week averaged 46.3 mg dry weight compared to an average of 51.4 mg dry weight for the control stigeocloniometers (Table 2). The same series of experiments were again performed on S. subsecundum cells grown in the stream for 48, 72, and 96 hr corresponding to initial average biomass values of 1.26, 2.12, and 2.80 mg dry weight respectively. As before, the slides grown in the stream for 48 hr failed to survive the 0.5 ppm free chlorine doses. The algal cells grown in the stream for 72 hr survived with final biomass values much lower than the control biomass values (Table 2). The algal biomass which existed on the stigeocloniometers after 96 hr in the stream also survived the intermittent chlorination and produced final biomass values which were either close to or equal to the control algal biomass values.

Thus, the biomass values of 2.31 and 2.12 mg dry weight (an average of 2.22 mg dry weight) per stigeocloniometer surface was taken to be the minimal resistant biomass value for Stigeoclonium subsecundum exposed to 0.5 ppm free chlorine every six hours for one week. A t-test was conducted on the final biomass values of the tests which determined the MRB and they (the final biomass values of the control and the chlorinated algal mats) were found to be very significantly different ($P > .005$). Final biomass values for the control and experimental

stigeocloniometers with initial biomass values of 2.85 and 2.80 mg dry weight were not found to be different from each other at any level of significance. These experiments are summarized in Table 2.

It was determined at this point in the study that two week old cultures of S. subsecundum gave higher biomass values of attached algae at the end of 12 days than did the one week old cultures. The first set of stigeocloniometers to be tested against 0.25 ppm free chlorine were placed in the stream for 24 hr after a 12 day attachment period and was found to have an average of 1.88 mg dry weight per stigeocloniometer surface. This biomass survived the intermittent chlorination and grew to a biomass equal to the control stigeocloniometers. At the time of the first test of 0.25 ppm, other aluminum pans containing stigeocloniometers had already received a suspension of two week old algal cells. Thus, when it was found that the initial 1.88 mg dry weight algal biomass survived, the decision was made to take the next set of stigeocloniometers out after 11 days in the pan and place them in the stream for 24 hr prior to testing. This algal biomass (an average of 1.01 mg dry weight per stigeocloniometer) survived, but exhibited a growth form (to be discussed later) and final biomass values which seemed to signal that a minimal resistant biomass had been reached. Thus, for the next test, the stigeocloniometers were removed from the pan after 10 days, placed in the stream for 24 hr and then tested against intermittent chlorine peaks of 0.25 ppm every six hours for one week. The average biomass of this set of slides was 0.512 mg dry weight per stigeocloniometer surface and did not survive the intermittent chlorination.

Thus, a MRB of 1.01 mg dry weight per stigeocloniometer had been determined.

The next series of tests to confirm the minimal resistant biomass for 0.25 ppm used exactly the same procedure for obtaining the attached algal biomass as was used for the previous 0.25 ppm tests. This produced average algal biomass values of 1.91, 0.880, and 0.556 mg dry weight per stigeocloniometer, corresponding to 12, 11, and 10 days in the aluminum pan followed by 24 hr in the stream. The algal mats with initial values of 1.91 and 0.880 mg dry weight survived while the mat with an initial biomass of 0.556 mg dry weight was killed. At the end of the test period, the mean biomass values for the stigeocloniometers with an initial average value of 1.91 mg dry weight and exposed to intermittent chlorination were not significantly different from the control. The final mean biomass value for the stigeocloniometers with an initial average of 0.880 mg dry weight were, however, significantly different ($P > .005$). Thus, a MRB value of 0.945 mg dry weight $(1.01 + 0.880/2)$ of Stigeoclonium subsecundum for 0.25 ppm free chlorine was determined.

Spectral scans (380-760 nm) of the pigment extracts from both chlorinated and control algal cells were exactly the same as were the ratios of biomass to pigment extract absorbance. When it was found that the biomass/pigment extract ratios for control and chlorinated algal cells were the same, the regression formula for algal cells in the battery jar after one week was used to determine the final biomass of the chlorinated algal mat. Therefore, the intermittent chlorination

did not effect either the composition or quantity of pigments in the algal cells.

The most important result of this study was the fact that the toxicity of free chlorine was affected by the biomass of the alga. Although this raises the question as to the relationship between toxicity and biomass in all toxicity tests, the more immediate question concerns periphyton or attached microorganisms in general. Is there a level or minimal resistant biomass for these attached organisms above which a specific level of toxicant will have little or no effect? The answer to this question would seem to be 'yes'. The key to survival in this study was the biomass present at the time the organism was first exposed to the chlorine. Thus, survival in nature would also depend upon the initial biomass of the attached organism or organisms. In addition, the speed with which any attached organism can adapt also would have an effect. If, in this study, the morphology of S. subsecundum had changed faster, the minimal resistant biomass values for 0.5 and 0.25 ppm free chlorine would have been lower. Different rates of change resulting from exposure to a toxicant would alter the number and kinds of attached organisms depending on their specific MRB for the level of toxicant.

An important feature of periphyton in nature is the fact that it is not constant, but changes seasonally especially in the temperate regions. While a particular attached organism may attain a biomass well above its MRB for a particular toxicant during one season, normal seasonal change could reduce its biomass below its MRB and thus permit

it to be destroyed.

Obviously, there are streams and rivers in this country which receive such a high level of toxic substances that no biomass of periphyton, however great, could withstand the toxicity. This study was aimed at the more nebulous environmental impact of low chlorine concentrations in a stream or river.

Effect of Intermittent Chlorination on Zoospore Production

Zoospore counts of the effluent from both the control and chlorinated battery jar test chambers were always less than one zoospore/ml of effluent for the first three to four days of a toxicity test. After that time, a sharp rise occurred in the number of zoospores in the control effluent. By contrast, the zoospore production of an algal mat surviving intermittent chlorination increased only slightly if at all. A comparison of the zoospore production of a control and chlorinated algal mat is shown in Figure 15. This algal mat had a biomass above the MRB level for the chlorine dose (0.5 ppm) and consequently zoospore production continued after the initial chlorination. With biomass values of the MRB level, zoospore production was evident only after about 72 hr into the toxicity test and never increased above one zoospore/ml throughout the remainder of the test period.

The large increase of zoospores in the control effluent was due to the development of increasing numbers of zoospore producing filaments (Fig. 14). A comparison of the normal vegetative filament with the zoospore producing filament is shown in Figure 13. Two quadriflagellate

zoospores were released from each cell of the zoospore producing filaments. The absence of these filaments in the chlorinated cultures account for the low numbers of zoospores in the effluent.

It was observed that single cells in a normal vegetative filament could also produce a single quadriflagellated cell. Whether these were gametes or zoospores was not determined; they were called zoospores for the purpose of this study. Their rate of production was never found to be very great and accounted for the initial low counts in the effluent of the control and chlorinated test chambers. The low rate of zoospore production from algal mats surviving intermittent chlorination was also due to the sporadic release of the single quadriflagellated cells. The viability of the zoospores from the chlorinated algal mats were not determined.

Zoospore production and viability of algal mats of S. subsecundum could be used as a biomonitor in a continuous flow system such as the one described in this study. An algal mat grown in a battery jar test chamber for two weeks reached a peak of 190 zoospores/ml of effluent (flowing at 125 ml/min). This kind of production is indicative of a healthy algal mat and interruption of zoospore production and the death of release zoospores can be looked upon as a sublethal indicator of stress to the entire algal mat.

Morphological Changes of the Algal

Mat Exposed to Intermittent Chlorination

The morphological changes which resulted from intermittent chlorination always took place within 72 to 96 hr after the first chlorination.

