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# PHYSICAL-CHEMICAL METHODS FOR THE CONTROL OF ALGAL SPECIES AND COMPOSITION IN ALGAL CULTURING FACILITIES



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## ABSTRACT

The bivalve molluscs C. virginica and M. mercenaria require specific species of algal food for growth. Therefore successful commercial culture of the hard clam and American oyster in a controlled environment depends on an ability to grow desirable algal species in the presence of potentially contaminating algal species.

Three methods are described for the selective culture of Thalassiosira pseudonana, a desirable bivalve food, in the presence of a contaminant species Phaeodactylum tricornutum, an undesirable food.

The first method takes advantage of differences in cell wall strength between the two types of algae. Treatment with ultrasonic waves of culture of T. pseudonana contaminated with 50% and 70% P. tricornutum (by cell numbers) yielded growing cultures of Thalassiosira which were free of Phaeodactylum cells. Treated controls consisting of cultures of 100% P. tricornutum and 100% T. pseudonana, showed no growth and normal growth respectively.

The second method involves manipulation of nutrient levels in a mixed culture of Thalassiosira pseudonana and Phaeodactylum tricornutum to eliminate one or the other species from the culture.

The third method utilizes a harvesting reseeding technique to maintain a relatively constant concentration of the slower-growing species in a semi-continuous mixed culture containing Carteria chuii and Thalassiosira pseudonana.

The results are integrated into a two-loop algal culturing process. One loop consists of a seed culture utilizing a small percentage of the influent water and a larger mass algae culture. The mass algae loop is continuously inoculated with new seed from the seed loop in sufficient quantities so that desirable species out-compete undesirable species. Water entering the seed loop is purified by an ultrasonic treatment process so that undesirable species may be eliminated from the seed cultures.

# INTRODUCTION

The bivalve molluscs Crassostrea virginica and Mercenaria mercenaria require specific species of microalgae in sufficient quantities in order to achieve rapid growth rates. For example, a mixed diet containing Phaeodactylum tricornutum, Rhodomanas sp. and Isochrysis galbana resulted in better growth than paired combinations of these same species (Hartman et al., 1973). A single marine algal species which yielded good growth rates when fed to these species is Thalassiosira pseudonana 3 H (Epifanio et al., 1975). However, efforts to culture this algal species on wastewater have resulted in substantial contamination of T. pseudonana cultures with P. tricornutum (Goldman and Stanley, 1974). Epifanio and Mootz (1975) reported that neither C. virginica nor M. mercenaria survived when fed a diet containing only P. tricornutum. It is clear that successful mariculture of bivalve molluscs requires methods for the control of the species of algae cultivated for their diets.

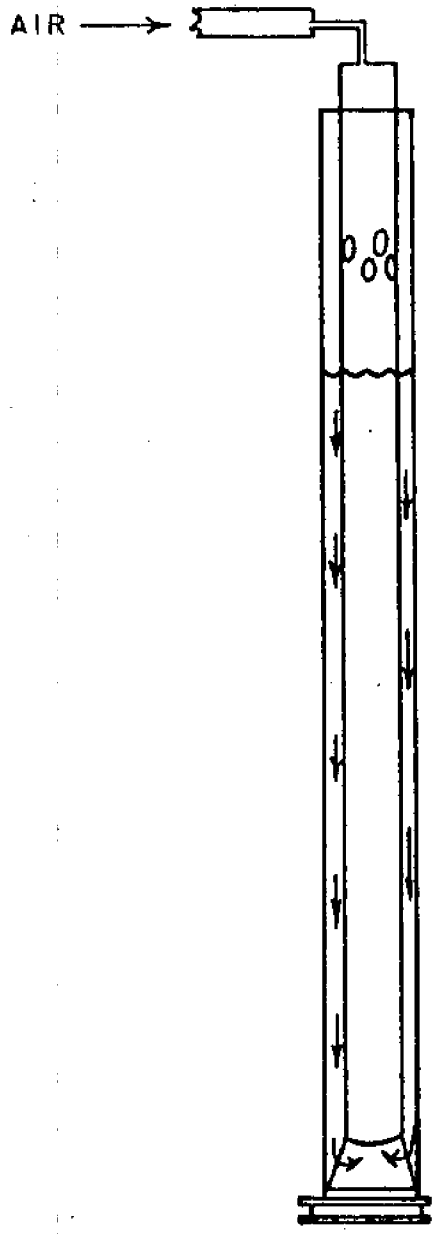
In addition to the necessity of maintaining species integrity in large scale algal cultures the productivity of these facilities is also important. Adult bivalves may require as much as $1 \times 10^9$ cells/animal/day for growth (Epifanio et al., 1975). Thus methods should be available to ensure that an outdoor algal culture on a December day provides as many cells as the same culture during July.

An examination of these problems led us to consider a variety of physical and chemical methods which might be applicable. Results from three types of experiments are reported.

The first experiment we performed involves the use of ultrasonic waves to remove selectively undesirable algal species from cultures of T. pseudonana. The second method utilizes a manipulation of the nutrient concentration in a culture, either to remove P. tricornutum from a T. pseudonana culture, or to permit this second species to become a component of the final algal harvest.

The third method involves the use of a continuous seeding process to compensate for the slower growth rate of one species of algae in the presence of a species which has a faster growth rate.

Figure 1. Tube used to culture algae



## MATERIAL AND METHODS

### I. Exposure of Algal Cultures to Ultrasonic Waves

A 280-watt Biosonik ultrasonics generator* with a standard horn was used at 100% power output to treat mixed algal cultures. In order to simulate conditions in which P. tricornutum has contaminated cultures of T. pseudonana, cultures were made which contained 0%, 34%, 60%, and 100% P. tricornutum in T. pseudonana. The mixed cultures were counted with a Coulter Counter**, model $Z_B$, fitted with a 100 μ aperture tube. Amplification and aperture current were set at 1/8.

Samples containing 50 mls of the mixed cultures were chilled to 15°C and then placed in an ice bath during the ultrasonic treatment. Exposure time to ultrasonic waves was two minutes so that the final temperature of the solution was kept below 25°C. The treated samples were placed in 1 liter of artificial seawater*** containing 2F medium (Guillard and Ryther, 1962). The growth of these cultures was monitored, using a Turner Model III fluorometer. Samples of the cultures were taken initially, after treatment, and after 48 hours of culture for photographing on a hemacytometer slide. From cell counts made from these photographs, estimates were made of the ratio of Phaeodactylum to Thalassiosira.

### II. Nutrient Composition as a Method of Controlling the Algal Species Composition

The vessel used to culture the algae in these experiments is shown in Figure 1. On a rack 50 cm away from these culturing tubes, six cool white fluorescent lights were mounted to provide continuous illumination. Three liters of artificial seawater containing 1F medium were inoculated with the appropriate algae. Nutrient analysis was done according to methods described in Strickland and Parsons (1968). Four of these culturing tubes were used in each experimental set. The first contained either pure

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*Bronwill Scientific, Rochester, New York, Model BP1

**Coulter Electronics, 590 W. 20th Street, Hialeah, Florida

***Instant Ocean Aquarium Systems, 33208 Lakeland Boulevard, Eastlake, Ohio

TABLE 1

| Initial % *P. tricornutum* in *T. pseudonana* | Initial counts cells $x10^6$/ml | % *P. tricornutum* after treatment | % *P. tricornutum* after culture | Final counts cells $x10^6$/ml |
|---|---|---|---|---|
| 0 | 3.00 | 0 | 0 | 3.53 |
| 34 | 2.96 | 0 | 0 | 2.09 |
| 60 | 3.08 | 0 | 0 | 1.90 |
| 100 | 3.29 | 0 | 0 | .03 |
| 34 | 3.20 | 0 | 0 | 3.94 |
| 60 | 2.89 | 0 | 0 | 2.27 |
| 34 (control) | 3.03 | - | 30 | 1.85 |
| 60 (control) | 2.95 | - | 55 | 2.81 |

T. pseudonana or pure P. tricornutum. The second, third, and fourth contained a 1%, 10% and 25% addition of P. tricornutum to a T. pseudonana culture. Tubes 1, 2, and 3 were batch cultured, although in one set of experiments extra nutrients were sequentially added to culture A.

Approximately 80% of the volume of culture 4 was harvested when the cell density reached $3.0 \times 10^6$ cells/ml. After harvesting, nutrients were added as well as sufficient artificial seawater to restore the initial volume.

### III. Repetitive Seeding Process for the Control of Algal Culture Composition

Four cultures were prepared in the culture tubes described above. The first tube contained Carteria chuii, the second and third a 50:50 (by cell count) mixture of Thalassiosira pseudonana and Carteria chuii; and the fourth Thalassiosira pseudonana.

At 20, 32, 56 and 68 hours the tubes were harvested by removing some of the culture and then restoring the nutrients and volume to their original levels. At 20 and 56 hours, however, $9.84 \times 10^8$ and $8.25 \times 10^8$ Carteria cells were added to tube 2 and 3 respectively in addition to the residual 400 ml of unharvested algal culture, fresh seawater and nutrients. The percent Carteria in the sample was determined by visual counts on a hemacytometer slide made from the mixture.

