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Ecological Research Series

DEVELOPMENT OF A SELECTIVE ALGAECIDE TO CONTROL NUISANCE ALGAL GROWTH

Office of Research and Development
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DEVELOPMENT OF A SELECTIVE ALGAECIDE TO CONTROL NUISANCE ALGAL GROWTH

By

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ABSTRACT

The objective of this project was to develop a compound which would effectively and economically control the growth of nuisance species of blue-green algae with a minimum impact on desirable forms of life in the aquatic environment.

A computerized structure search of more than 100,000 compounds was made to select the analogs of the following four Phase I prime candidates: 2,5-Dichloro-3,4-dinitrothiophene; [5-Chloro-2-(p-nitrophenoxy)phenyl]phenyliodoniumchloride; 4-Amino-2,5-dibromophenylthiocyanate; and 1,1-Dimethyltetradecylamine, hydrochloride. This endeavor resulted in the selection of 1309 analogs, which were each subjected to rapid agar-plate screening tests. Forty-one compounds emerged from these tests as candidates for final laboratory screening. At the conclusion of Phase II, compounds No. 23 (2,5-Dichloro-3,4-dinitrothiophene) and No. 73 [(p-Chlorophenyl)-2-thienyliodoniumchloride] were selected as final candidates, based on superior algaecidal activity, environmental acceptability, economic feasibility and freedom from human health and handling hazards.

A non-pigmented flagellate, *Ochromonas ovalis*, which exhibited phagocytic activity against the blue-green alga, *Microcystis aeruginosa*, was discovered during Phase I.

Three additional species of *Ochromonas* were discovered during Phase II which also exhibited phagocytic activity against *Microcystis aeruginosa*. The original species discovered during Phase I, exhibited the greatest activity and showed some improvement in the presence of certain test compounds.

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SECTION I

CONCLUSIONS

A computerized structure search of over 100,000 compounds revealed 1309 analogs of the four Phase I candidate compounds. As a result of rapid agar-plate screening tests, 41 of these compounds were selected for more detailed testing in the laboratory.

Although six of these compounds showed high levels of activity against the target blue-green algal species, *Anabaena flos-aquae*, four of them proved unacceptable because of poor economic feasibility, environmentally unacceptable properties or difficulty or danger in compound production.

The following conclusions are reached:

1. Compound No. 23 (2,5-Dichloro-3,4-dinitrothiophene) is 100 percent active against *Microcystis aeruginosa* at concentrations to 0.2 ppm and 100 percent active against *Anabaena flos-aquae* at concentrations as low as 0.8 ppm. This compound is safe to applicators, if ordinary safe handling procedures are observed, and exhibits no known environmentally hazardous properties. The estimated cost, for the control of *Microcystis aeruginosa*, is only 0.51 that of copper sulfate.

2. Compound No. 73 (p-Chlorophenyl-2-thienyliodoniumchloride) is 94 percent active against *Microcystis* at 0.2 ppm and 100 percent against *Anabaena* at 0.8 ppm with low environmental and human handling hazards.

3. *Ochromonas ovalis* was the most voracious, in its attack on *Microcystis aeruginosa*, of the several species of *Ochromonas* which were determined to have phagocytic properties.

4. Compound No. 6 (1,1-Dimethyl-2-(α,α,α-trifluror-p-tolyl)-urea) produced about 27 percent increase in *Ochromonal* phagocytic activity.

SECTION II

RECOMMENDATIONS

Since the research efforts in Phases I and II have indicated that the two selected candidate compounds are relatively specific, technically effective and economically competitive, it is recommended that investigations of their algaecidal activity under natural field conditions and determination of of persistance, degradability and toxicity, be undertaken.

Phase III of this effort should include:

1. Re-synthesis of multi-pound lots of the two candidate compounds.

2. Testing of the compounds under natural field conditions in various sectors of the country. These tests should take place in small lakes or ponds having naturally occuring blooms of nuisance algae.

3. Toxicity studies on fish and mammals, as well as gross pathology, acute oral toxicity and human primary skin irritation tests.

4. Development of analytical test procedures, in order that persistence, adsorption and degradability rates can be followed during and after treatment to determine the environmental stability of the compounds.

5. Further development of biological-chemical control systems in the laboratory, and testing for efficacy in naturally occuring field situations.

SECTION III

INTRODUCTION

The control of algae and aquatic weeds has become of major concern to the Environmental Protection Agency in a national effort to improve "the quality of life". Due to the great diversity of species and the ubiquitous nature of many species, there is hardly a body of water or moist spot on the face of the earth which is devoid of algae (Prescott, 1970).