When algal mats were at the MRB level, it was readily apparent, due to the bleaching of the pigment, that chlorine was killing most of the attached cells. However, small loci of cells attached to the glass surface would survive and could be seen as tiny green spots on the stigeocloniometer surface. These loci would not appear to increase in size until after 96 hours, then they grew rapidly. From observations of the reaction of algal mats with biomass values above the MRB for a given chlorine dose, it was obvious that the terminal cells on the filaments were the first to be bleached and killed. Although dead or dying, these terminal cells helped provide a layer of water beneath them which did not receive the full concentration of chlorine present in the rest of the test chamber. The resulting growth produced a carpet-like appearance of the mat; a smooth, unbroken, flat surface, neatly trimmed around the edges. In contrast, the ragged appearance of the control mat was due to a great many filaments of unequal length which became more branched with increasing distance above the stigeocloniometer surface. When the initial algal biomass was so small that even some of the basal cells on the glass surface were killed, the surviving basal cells produced short, highly branched filaments (Fig. 11). Microscopic examination of these basal cells during preliminary tests showed them to be surrounded by basal cells and short filaments which were dead. Even though they were dead, however, the protection afforded the surviving cells was enough to allow it to produce short highly branched filaments which continued to grow. The filaments which radiated from the surviving basal cells were so

compact that they provided a layer of water which did not mix well with the over lying water. Hence, the cells in the filament below the terminal cell did not receive the full impact of chlorination. If the basal cells which produced the highly branched filaments were sufficiently isolated from other surviving cells, a hemispherical, dome-shaped colony was formed on the glass surface.

Rounded basal cells which produce both the rhizoids and erect filaments (Fig. 8) were easily obtained from the control stigeocloniometers. However, the basal cells of algal mats surviving intermittent chlorination were so densely packed (Figs. 9 & 10) that rhizoids were never observed.

The ability to grow in water receiving intermittent chlorination seemed to be a result of a change in the alga's growth and morphology. The algae receiving intermittent chlorination was never observed to produce mucilage and no experiments were conducted to ascertain any physiological changes in the alga cells. After the terminal cells were killed there was increased multiplication of the basal cells on the glass surface which in turn produced more erect filaments. As the number of filaments increased, the water amongst the filaments became increasingly isolated from the overlying water. At the same time the dead terminal cells were pushed outward by the growing intercalary cells, thus allowing the colony to grow in water which did not receive the full impact of the introduced chlorine solution. Although the change in morphology was observed, the intracellular mechanisms which caused the changes are not known.

Changes in morphology or polymorphism in freshwater algae was recently reviewed by Trainor, et al. (1971). However, each of the cases cited in the aforementioned paper were due to environmental or culture conditions, not by exposure to toxic substances. Among the green algae, the genus Scenedesmus was studied the most regarding polymorphism (Trainor, 1969; Trainor and Roskosky, 1967; and Trainor and Rowland, 1968). Using several strains of Scenedesmus, Trainor and Shubert (1974) were able to control the formation of the typical colony (as opposed to unicells) by adjusting the total concentration of dissolved inorganic salts in the algal medium. This research culminated in the first species description of a freshwater polymorphic alga (Shubert, 1975) using a combination of morphological and physiological attributes. Shubert and Trainor (1974), however, pointed out that nothing is known about the mechanisms within the cells themselves which cause their morphology to change.

Although much is known concerning the toxicity of metals to algae (Whitton, 1970), there is only one report of morphological change due to sublethal levels of toxic metals (Thomas, 1962). In this case, both Cladophora glomerata and Rhizoclonium hieroglyphicum were found to exhibit abnormal cell wall development when exposed to sublethal levels of copper and zinc.

In the paper by Trainor, et al. (1971), the authors emphasized the problems in taxonomy caused by differing environmental conditions. The alteration of the aquatic environment by man-made pollutants such as chlorine has been demonstrated to alter the morphology of Stigeoclonium subsecundum and thus potentially create even more taxonomic problems.

The Mode of Action of Chlorine

The action by which chlorine kills bacterial cells was demonstrated by Green and Stumpf (1946) and by Knox, et al. (1948). The report by Green and Stumpf was an abbreviated version of the more extensive work by Knox, et al. The latter paper demonstrated that the bactericidal action of chlorine was due to the oxidation of sulfhydryl enzymes, specifically those of the Embden-Meyerhoff pathway of glycolysis. Once such enzyme in E. coli, aldolase, (reacts with fructose-1, 6-diphosphate to produce dihydroxyacetone phosphate and glyceraldehyde-3-phosphate) was found to have the same sensitivity to chlorine as did the entire glycolytic cycle. Other reagents which were known to oxidize sulfhydryl groups were also used to identify the manner in which chlorine exerted its effect.

The Embden-Meyerhoff pathway of glycolysis has been identified in all algal species studied with respect to dark respiration (Lloyd, 1974). Therefore, the enzyme system of glycolysis in Stigeoclonium subsecundum was undoubtedly attacked by the free chlorine. The bleaching of the algal cells during the intermittent chlorination was always very evident, so the chlorine obviously attacked the chlorophyll molecules as well as the enzyme systems. Although there is nothing in the literature concerning the effect of chlorine on the chlorophyll molecule itself, speculation can be made on the possible interaction. Chlorine, being a strong oxidizing agent, could oxidize the numerous double bonds which occur in the chlorophyll molecule. These oxidations could alter the shape and structure of the chlorophyll molecule to such an extent that it could no longer function to absorb light.

CONCLUSION

A continuous flow algal bioassay utilizing Stigeoclonium subsecundum attached to removable substrates (stigeocloniometers) inside a battery jar test chamber was employed in a study of the toxic effects of chlorine on an attached, filamentous alga. Preliminary experiments were conducted to determine 1) the best conditions for attaching the algal cells to the stigeocloniometers, 2) the best method for increasing the algal biomass on each stigeocloniometer prior to testing, and 3) the determination of the mathematical relationship between the pigment extract and the dry weight biomass.

The stigeocloniometers, with attached algal cells were arranged inside a battery jar test chamber to form a continuous algal mat of 42 in² (271 cm²). Free chlorine in the form of a calcium hypochlorite solution was introduced into the test chamber by a peristaltic pump every six hours for one week. Two peak chlorine concentrations (0.5 and 0.25 ppm) were used to test the response of different algal biomass levels.

The term 'minimal resistant biomass' or MRB was coined to describe the response of the alga to intermittent chlorination. Average algal biomass values above the MRB survived the intermittent chlorination and grew to equal the biomass of the control algal mat at the end of one week. Average biomass values below the MRB were destroyed by the intermittent chlorination. The MRB for Stigeoclonium subsecundum was 2.22 and 0.945 mg dry weight per 3 in² (19.4 cm²) for exposures at six hour intervals to 0.5 and 0.25 ppm respectively peak free chlorine.

The probable cause of the MRB response was the morphological change of the algal mats exposed to intermittent chlorination. The production of mucilage by either the control or chlorinated algal mats was not observed. The morphological change was of two types. If the initial algal biomass was above the MRB, then the mat simply became more compact and dense than the control. At the MRB level, a few surviving cells would produce highly branched filaments radiating from a central region of compact basal cells. These colonies took on the shape of a hemisphere or dome on the surface of the stigeocloniometer.

Although cessation of intermittent chlorination allowed the alga to return to its normal morphology, the study did show that low concentrations of man-made pollutants could change the morphology of attached organisms to the extent that known taxonomic criteria would not apply.

The zoospore production was also effected by the intermittent chlorination. The zoospore producing filaments which were responsible for the increased production of zoospores in the control test chambers were never observed in the chlorinated algal mats. Thus, the zoospore counts from both the control and chlorinated algal mats always began at the same level. However, after four days the control algal mats liberated zoospores at a much greater rate than the mat receiving the intermittent chlorination.