## RESULTS

### I. Exposure of Algal Cultures to Ultrasonic Waves

Results of experiments in which pure and mixed cultures of the microalgae P. tricornutum and T. pseudonana were exposed to ultrasonic radiation are shown in Table 1. Two control cultures which were not treated are also shown. The initial cell concentration of each culture was approximately the same prior to treatment. After treatment with ultrasonic waves, no Phaeodactylum cells could be found in any of the cultures.

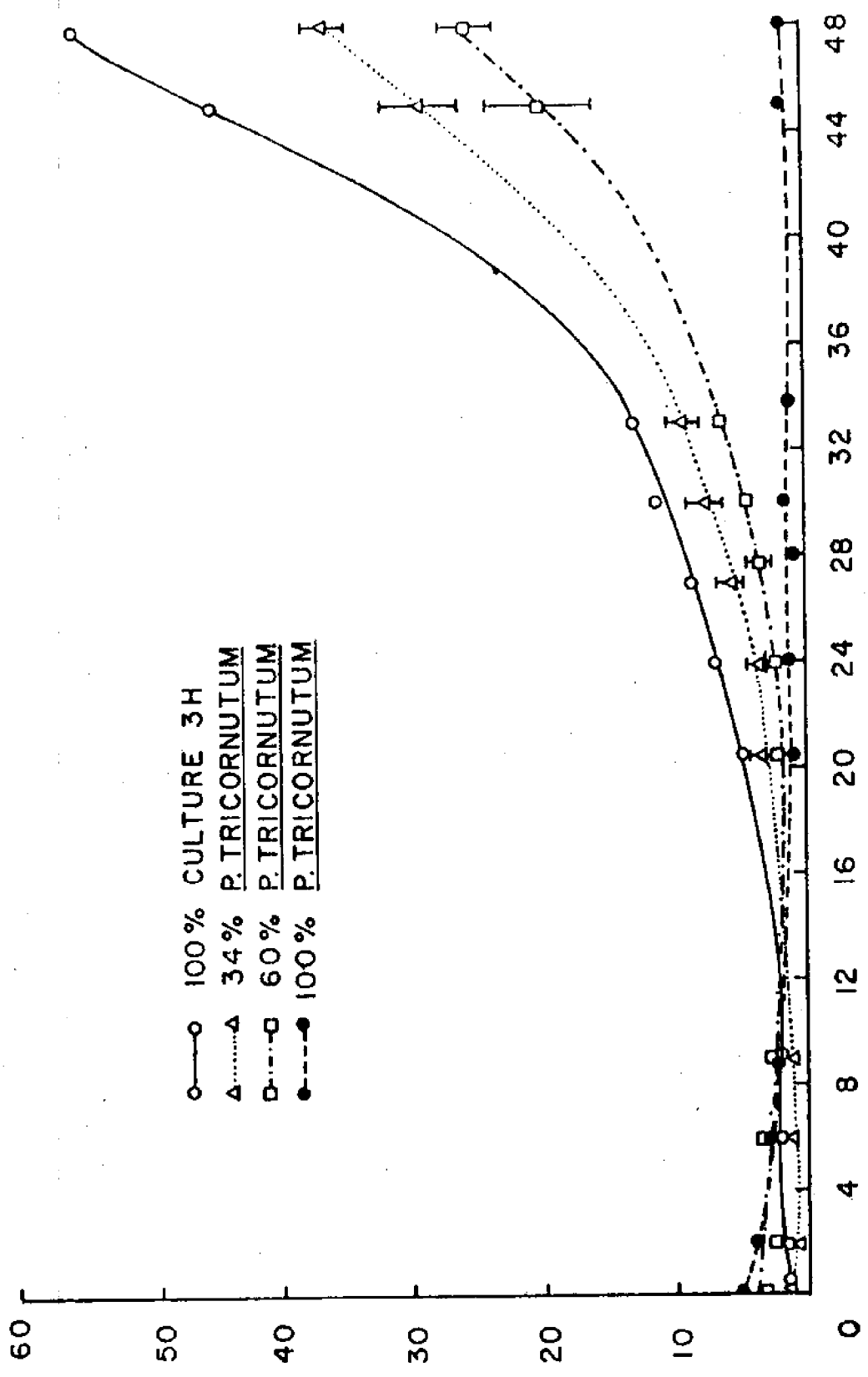



Figure 2. Growth curves for cultures treated with ultrasonic waves

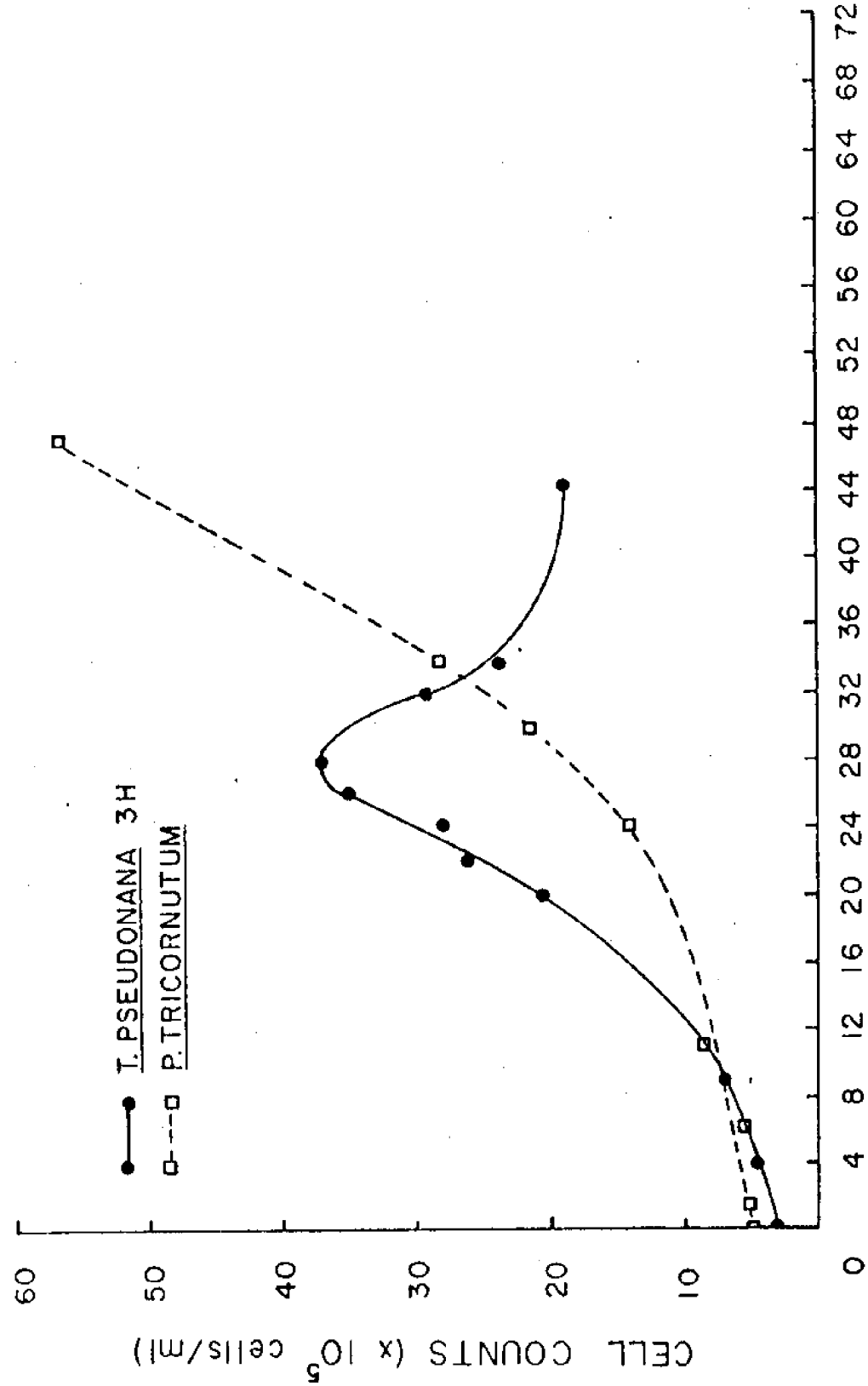



Figure 3. Growth curves for monospecific cultures of Thalassiosira pseudonana 3H and Phaeodactylum tricornutum

Treated and untreated 50-ml samples, were transferred to a 1-liter seawater solution containing 2 F nutrients. All of the inoculated cultures except the one originating from a treated 100% P. tricornutum solution showed increased fluorescence and cell counts, as recorded in Figure 2 and Table 1.

Phaeodactylum did not appear as a component in any of the final cultures, while Thalassiosira cells remained in the inoculum sample and were capable of reproduction.

## II. Nutrient Composition as a Method of Controlling the Algal Species Composition

Figure 3 is a plot of the growth curves for P. tricornutum and T. pseudonana. A fit of these data to the equation $\ln c = kt + \ln c_o$; where K is the growth rate, c is the cell concentration at time t and $C_o$ is the initial concentration of cells, yields growth rates, k, of .111 for Thalassiosira pseudonana and .049 for Phaeodactylum tricornutum. These values correspond to 3.84 and 1.70 doublings per day, respectively.

T. pseudonana has a more rapid growth rate than P. tricornutum, but the final cell density and life of the P. tricornutum culture, as shown in Figure 3, was greater. This suggests nutrient limitation of growth. Silicate analysis of the two mixtures at 28 hours showed zero silicate remaining in the T. pseudonana medium, while the P. tricornutum medium still contained 20 μ moles of silicate. This indicates different nutrient requirements for the two species.