Algae are common and normal inhabitants of all surface waters and are encountered in virtually all waters which are exposed to sunlight. To date more than 18,000 species have been identified (Palmer, 1962). Unlike the other groups of small or microscopic organisms, all species of algae possess chlorophyll and are responsible for an estimated 90 percent of all photosynthetic activity on the earth (Meyer, 1971). One pound of algae growth produces about 15 pounds of oxygen (Mackenthun and Ingram, 1964).

The need for control of certain nuisance algal species has arisen in relatively recent times as a result of excessive nutrification of surface waters due to an increasing human population and to indiscriminate use of available supplies of water.

Eutrophication of inland waters is caused by concentrations of human population in urban areas and by inadequate treatment of the resulting sewage runoff from urban areas. Additional eutrophication occurs as a result of runoff from fertilized agricultural lands from the natural processes of precipitation from the atmosphere, and interchange

of bottom sediments which occurs periodically in lakes and reservoirs (Mackenthun and Ingram, 1964).

Although there is no universal agreement among scientists and environmentalists as to the particular elements which are responsible for excessive algal populations, most commonly suspected are phosphorus and nitrogen. Algal blooms are usually associated with waters which receive sewage effluents which are rich in phosphorus and nitrogen. For example, Mackenthun and McNab (1961) made studies of Wisconsin waste stabilization ponds and concluded that the annual per capita contributions were 4.1 pounds of inorganic nitrogen and 1.1 pound of soluble phosphorus.

Increasing urbanization and changes in ground cover and surface soil due to such processes as fires, overgrazing, deforestation and agriculture have increased surface runoff and reduced soil seepage to the extent that, as estimated by some authorities, the water table in the Eastern half of this country has been lowered about 60 feet in the last 50 years (Palmer, 1962). This, in addition to a large increase in the use of ground waters, has created severe water shortages in many areas. Thus, as population and industrial demands increase, attention has, of necessity, turned more and more to lakes, streams and reservoirs to meet these needs.

In view of the increasingly heavy demands on our existing water supplies, it is of vital importance that the quality and availability of these waters be preserved. Waters containing dissolved and suspended materials not only support the growth of algae, but many other kinds of aquatic life, the numbers and abundance of which are governed by the

amounts and kinds of nutrients available. Since the utility of natural waters is often determined by the abundance and type of biota, adequate control of noxious species becomes a major concern.

When the nutrients, temperature and light condition becomes optimal for the growth of a particular species of algae, proliferation may take place in such abundance as to produce a visible agregation of algal masses. To produce such "algal blooms" the water and environmental conditions usually become optimal for only one species at a time. Thus, a bloom usually contains a heavily predominant species of algae. Many algae, particularly blue-greens, may impart obnoxious tastes and odors to the waters, clog intake screens and rapid-sand filters of water treatment plants. In addition, they may produce unsightly floating masses or collections of debris on shores making the waters unsuitable for many purposes.

Such nuisance algae may shorten filter runs in water treatment plants, or otherwise hamper industrial and municipal water treatment processes, impair areas of picturesque beauty, lower waterfront property values, and, in some cases, be toxic to certain warm-blooded animals which ingest the water (Mackenthun and Ingram, 1964). Certain species of Cyanophyta (blue-green algae) such as *Microcystis*, *Aphanizomenon*, and *Anabaena* are known to have caused animal deaths by the production of toxic substances, particularly in areas where the wind has concentrated the algae in lee-shore areas (Mackenthun and Ingram, 1964).

Fish kills have resulted from the depletion of dissolved oxygen in waters where the algal population has been very

dense. Even though algae do impart oxygen to the water through photosynthesis during daylight hours, the metabolic and catabolic processes within plant cells utilize oxygen continuously, day and night. If a heavy cloud cover should reduce oxygen-producing photosynthetic activity to a low level over a several-day period, the amount of dissolved oxygen during subsequent nights may fall below the critical level for many species of fish and fish kills may result. The pH of the water will also tend to increase as the algae increase their requirements for carbon dioxide during daylight hours. This process of continuous removal of carbon dioxide from the water results in an alteration in the relative amounts of soluble carbonic acid, intermediately soluble bicarbonates and nearly insoluble monocarbonates, often causing some of the latter to precipitate. The corrosive activity of the water is also often increased as a result of algal growth, causing water storage and transport problems due to the depolarizing action of the oxygen which is produced (Palmer, 1962).