The toxic effect of chlorine on the algal cells was manifested visibly by the bleaching of the pigment. The bleaching was probably due to the action of the chlorine on the chlorophyll molecule,

specifically the oxidation of the double bonds and subsequent alteration of the molecular structure and light absorbing capacity. In addition, chlorine is known to attack sulfhydryl groups on enzymes, thus terminating such enzymatically controlled reactions as occur in the glycolytic pathway.

The results of this study demonstrate that Stigeoclonium subsecundum can modify its growth to such an extent that it can survive and grow while being exposed to intermittent chlorination. The key to survival, however, is the amount of algae present at the time of the first chlorine exposure. In temperate latitudes, the increase and decrease of the biomass of benthic algae (especially attached, filamentous algae) is easily observed during seasonal changes. The decrease in biomass during the Fall and Winter could then lower the algal biomass to a point at which it could not survive the intermittent chlorination.

The observed morphological change in Stigeoclonium subsecundum raises the question as to possible morphological changes occurring in other attached algae and periphyton in general when subjected to pollutants. The bioassay system developed in this study could be used to answer these questions by using organisms other than Stigeoclonium subsecundum which would attach to the stigeocloniometer surface.

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Table 1: Typical chemical analysis of Blacksburg dechlorinated tap water after leaving the charcoal columns. Zinc, copper, and iron were below detectable limits.

pH	7.4 ppm
Phosphate	
Ortho	0.2 ppm
Condensed	1.5 ppm
Nitrate	3.0
Alkalinity	40.0 ppm
Hardness	64.0 ppm
Silica	3.5 ppm
Chloride	5.8 ppm
Ammonia	0.2 ppm

Table 2: Initial and final average biomass values in mg dry weight per stigeocloniometer surface (3 in^2) tested against 0.5 ppm peak free chlorine every six hours for one week. Dash indicates death of all cells on stigeocloniometers. Parentheses enclose 95% confidence limits.

Initial Average Algal Biomass	Final Average Algal Biomass	
	Control	Chlorinated
1.26 (\pm 0.122)	40.6 (\pm 1.57)	-
1.51 (\pm 0.125)	40.4 (\pm 1.63)	-
2.12 (\pm 0.218)	50.7 (\pm 5.45)	34.3 (\pm 3.46)
2.31 (\pm 0.204)	51.7 (\pm 5.59)	26.5 (\pm 3.09)
2.80 (\pm 0.219)	51.0 (\pm 1.40)	45.6 (\pm 2.19)
2.85 (\pm 0.215)	51.4 (\pm 1.23)	46.3 (\pm 2.32)

Table 3: Initial and final average biomass values in mg dry weight per stigeocloniometer surface (3 in^2) tested against 0.25 ppm peak free chlorine every six hours for one week. Dash indicates death of all cells on stigeocloniometers. Parentheses enclose 95% confidence limits.

Initial Average Algal Biomass	Final Average Algal Biomass	
	Control	Chlorinated
0.512 (+ 0.146)	43.7 (+ 1.55)	-
0.556 (+ 0.082)	44.1 (+ 1.37)	-
0.880 (+ 0.635)	43.6 (+ 2.70)	24.1 (+ 4.35)
1.01 (+ 0.073)	47.5 (+ 5.01)	33.6 (+ 6.23)
1.88 (+ 0.067)	50.4 (+ 2.94)	46.8 (+ 1.89)
1.91 (+ 0.059)	50.7 (+ 2.87)	46.8 (+ 1.75)

Figure 1. Diagram showing procedure used to obtain attached algae on stigeocloniometers prior to testing. Number 1 represents flask culture of the alga which was then concentrated by settling (2) and blended (3). The blended suspension was used to inoculate flasks (1) as well as aluminum pans (4) containing stigeocloniometers. After the attachment period was completed in the aluminum pan, the stigeocloniometers were placed in an artificial stream (5) to increase the algal biomass on each stigeocloniometer. The stigeocloniometers were then removed from the artificial stream after a specific time and placed in three battery jars (6) for testing against a specific chlorine concentration.

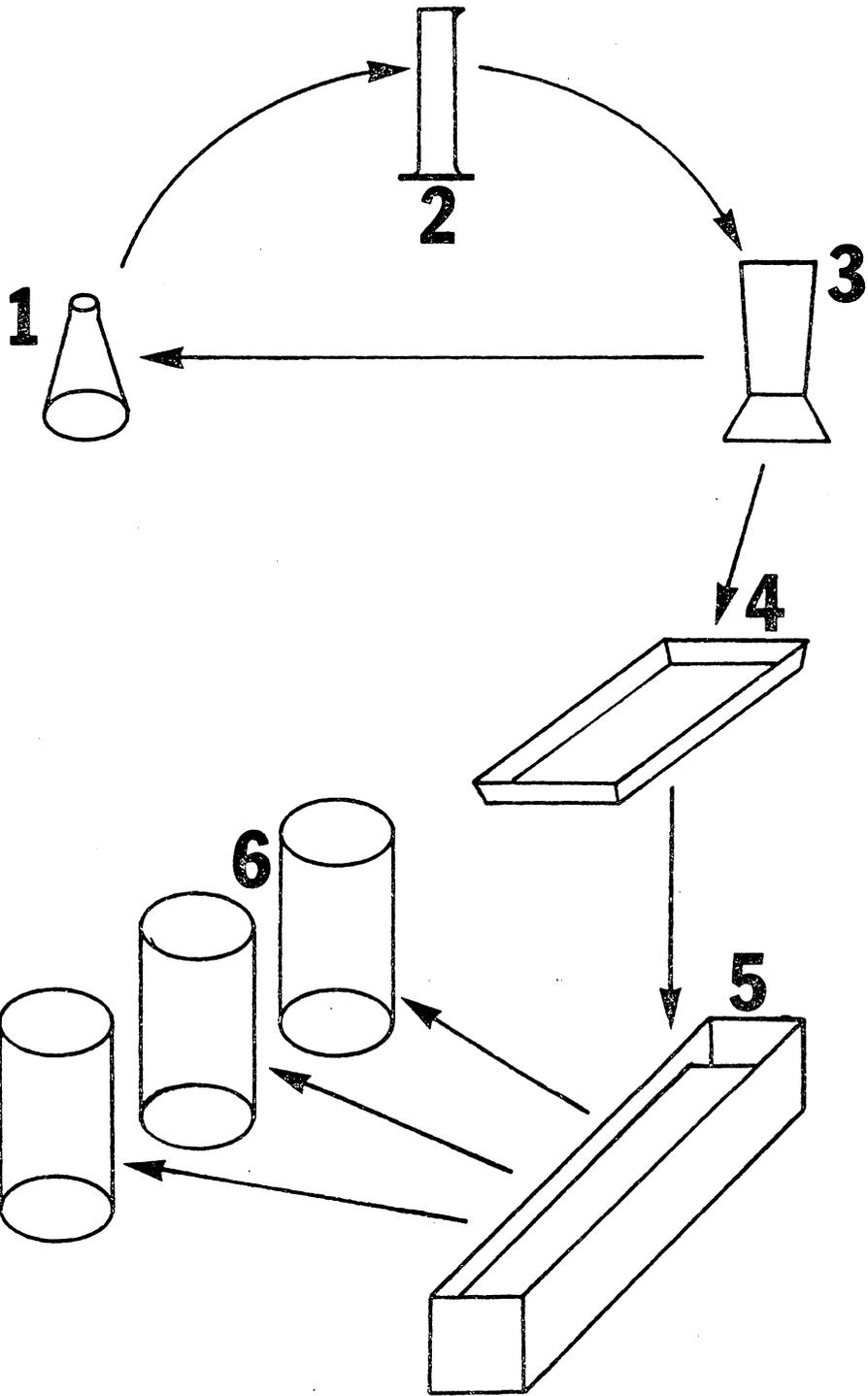


Figure 2. The stigeocloniometer is constructed from four 5 mm glass rods attached to a 1 x 3 in. (2.54 x 7.62 cm) glass microscope slide (the top). The bottom is one-half of a glass microscope slide centered between the ends of the stigeocloniometer. This construction allows the stigeocloniometer to be placed on a 5 mm glass rod attached inside the test chamber.