Figure 4 is a plotting of cell density versus time for batch cultures inoculated with 1% Phaeodactylum (by cell count). Culture 2A had extra nutrients (F concentration) added at 20, 46 and 56 hours. Figure 5 shows the percent of Phaeodactylum in these same cultures when measured at different times.

After 24 hours, the percent of Phaeodactylum increased in cultures 2B and 2C. Culture 2A which had extra nutrient added contained no Phaeodactylum cells after 72 hours of growth.

The results from Thalassiosira cultures with 10% added Phaeodactylum are shown in Figure 6; Figure 7 gives the percent composition of these cultures when determined at different times. After 24 hours, culture 3B and 3C showed increases in Phaeodactylum. After 48 hours, culture 3A also showed an increase in the Phaeodactylum component.

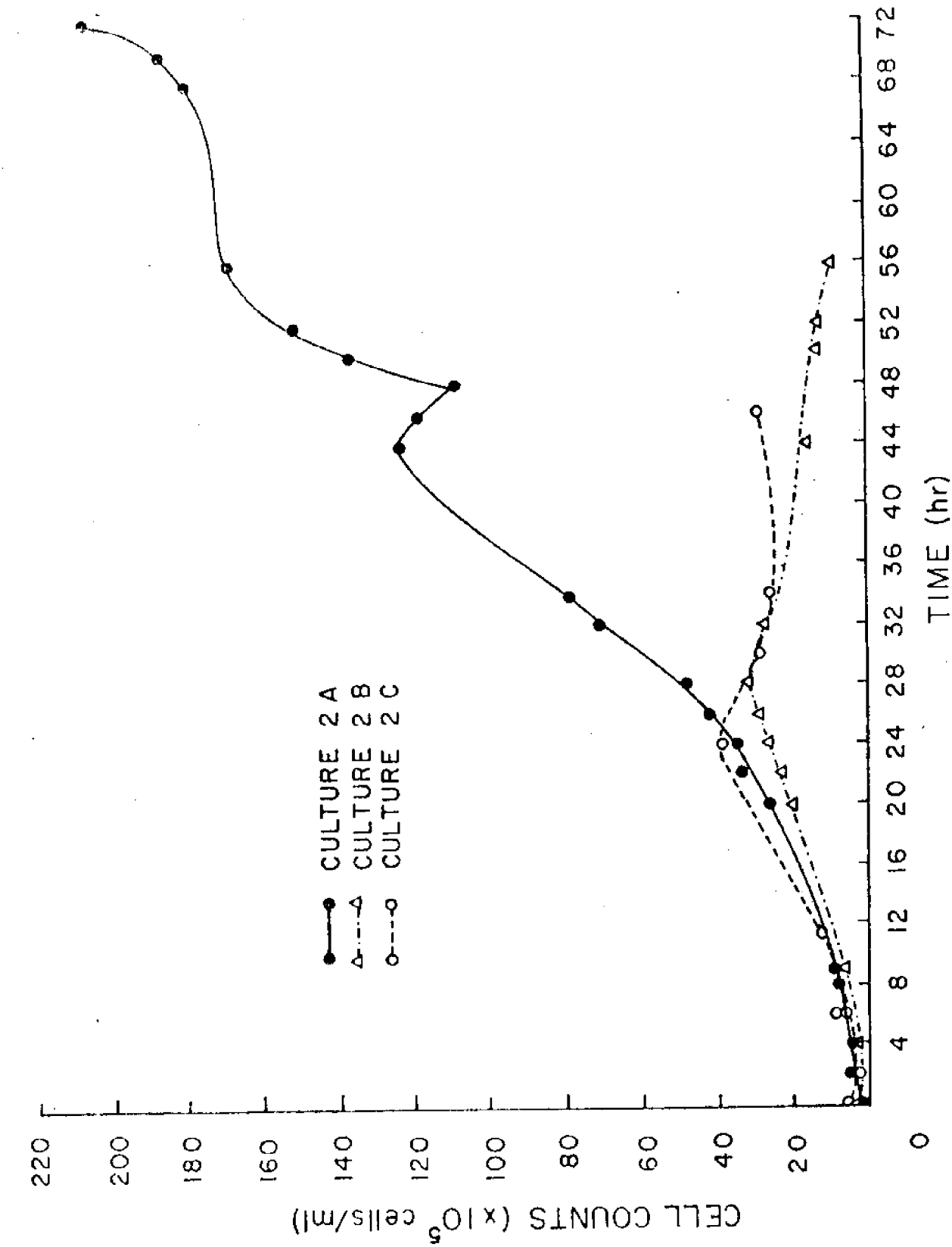



Figure 4. Growth curves for mixed cultures containing one percent Phaeodactylum tricornutum in Thalassiosira pseudonana 3H

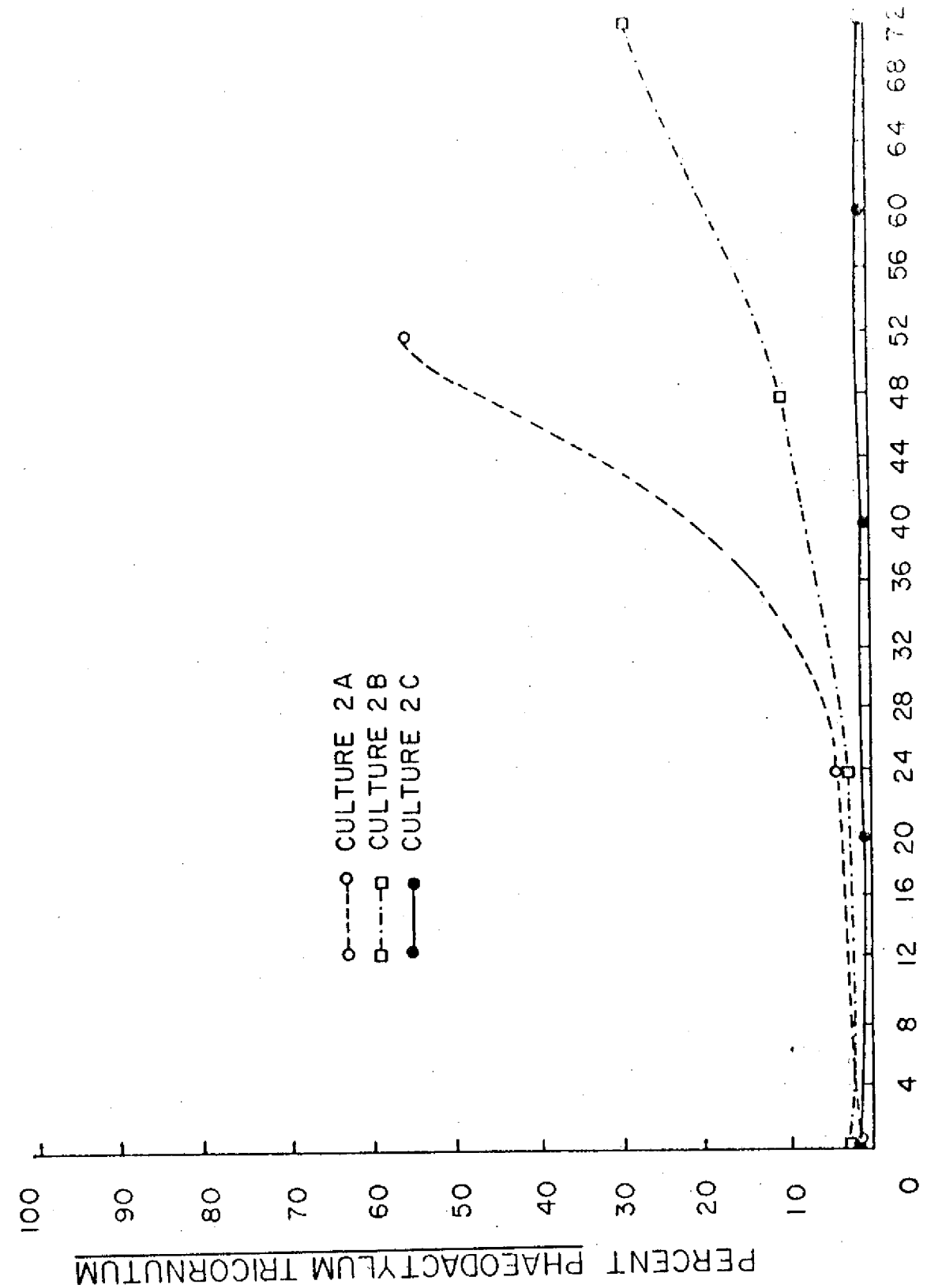
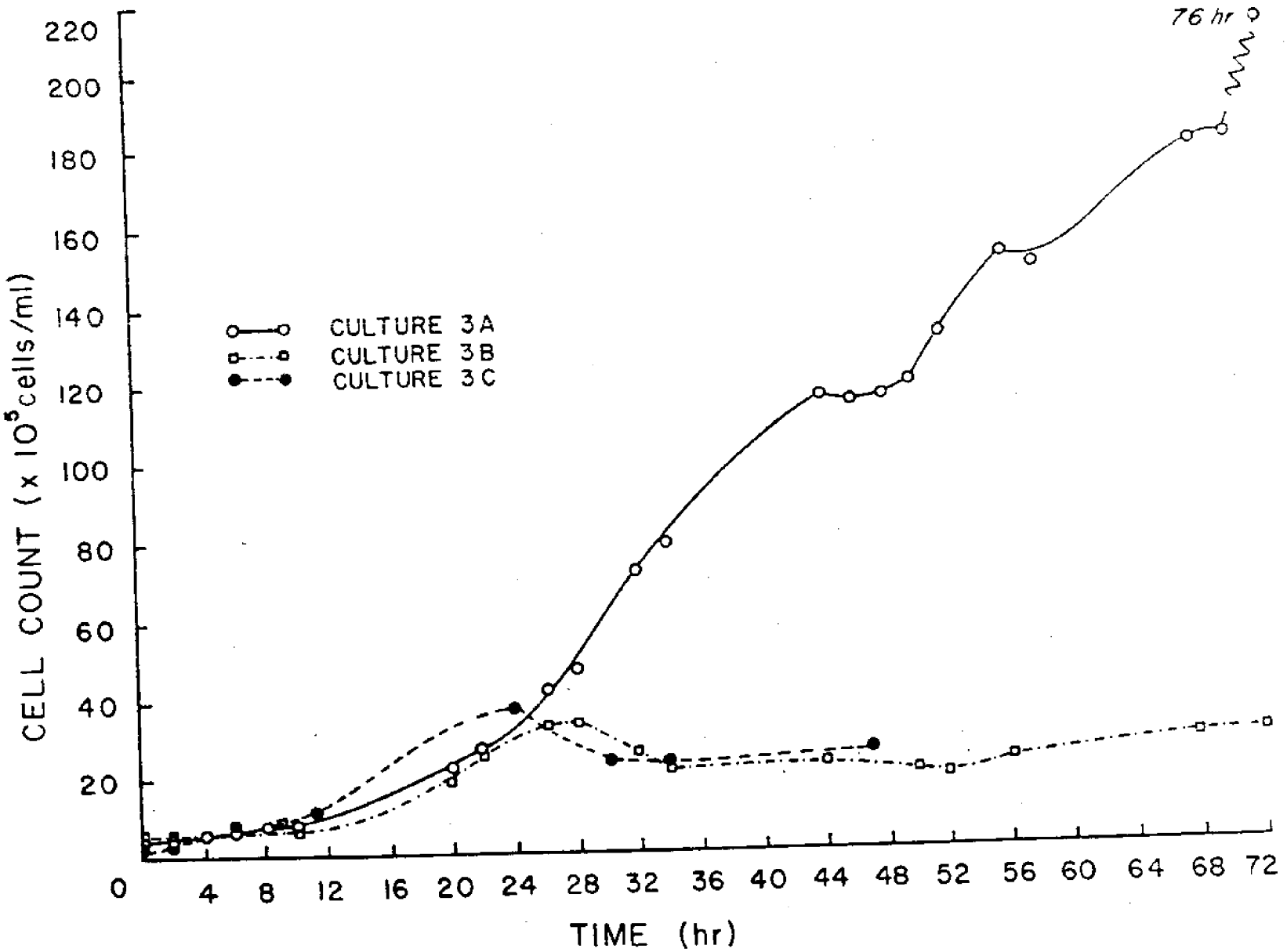