Many inland waters have been seriously impaired in recent years as a result of algal growths. For example, Lake Washington in 1959 contained a maximum of 1.5×10^6 μ^3/ml of total phytoplankton volume, of which only 15 percent was blue-green algae. By 1963 the phytoplankton population had shown a ten-fold increase, of which 95 percent consisted of various species of blue-green algae (Bartsch, 1967).

The blue-green algae are of particular concern for a number of reasons. In contrast to most species of non-flagellated algae which settle to the bottoms of lakes in calm weather, many planktonic blue-greens exhibit an "upside-down" characteristic and accumulate as dense scums on the surface which

can then be blown into thick windrows and piled upon the shore. Decomposed materials often produce an offensive "pig-pen" odor (Mackenthun and Ingram, 1964). These products as well as the substances produced by several living species may impart tastes to the water and be toxic to animals and humans alike (Bartsch, 1967).

Several approaches to the problem of nuisance algal control are being investigated under the auspices of the National Eutrophication Program of the Environmental Protection Agency. Basically the approaches fall into four broad categories: (1) mechanical, (2) biological, (3) ecological and (4) chemical. Mechanical approaches involve the design and engineering of machines for underwater mowing, raking and harvesting of aquatic weeds and algae. Biological control mechanisms being investigated include viruses, insects, algae-eating fish and phagocytic organisms. Ecological approaches include diversion of nutrient-rich waters, flushing lakes and ponds with nutrient-poor water, removal of nutrients by sewage treatment processes and nutrient removal from bodies of water by aquatic plant crops or other means.

Chemical approaches to the control of nuisance algae have not been investigated thoroughly, even though much effort has been expended in other aspects of pest control. The problem is complicated by difficulties encountered in developing an algaecide which will selectively kill or stop reproduction of the target species without adversely affecting the desirable forms of aquatic life.

Copper sulfate has, for a number of years, been the chemical of choice for the control of algae. Despite its extensive usage, such shortcomings as its toxicity at higher concen-

trations to desirable aquatic life, non-biodegradability, accumulation as copper salts in bottom muds and its corrosive properties to paint and equipment (Bartsch, 1954), leave room for the development of a more ideal algaecide. Hasler (1947) and Kunkel (1969) point out the possible deleterious effects of lake bottom accumulations of copper sulfate on lake ecology, and investigations in Wisconsin and Minnesota conducted by Moyle (1949) indicate that certain algae, particularly the blue-green algae *Aphanizomenon*, seem to have acquired an increased tolerance to copper sulfate as a result of many years of treatment; two to five times as much copper sulfate was required for control as was required 20 years earlier.

There is, at present, no federally registered chemical available which is effective against nuisance species of blue-green algae in surface waters from which drinking water may be prepared. In recent years a considerable amount of effort has been expended in search of a selective compound, to replace copper sulfate, which would be safe to non-target organisms, non-cumulative and economically competitive (Mackenthun, et al, 1964).

Although it is likely that no single approach will be found which will serve in all cases, it is possible that a chemical approach to the control of nuisance algal growths will be suitable for many situations, particularly where algal "blooms" are evident and an ecological balance needs to be restored.

It should be emphasized that the development of a chemical for widespread use in the environment, particularly where recreational and culinary waters are concerned, is not a short-term project. After a compound has been selected for long-range development, as a result of proven activity,

economics and initial environmental safety projections, larger-scale field tests and complete toxicology studies, together with environmental, persistence and handling hazards tests, must be conducted. To avoid misleading generalizations, experiments must be designed to give specific answers related to the compound in question. The chances for success for a candidate compound must be very good at this point to justify the time and expense necessary to meet federal clearance requirements.

Phase I of this contractual effort to "Develop a Selective Algaecide to Control Nuisance Algal Growth" was initiated April 5, 1969. The primary objective was to develop a compound which would control the growth of various species of blue-green algae effectively, safely and economically, while exhibiting a minimum impact on other forms of life in the aquatic environment.

The approach to the problem involved initially making a computer search of some 80,000 compounds in Dow's computer listing of compounds in order to select those having the highest probability of meeting the established criteria for an ideal algaecide. Most of these compounds had already been screened against at least one species of algae and also for activity against higher aquatic plants, fish and some terrestrial plants and animals. A final hand selection was then made from the computer printout, eliminating those compounds which contained heavy metals and those which where likely to be costly, or which possessed inherent or demonstrated undesirable properties, such as high toxicities to fish, terrestrial plants or mammals. The candidate compounds, thus selected, were then divided into several priority groups according to the established selection criteria.