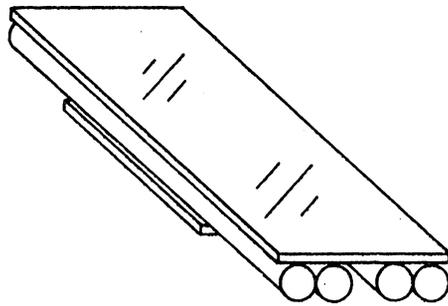


Figure 3. Artificial stream with near side and middle section removed. Water entered through rigid plastic tube (A) and flowed over barrier (B). Stigeocloniometers were placed in area C. Water flowed over wier (D) which maintained water depth (dotted line) over stigeocloniometers, and then out of stream through E.

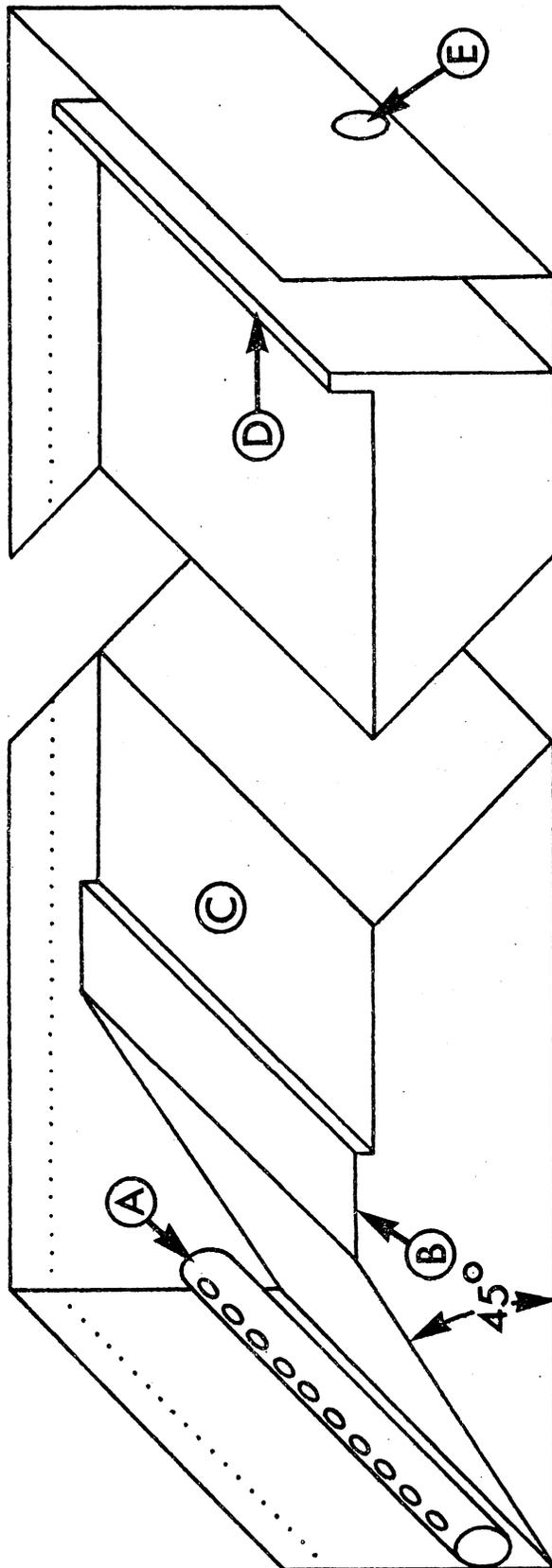


Figure 4. Battery jar test chamber with 5 mm glass rods (A) cemented inside with silicone rubber sealer (B). The plywood top (C) has three one inch holes (D) and a center 2.375 in. hole (E). 'Stops' made of 7 mm glass rods (F) and attached to the 5 mm glass rods are also shown. The top is held in place by a piece of plastic tubing (G) attached around the underside of the top with rubber sealer.

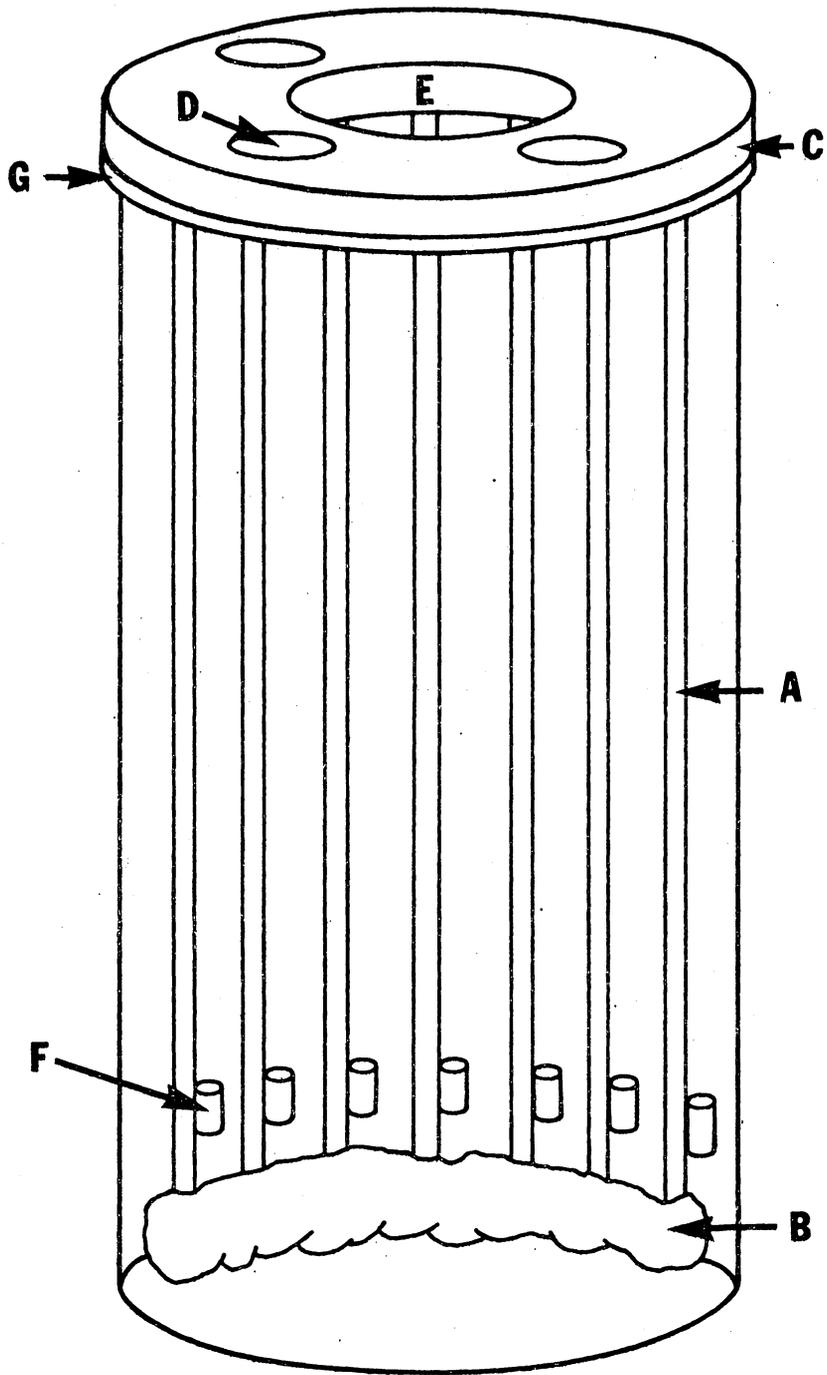


Figure 5. Battery jar connected to continuous flow system in front of fluorescent lights (A). The vertical glass rods shown in Fig. 4 were not shown. Top contains thermometer (B), influent tube with stopcock (C), and effluent tube (D) which is connected to smaller 4 mm I.D. siphon tube (K). Siphon tube terminates in no. 10 rubber stopper (L) which rests in plastic funnel (E). Hole in no. 13 rubber stopper (H) allowed introduction of chlorine into vortex of water created by paddle (J). Arrow indicates direction of paddle rotation.