Figure 5. Percentage Phaeodactylum tricornutum in mixed cultures

Figure 6. Growth curves for mixed cultures containing ten percent Phaeodactylum tricornutum in Thalassiosira pseudonana 3H



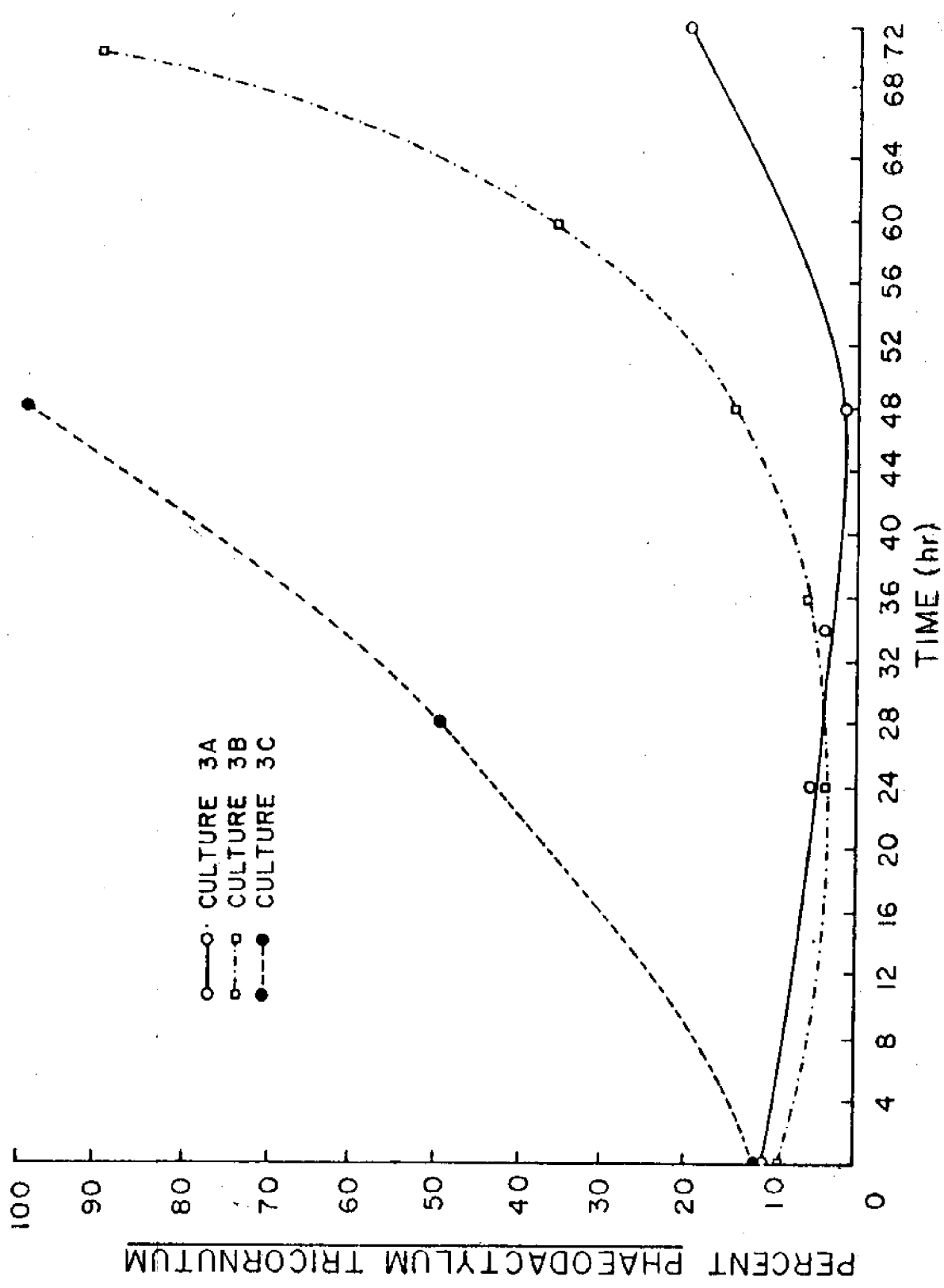



Figure 7. Percentage Phaeodactylum tricornutum in mixed culture

Figures 8, 9, and 10 show results from a semicontinuous culture. The value shown in parenthesis is the percent Phaeodactylum at the time of harvest. The percent of P. tricornutum in the cultures decreases with time as expected, since its growth rate is slower than T. pseudonana.

III. Repetitive Seeding Process for the Control of Algal Culture Composition

An equal mixture (by cell count) of Thalassiosira pseudonana and Carteria chuii were cultured semi-continuously by harvesting at appropriate intervals. The results of replicate experiments are shown in Figure 11. At 20 and 56 hours after harvest, the cell ratio was restored to the original 50:50 ratio by adding a sufficient quantity of the slower-growing species, Carteria chuii, from a seed culture.

In this manner, it was possible to harvest a mixed algal culture containing about 25% Carteria chuii for the 72-hour duration of the experiment. After 72 hours, in the absence of a fresh addition of Carteria cells, Thalassiosira dominates the culture, as shown by the values in parenthesis on Figure 11.

## DISCUSSION

Ultrasonic waves appear to be highly selective in their action on mixtures of Phaeodactylum and Thalassiosira. Apparently differences in the composition of the cell walls of the two species account for the quantitative destruction of Phaeodactylum. Thalassiosira cells not only remained intact after sonication but were capable of reproduction as shown by the family of growth curves in Figure 2.