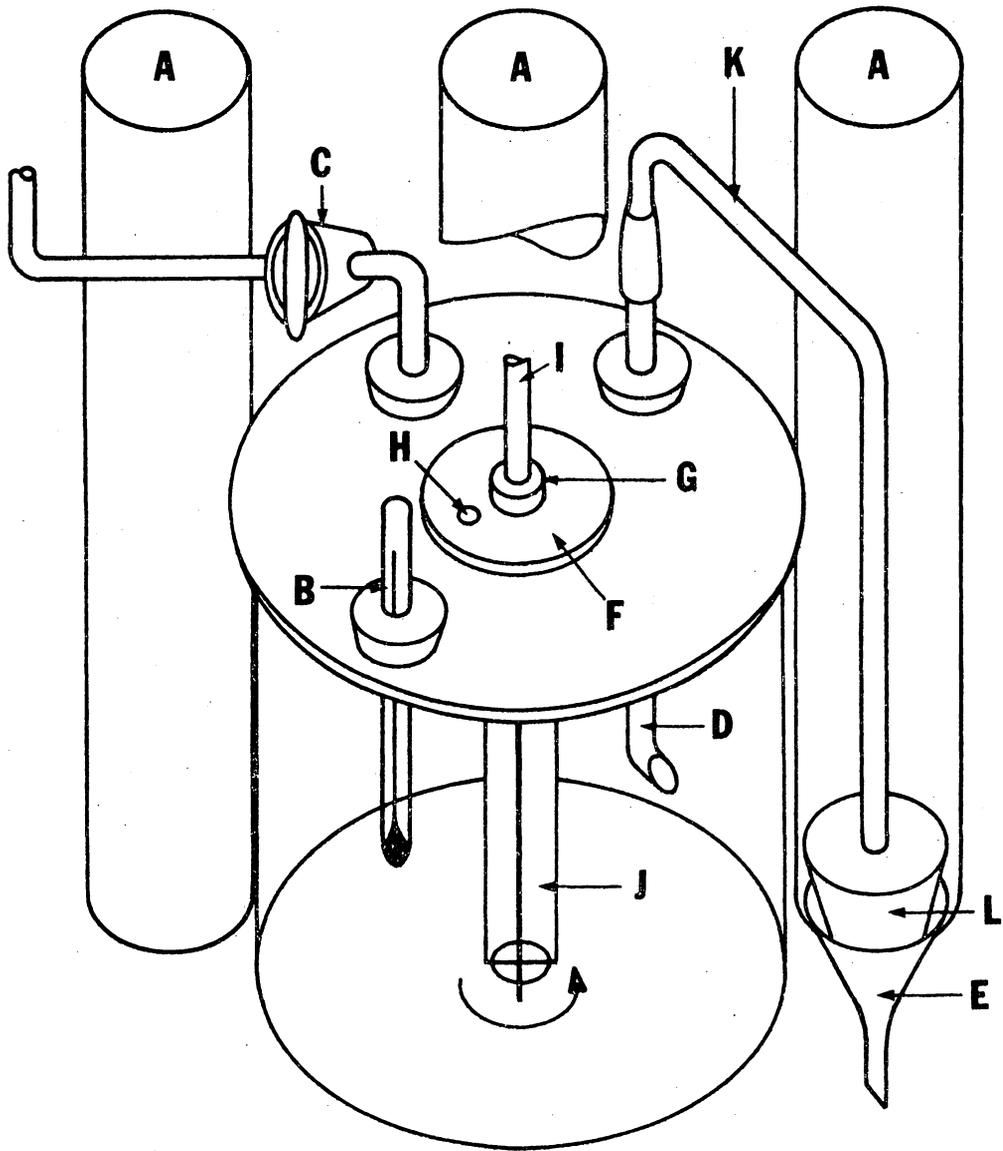


Figure 6. Graphs illustrating the shape of the curve of chlorine concentrations as one liter of hypochlorite solution was pumped into the test chamber and subsequently flushed out. One liter of 3 ppm free chlorine produced a peak of 0.25 ppm total chlorine in the test chamber at the end of 30 min. One liter of 10 ppm free chlorine produced a peak of 0.5 ppm total chlorine.

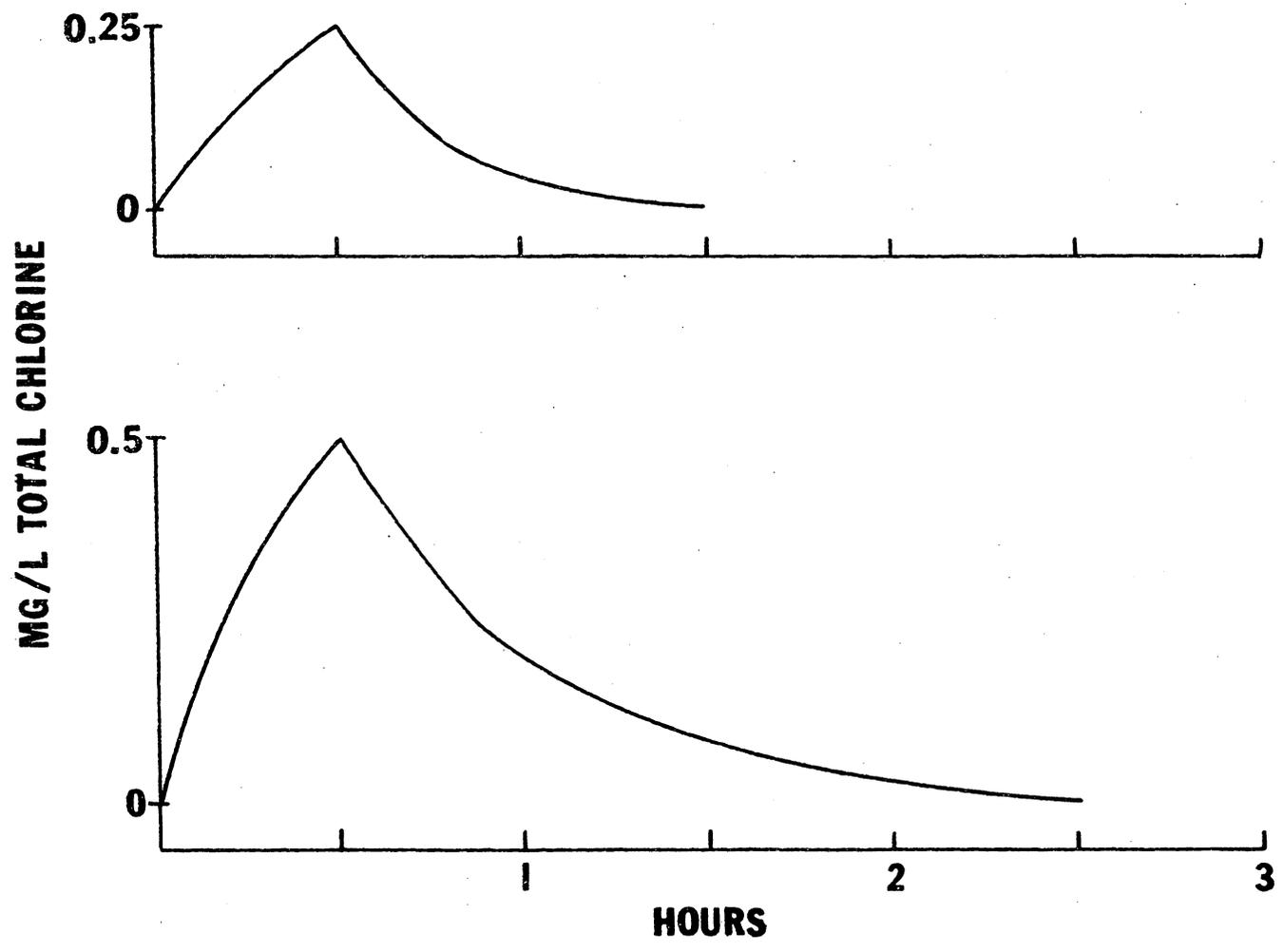


Figure 7. Hemispherical colony with radiating filaments which survived intermittent chlorination. Colony is next to larger algal mass and was slightly damaged (white area) in transfer from stigeocloniometer to microscope slide. (100X)

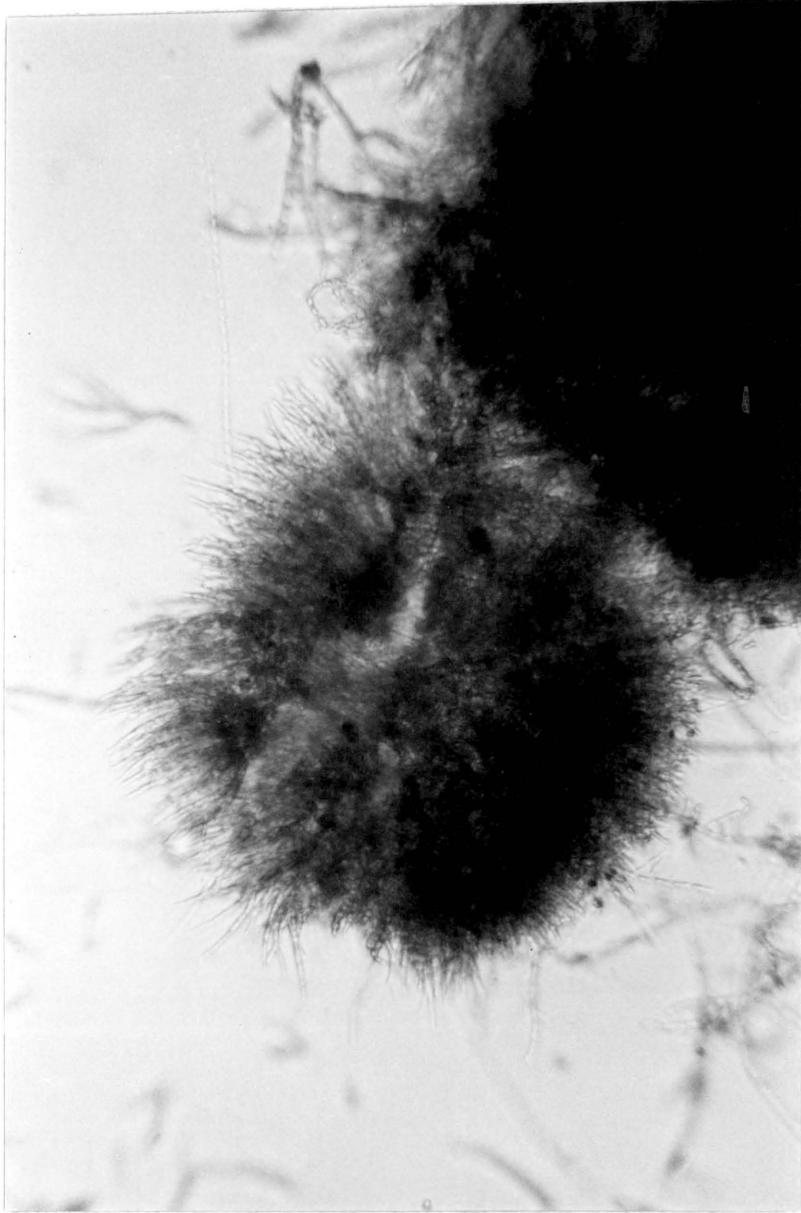


Figure 8. Basal cells (BC) from which extend erect filaments (EF) and rhizoids (R) present on control stigeocloniometer. Compare with Fig. 10. (400X)

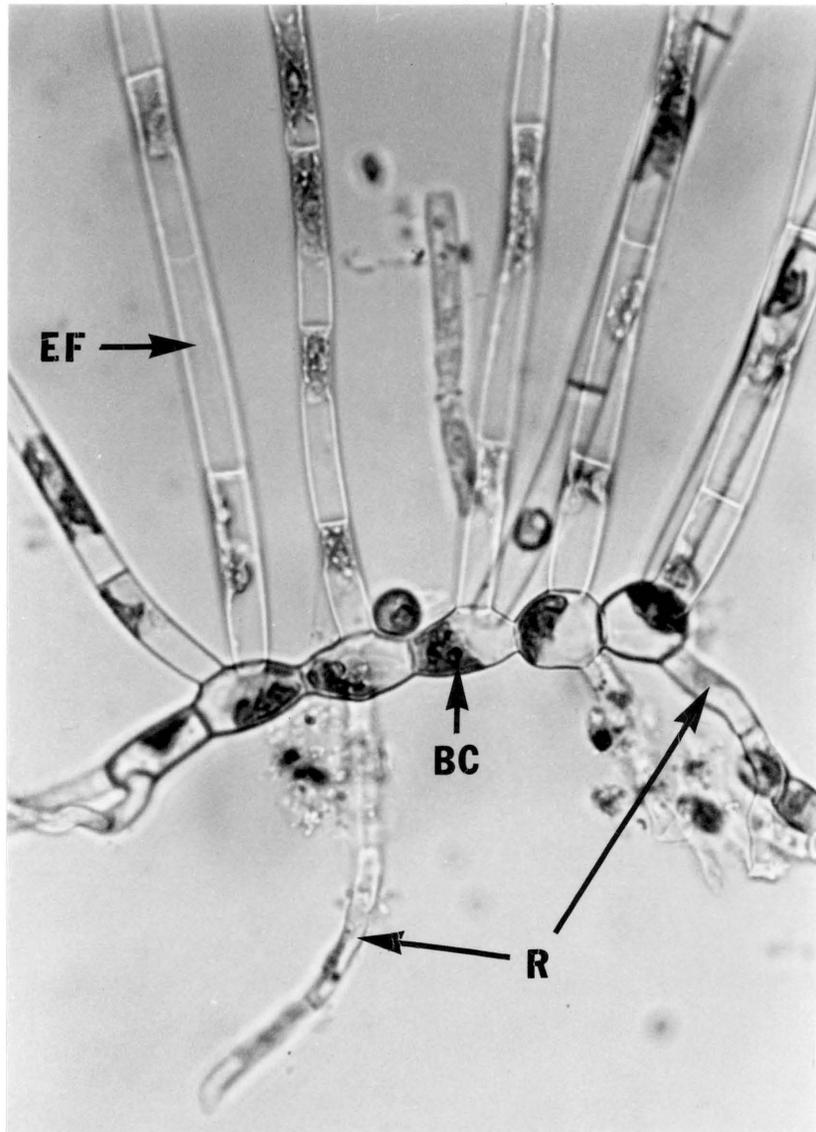


Figure 9. Compact filaments from algal mat surviving intermittent chlorination. (250X)