From a practical standpoint, the sonication treatment to remove undesirable microrganisms has several useful characteristics. It utilizes simple, readily available commercial instrumentation. It does not require the introduction of foreign materials into the water and, except for the release of cell contents from disrupted cells, does not alter the

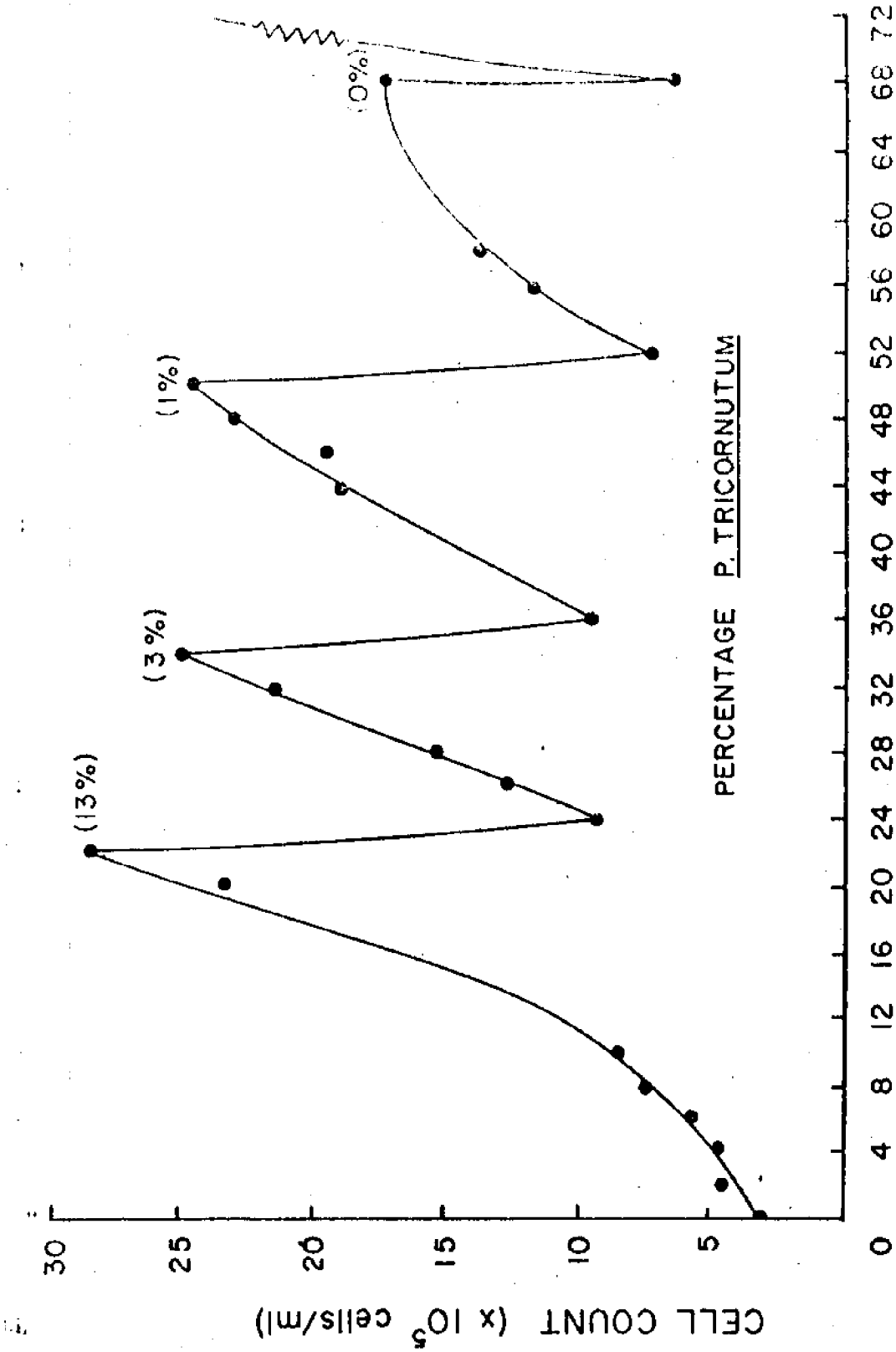
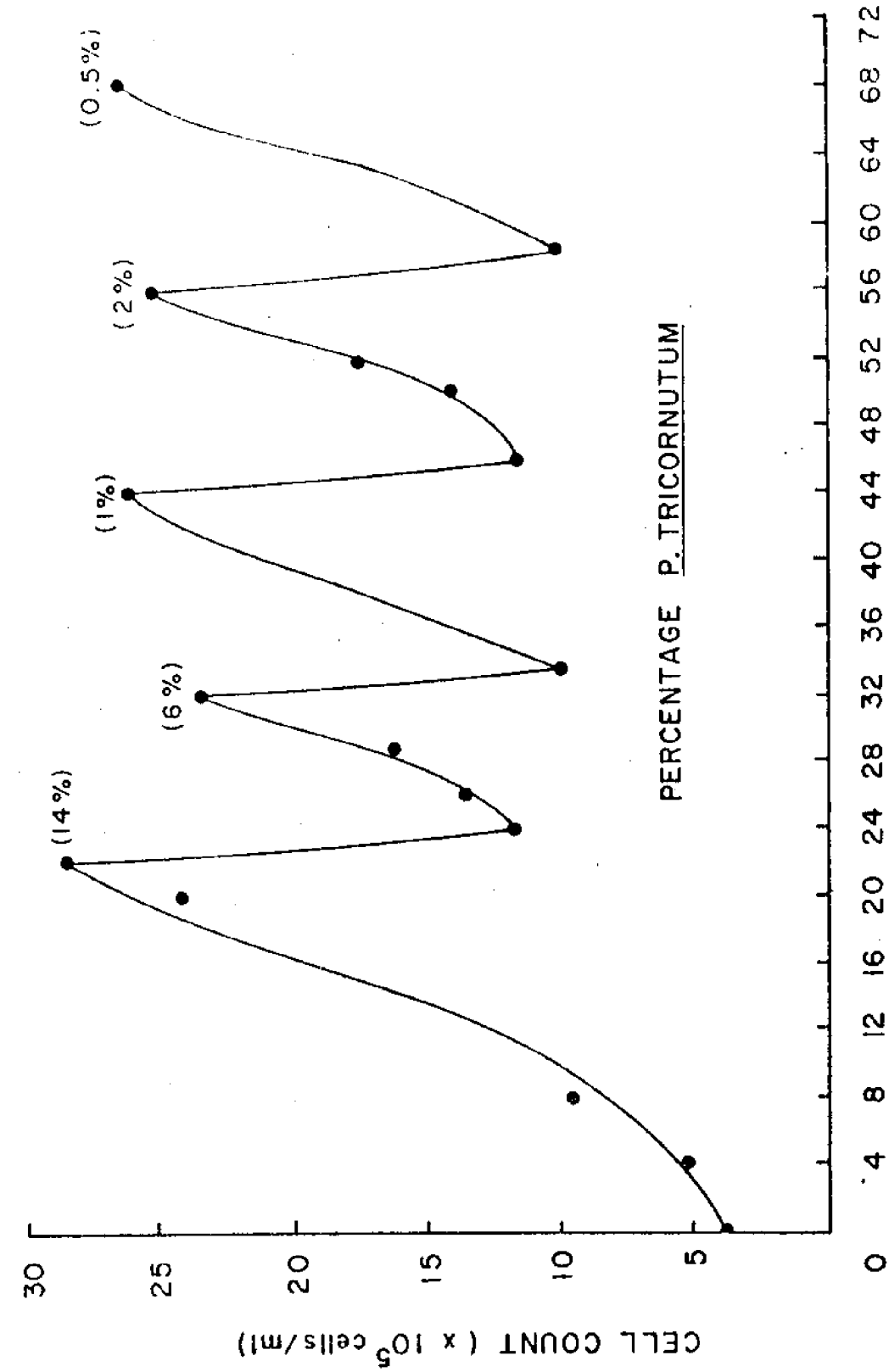



Figure 8. Decrease in percentage of Phaeodactylum tricornutum in semi-continuous Thalassiosira pseudonana culture

Figure 9. Decrease in percentage of Phaeodactylum tricornutum in semi-continuous Thalassiosira pseudonana culture