Figure 10. Compact radiating filaments from dense basal cell mass which survived intermittent chlorination. Compare with Fig. 8. (400X)



Figure 11. Portion of hemispherical colony which survived intermittent chlorination. This was the most extreme morphological change observed. (250X)

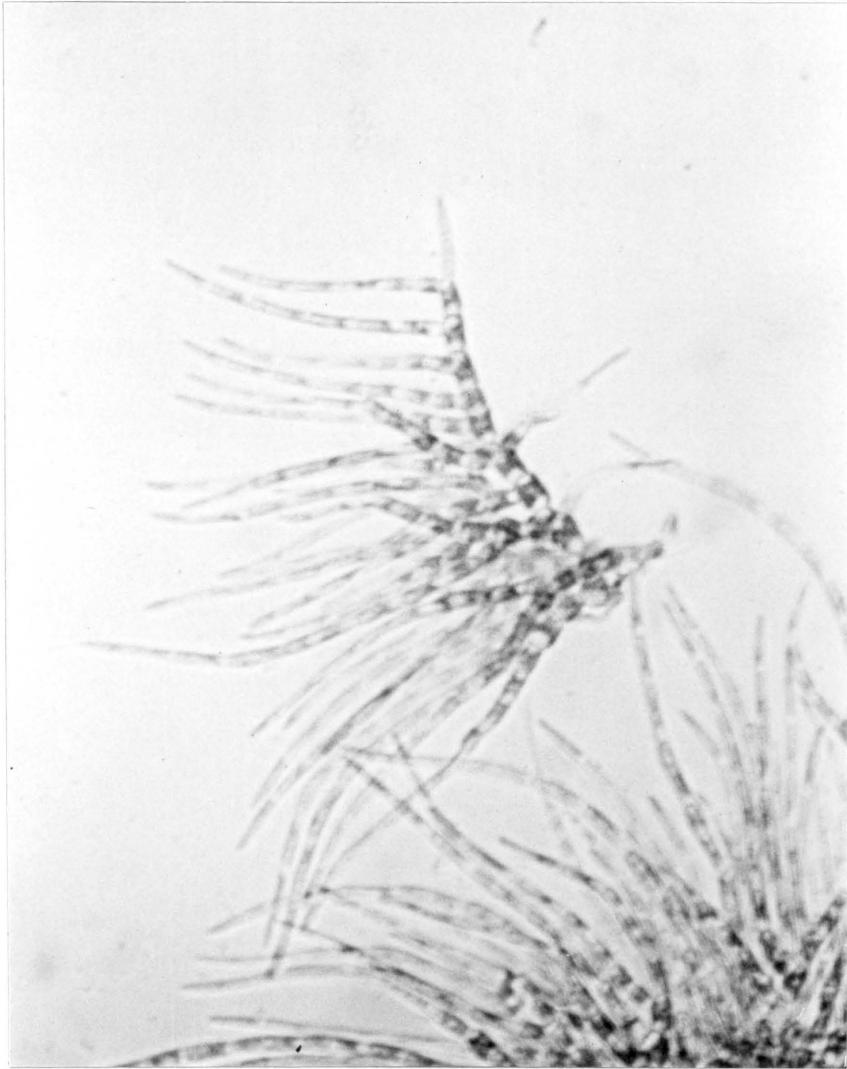


Figure 12. Filamentous cells from control algal mat. Very little filamentous branching occurred in the control cultures during the one week testing period. If allowed to continue growing, braching became much more common. (400X)



Figure 13. Comparison of normal vegetative filament cells (V) and zoospore producing filament cells (ZP). (250X)

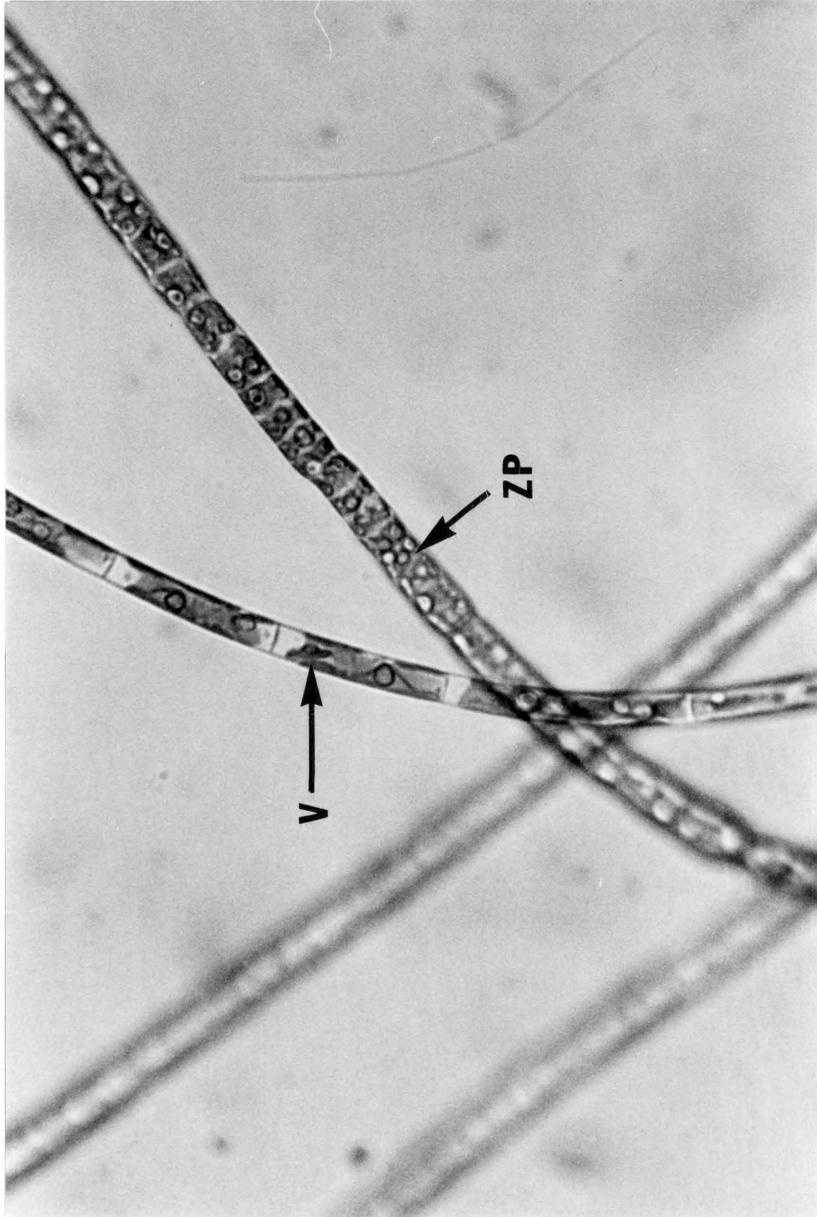


Figure 14. Zoospore producing filament, a branch of which (C) has just released several zoospores. Also shown is newly released zoospore (Z). (250X)