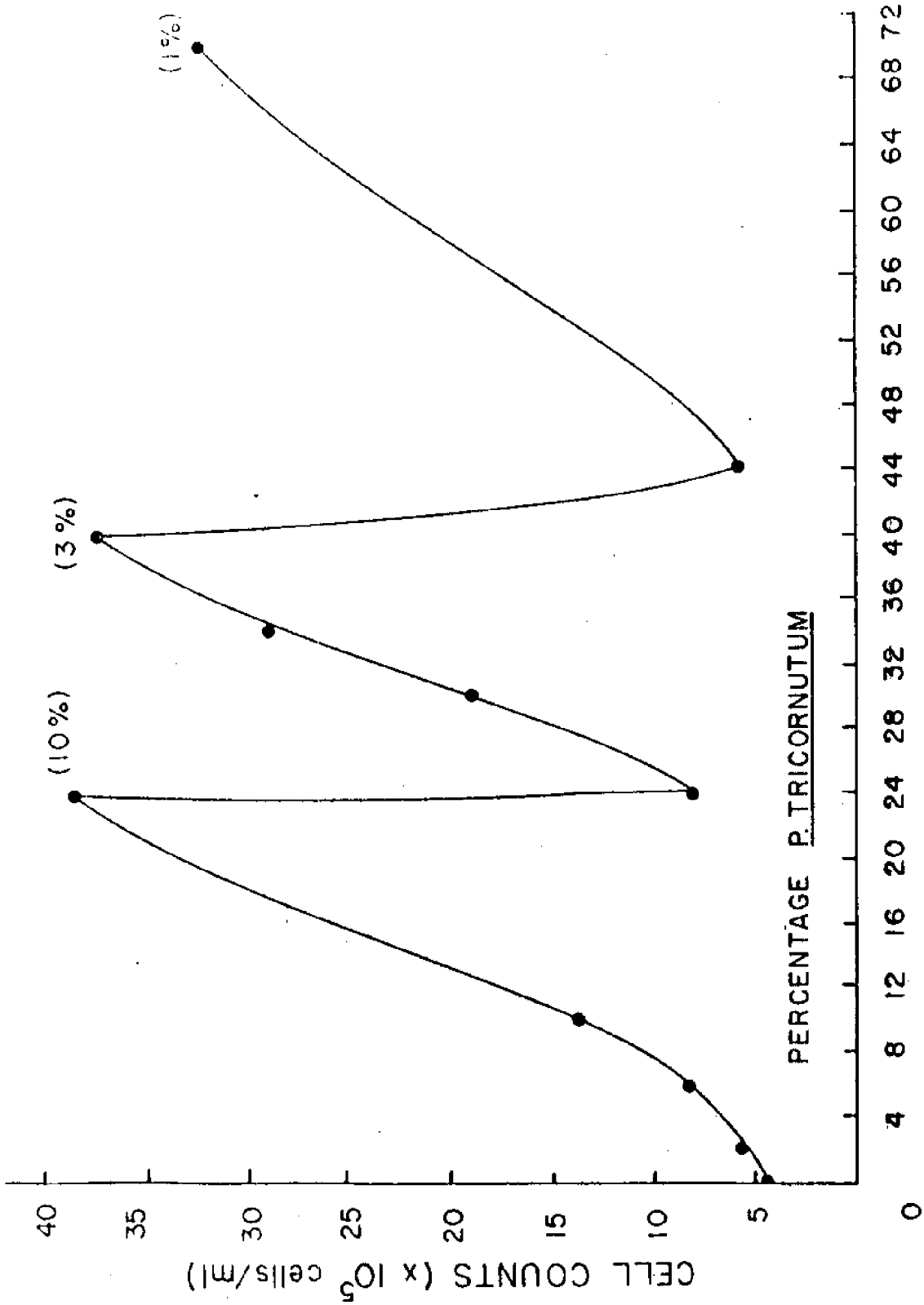



Figure 10. Decrease in percentage of Phaeodactylum tricornutum in semi-continuous Thalassiosira pseudonana culture

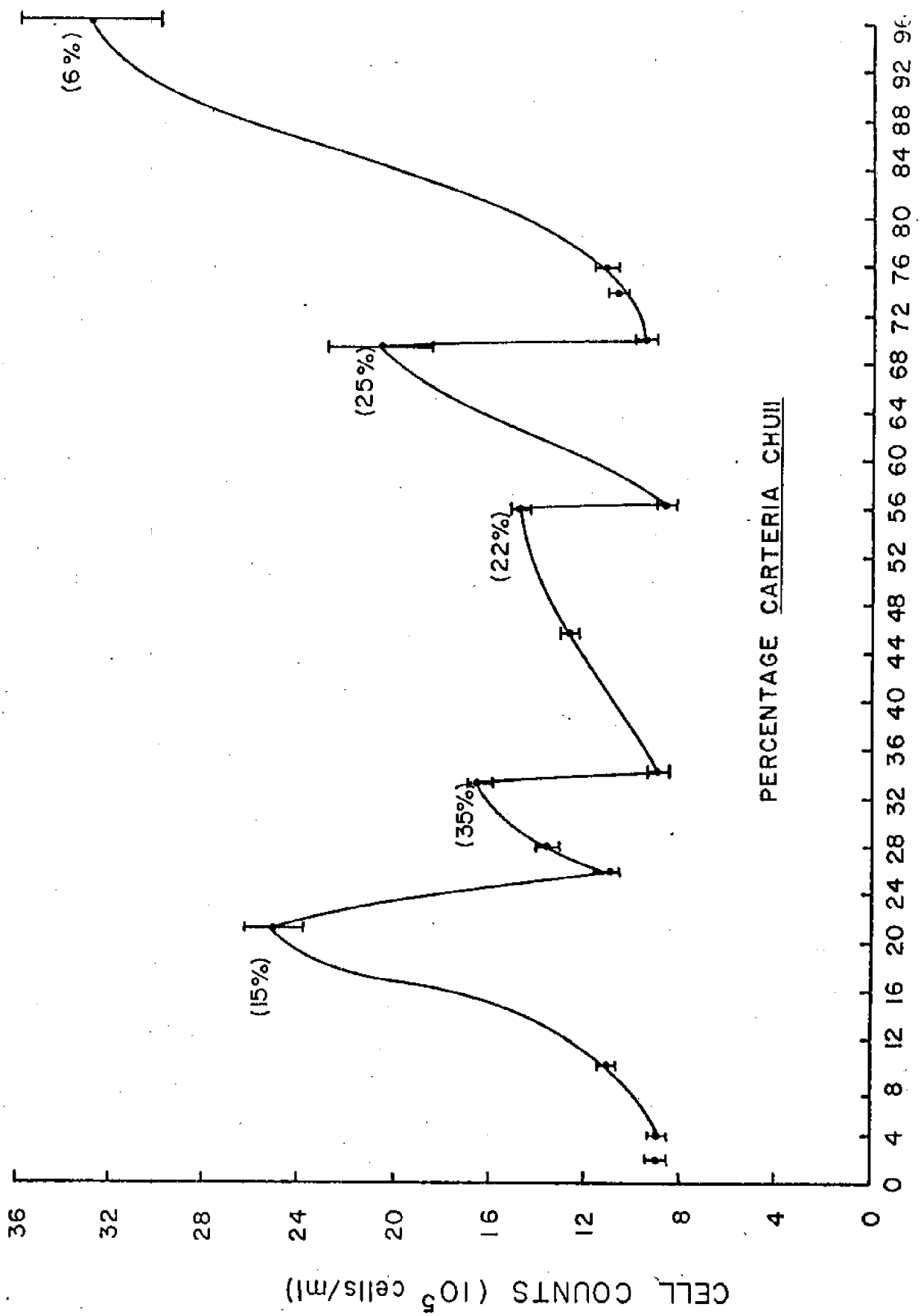



Figure 11. Growth curve and percentage composition of mixed cultures of Carteria chuii and Thalassiosira pseudonana 3H

composition of the water. The treatment may be used continuously or intermittently as required. It is likely that sonication will also be effective in destroying larvae or bacteria which may enter an algal growing tank.

The careful regulation of a limiting nutrient may also be employed to control the species of algae which are grown in an algal culturing facility. Figure 3 shows the effect of the inadequate silicate content of F nutrient media for the growth of Thalassiosira pseudonana. Figures 4 to 7 show the result of using this nutrient formulation on mixed cultures containing both Phaeodactylum and Thalassiosira. Although Thalassiosira quickly becomes the dominant species, Phaeodactylum forms a significant percent of the final composition of the cultures, since its growth is not limited by the reduced silicate content of the water. Thus an algal species whose growth rate is only half that of its competition may become dominant if the nutrient content of the cultures is inadequate for the faster-growing species. It may be possible to take greater advantage of this phenomenon in continuous cultures. If the first in a series of interconnected growing ponds were seeded with a slow-growing species which did not require large quantities of silicate, its concentration could reach high levels near the middle of the series of ponds. At that point, more silicate and seed cultures of Thalassiosira (a fast-growing species) could be introduced. The product culture would then be a mixture of two desirable species having considerably different growth rates.

The growth rate of algal cultures during the "log phase" may be adequately represented by an exponential equation of the form, $c_t = c_o e^{kt}$, where $c_t$ is the concentration of algae at time t, k is the growth rate, and $c_o$ is the initial concentration of algae. Two species having different values of k may be grown in the same vessel and then harvested to some percentage of their final cell density at the time t. The initial and final ratios of the two species in the culture will reflect the time at which they were harvested as well as the differences in values of k. If after harvesting a culture, sufficient new inoculum of one species is added to restore the original ratios, the system will maintain the same