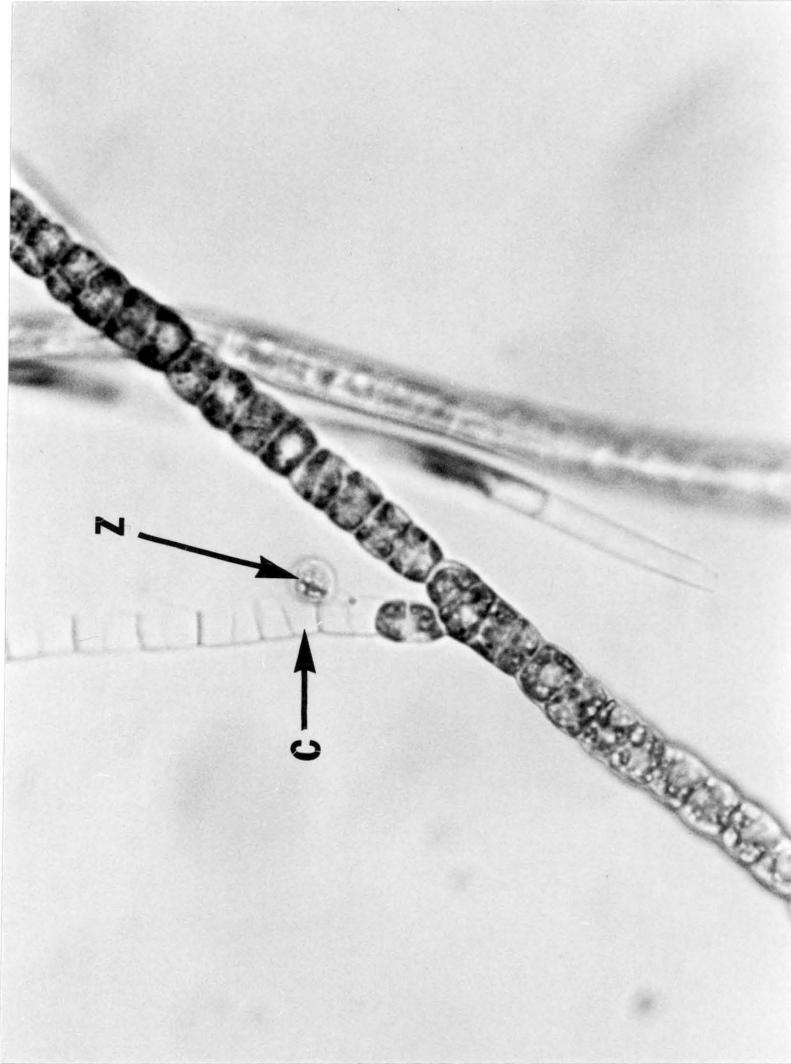
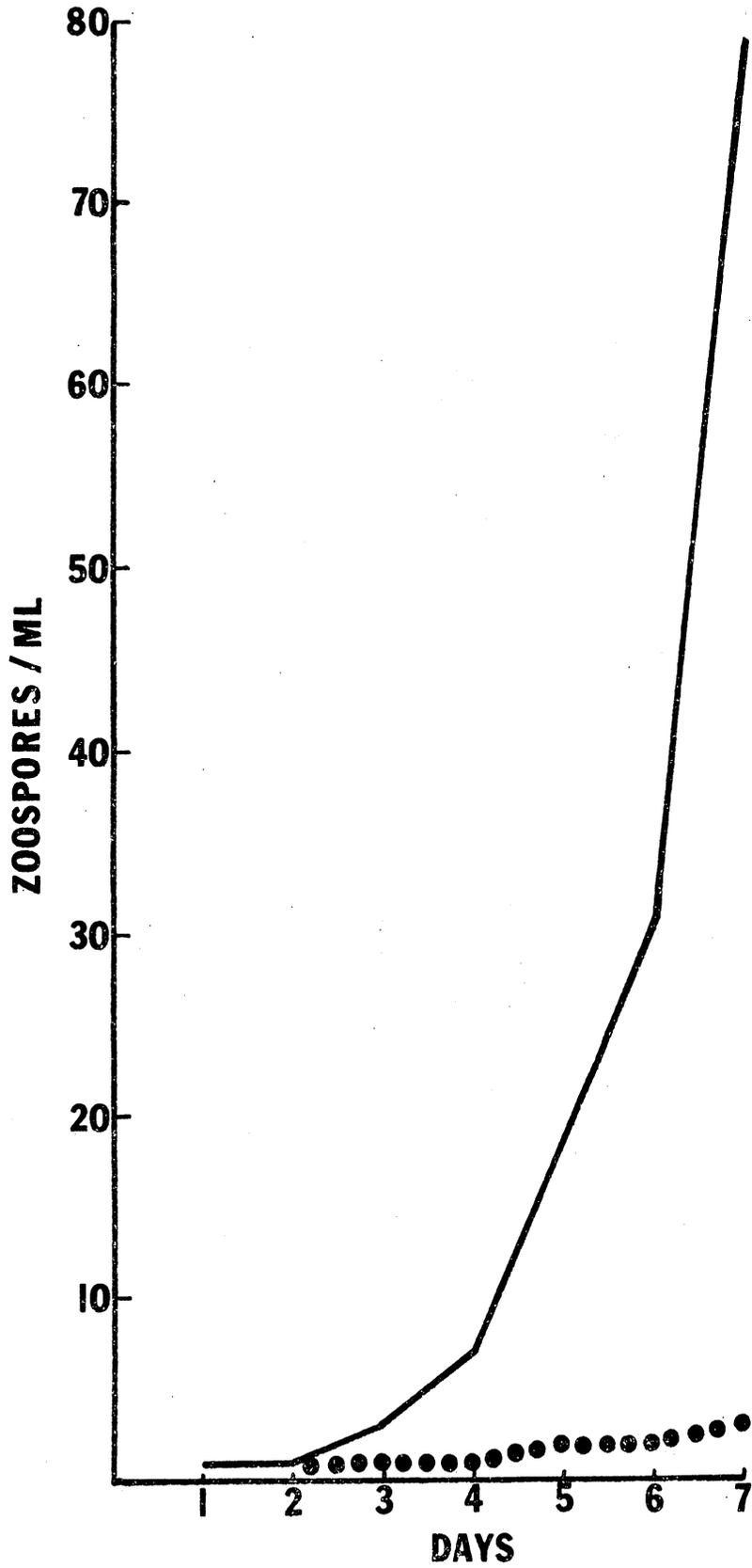


Figure 15. Zoospore production of control algal mat (solid line) and an algal mat surviving intermittent chlorination (dotted line).



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THE DEVELOPMENT OF AN ALGAL BIOASSAY PROCEDURE
USING STIGEOCLONIUM SUBSECUNDUM AND THE DEMONSTRATION
OF THE EFFECT OF INTERMITTENT CHLORINATION ON AN
ATTACHED FILAMENTOUS ALGA

by

Dennis Mitchell Trotter

(ABSTRACT)

A continuous flow algal bioassay system using the filamentous alga Stigeoclonium subsecundum attached to removable substrates (stigeocloniometers) was used to determine the effects of intermittent chlorination on an attached alga. Preliminary experiments were conducted to determine 1) the best conditions for attaching the algal cells to the stigeocloniometers 2) the best method for increasing the algal biomass on each stigeocloniometer prior to testing and 3) the determination of the mathematical relationship between the pigment extract and the dry weight biomass. The term 'minimal resistant biomass' (MRB) was coined to describe the reaction of various levels of algal biomass to a given chlorine dosage. An MRB of 2.22 mg dry weight/3 in² survived 0.5 ppm peak free chlorine at intervals of six hours but, biomass values below this were destroyed. The MRB for 0.25 ppm peak free chlorine at six hours intervals was 0.95 mg dry weight/3 in². Zoospore production was also drastically reduced in the algae receiving intermittent chlorination. The apparent cause of the minimal resistant biomass phenomenon was the morphological change which occurred in the algae

surviving intermittent chlorination. This change was of two types. When sufficient algae was present to withstand chlorination, the mat became much more compact and dense. When all but a few cells were killed by the first 96 hr, a hemispherical or dome shaped colony composed of highly branched filaments was produced. Both changes produced such compact algal mats, that the water amongst the filaments was somewhat isolated from the water above the mat, and hence did not receive the full impact of chlorination.