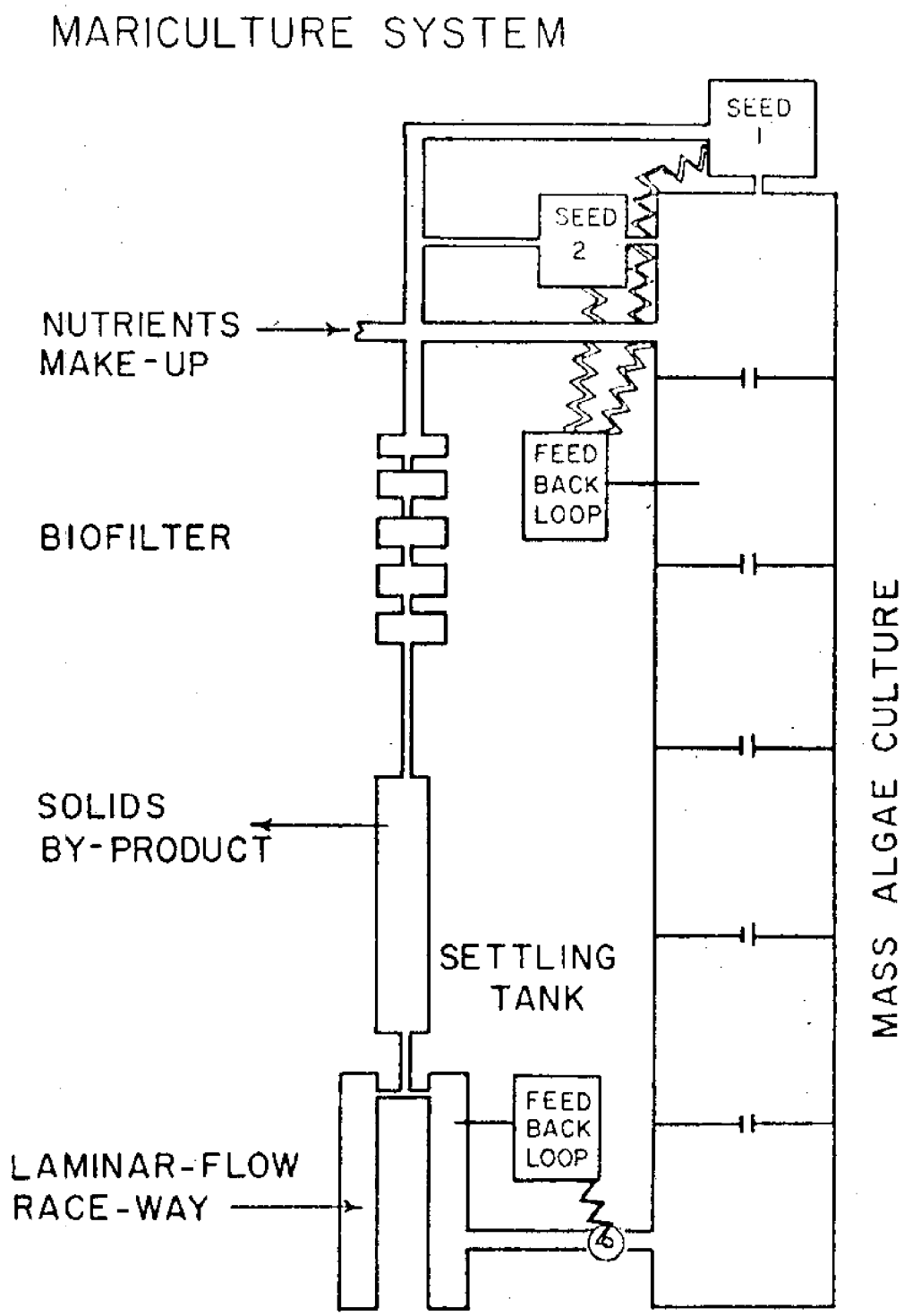



Figure 12. Schematic of a bivalve culturing facility

ratios of algal cells over time. If quantities of the slower-growing species are added in excess of that required to restore the original ratio at the time of each harvest, then the slower-growing species will eventually dominate the cultures.

Constraints on this process will be the cell density to which the slower-growing species can be cultured in the seed cultures as well as the cell density of the mixed species culture at the time of harvest.

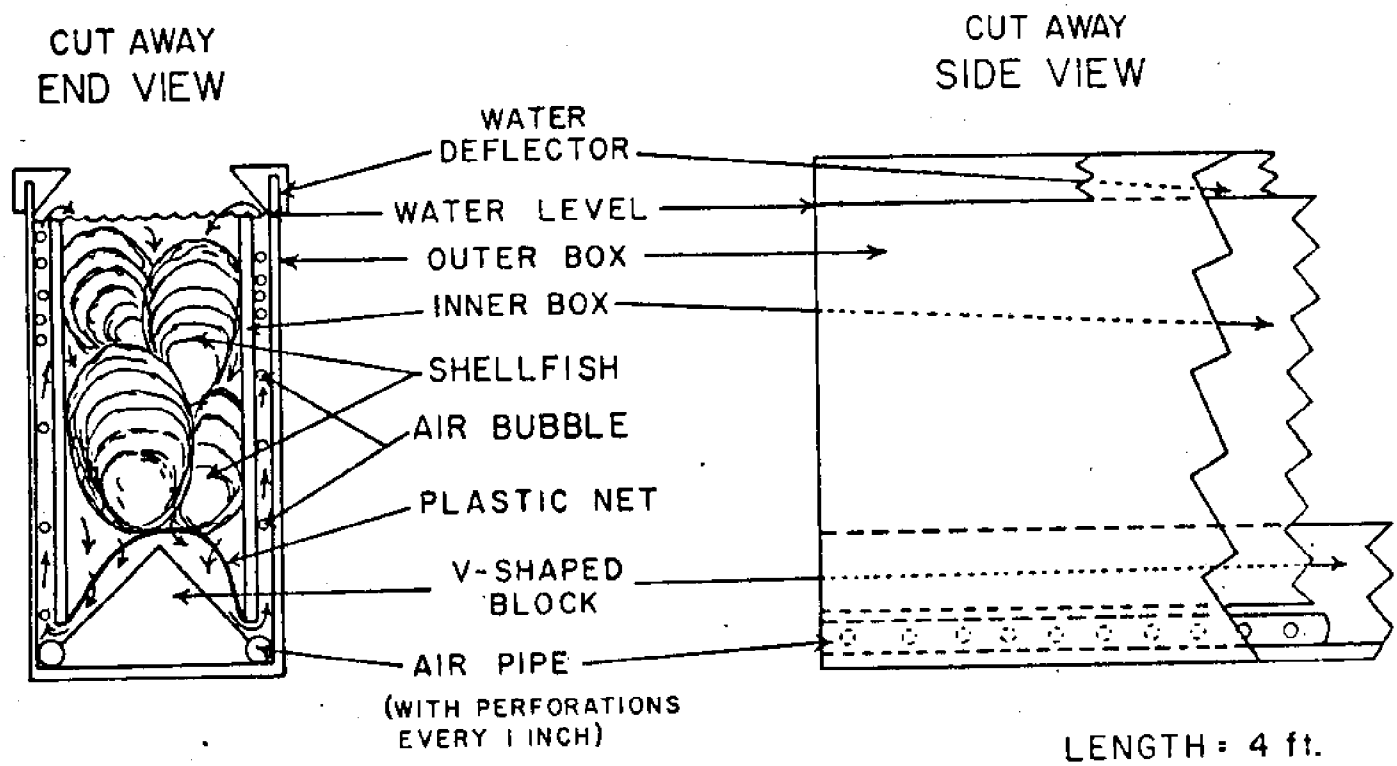
This technique of continuous seeding of a culture represents a powerful method for control of culture composition, since contaminant species which grow faster than the desirable species can be out-competed by sufficient additions from seed cultures of a slower-growing species.

The first module in a continuous culture sequence is the logical place to exercise this control since it is easiest to exceed cell numbers of contaminant species at this point. A refinement of this technique leads to mixed cultures of species growing at different rates to a predetermined composition at harvest.

In addition to removal of contaminant species from water, regulation of the seed input can control the quantity of algae present in the mass algae units. Short-term adverse growing conditions in the mass algal tanks, which lead to less productivity at harvest, can be compensated for by the addition of excess seed culture during these periods.

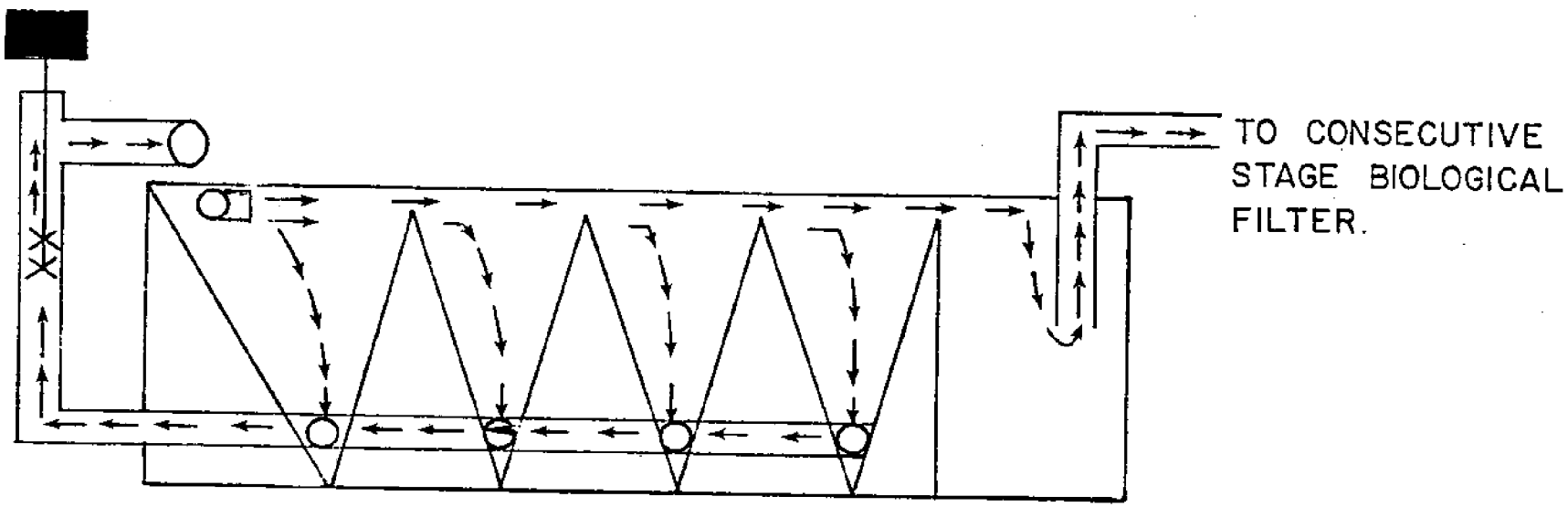
These three methods of controlling algal cultures may be integrated into a single recirculated seawater mariculture facility. An example of this is shown in Figure 12. In this configuration, the shellfish are grown in laminar flow raceways. The design of the individual tanks is shown in Figure 13. Algae and solid wastes are kept in suspension by the combined action of airlift pumps located next to the walls of the tanks and an inverted v-shaped bottom. Water circulates continuously around a number of these interconnected units until a sensor determines that most of the algae has been cleared from the water. This triggers a pump which brings water from the mass algal tank to the growth tanks until the desired concentration of algae in the growth tanks has been achieved. Overflow water from the growth tanks passes into a settling

Figure 13. Shellfish growth tank




SHELLFISH GROWTH TANK UTILIZING 2 AIR LIFT PUMPS AND V-SHAPED BLOCK TO PREVENT SETTLING OUT OF SOLID WASTE AND ALGAE

Figure 14. Solid waste settling tank



tank (shown in Figure 14) where solids are continuously removed. The solid wastes are a valuable by-product of the bivalve mollusc culturing process (Srna et al., 1975). The water then passes into a modular biological filter (Srna, 1975) for treatment of dissolved wastes (Figure 15). Most of the water is then returned to the algal growing tanks. Makeup water may be added at this point to compensate for evaporation and other factors. A smaller portion of the water is pumped to one of several seed culture facilities (shown in Figure 16). Here water is treated by sonication to remove living contaminants before entering a small-scale continuous culture. This step is critical since without it contaminant species would soon dominate the seed cultures. The environment for the continuous algal culture in these seed-growing units is kept at an optimum temperature by the use of supplemental heating or cooling, light, and nutrient additions as necessary. Seed cultures containing excess nutrients are continuously added to the mass algal growing facility in quantities sufficient to respond to adverse growing conditions in the large tanks or to the presence of contaminating species in the mass algal culture.

If more than two species of algae are desired, two types of seed algae may be introduced, either at the beginning of the mass algal sequence, or a faster-growing species may be added several tank modules after the beginning tank in the process. Appropriate feedback information on the composition of the water controls nutrient and seed inputs from the seed loops so that the system is responsive to changes in environmental conditions.

The system incorporates a number of feedback loops which automatically stabilize the algal production process. This may prove to be an extremely important concept in maricultural efforts in which, typically, organism densities and species diversity have been altered from those which occur in nature. Thus many of the natural pathways which stabilize natural ecosystems are no longer present in a mariculture facility.

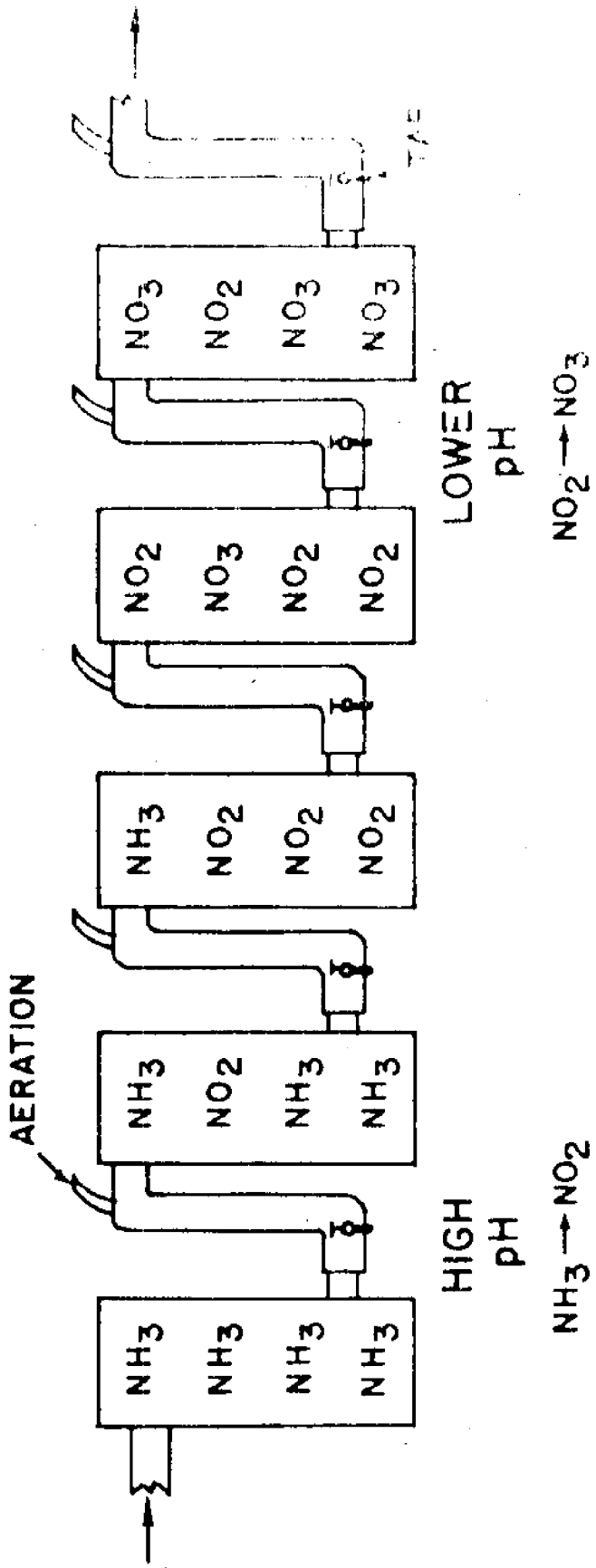
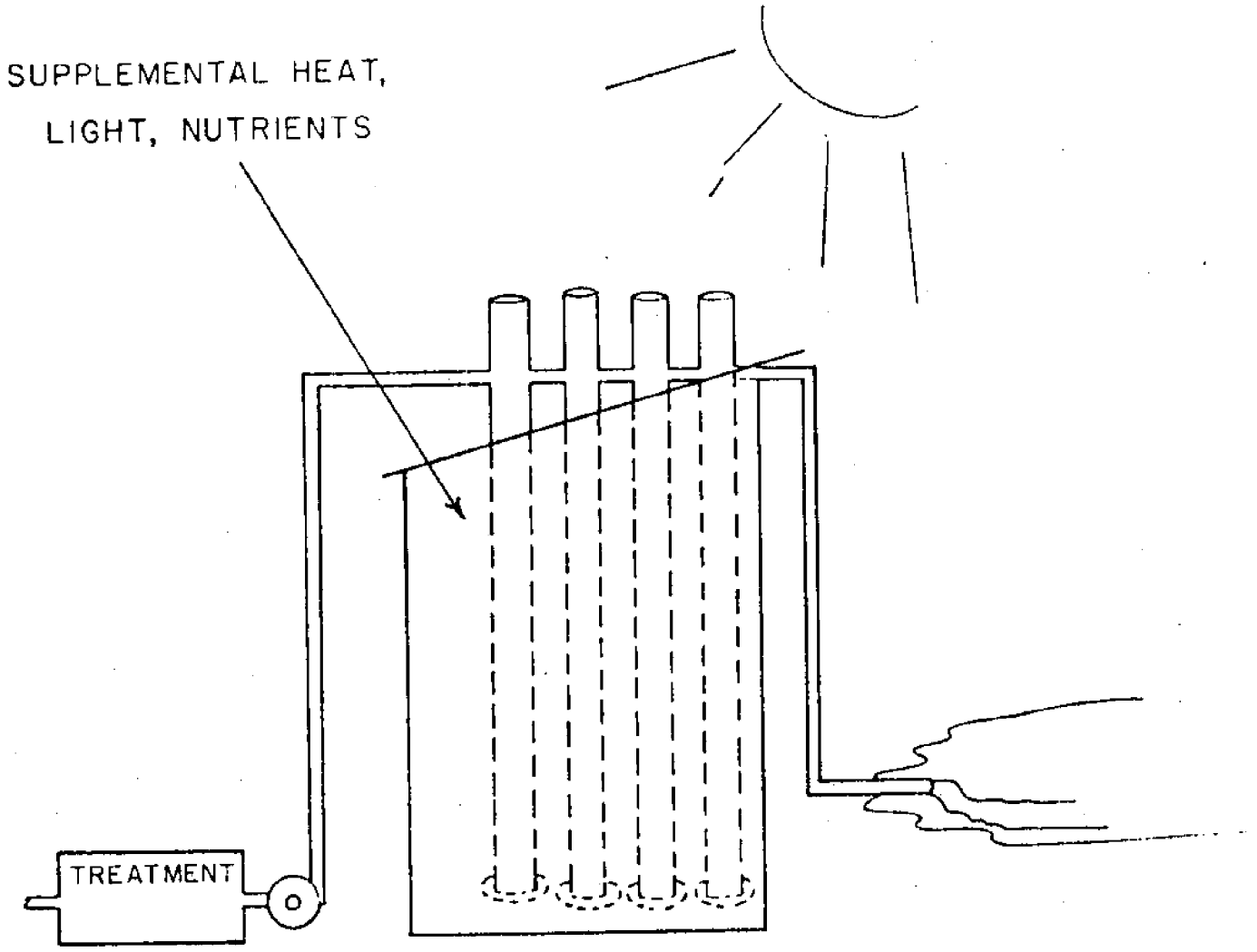



Figure 15. Consecutive modular nitrification filter

Figure 16. Schematic of algal seed-growing facility



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