Within the 12-month contractual period, thirty-three selected compounds were screened using the two target species of blue-green algae suggested by EPA's Federal Water Qaulity Office, Corvallis, Oregon. Each compound was tested against cultures of *Microcystis aeroginosa* and *Anabaena flos-aquae* at a concentration of 2.0 ppm in constant-temperature water bath shakers (24°C) at 80 oscillations per minute under a cool-white fluorescent light intensity of 100 foot-candles. Compound activity was expressed as percent control, as compared to control cultures which were run simultaneously under identical conditions. Cell growth was monitored by visual inspections, cell counts and relative intensity readings using a fluoro-microphotometer. Those compounds which passed the first screening test with at least 80 percent control at 2.0 ppm against both test species were selected for more detailed testing at 1.0, 0.5 and 0.1 ppm. Of the 33 compounds tested, Compound Nos. 23 (2,5-Dichloro-3,4-dinitrothiophene), 15 (5-Chloro-2-(p-nitrophenoxy)phenyl)phenyl iodonium chloride), 8 (4-Amino-2,5-dibromophenylthiocyanate) and 24 (1,1-Dimethyltetradecyamine, hydrochloride) were effective at these concentrations and were selected as the prime candidate compounds for further research through Phase II of the long-range algaecidal development program.

A phagocytic organism, identified as *Ochromonas ovalis*, was discovered which showed promise as a means of controling *Microcystis*. In addition, it was found that the growth and activity of *Ochromonas* was enhanced by low concentrations of some of the test compounds being studied.

In the work statement, as included in the contract agreement, efforts were to be directed toward the long-range

goal of developing an algaecidal compound which would: (1) be safe to non-target organisms, (2) exhibit a high degree of specificity for the target algae, (3) be economical, (4) be safe to applicators and (5) be non-persistent in aquatic systems.

Included in the Phase II effort was to be the screening of analogs of compounds that had exhibited good algaecidal activity in the initial screening. This was to be done because of the possibility that analogs of these compounds might possess potential algaecidal activities equal to or greater than the initial compounds. Also, the physical and chemical properties of the selected compounds were to be analyzed more completely in an effort to develop the optimum formulations in terms of use in the aquatic environment.

The research was to continue in the identification of other biological chemical systems and to continue the effort toward optimizing the *Ochromonas* system discovered as specified under Contract No. 14-12-814. Phagocytic algae, nemotodes and protozoa were to be obtained and tested against *Microcystis* and *Anabaena*. Those showing promising algaecidal activity were to be tested with algaecidal chemicals in an effort to develop a feasible integrated control technique. The three compounds found to be algaecidal in the initial screening were to be tested on a pilot-scale, using plastic wading pools. Analytical procedures for suitable and rapid monitoring of the test compounds were to be developed. Preliminary mammalian and fish toxicities were also to be conducted on the selected test compounds.

SECTION IV

EXPERIMENTAL PROCEDURES

The primary objective of the presently reported effort was to include all of the available analogs of the four candidate compounds chosen through the initial screening program and then to make a final selection of the prime candidates for large-scale field testing. Specific efforts were to be directed toward further defining an effective chemical or biological-chemical system which would control the growth of blue-green algae safely and economically, and yet would be non-persistant in the environment and exhibit a high degree of specificity for the target algae.

Analog Search

A computerized search was conducted to select from Dow's library of over 100,000 compounds those which were analogs of the four candidate compounds and were selected as a result of the Phase I effort. It was felt that there would be a good probability of finding other active algaecides among those compounds with structures closely related to the Phase I prime candidates.

The analog structure search was undertaken by personnel at Dow's Computation Research Laboratory in Midland, Michigan. The computer search revealed 1309 analogs which were screened in a rapid test for algaecidal activity at Dow's Western Division Agricultural Research Center, Walnut Creek, California.

Laboratory Tests

Rapid Screening

Stock cultures of *Anabaena flos-aquae* were also sent to the Western Division for use in an agar plate screening test which was designed for rapid screening of the selected analogs. Gorham's "1x" nutrient medium was prepared with agar, poured into culture plates, and then spotted with test compounds at 1.0 and 0.2 ppm. Those which proved positive within seven days at these concentrations were tested at lower concentrations to the "break point" where the compounds were only 50 percent active. It was not necessary to test any compound at concentrations lower than 0.04 ppm. With the facilities and space available, about 200 compounds were tested per week until screening data had been completed on all of the compound analogs.

Fine Screening

Those compounds which exhibited complete activity at 1.0 ppm or less were tested according to the previously established screening test procedures, using Gorham's medium.

Early in the inception of Phase II the decision was made, with the approval of the Project Officer, Dr. T. E. Maloney, to use only *Anabaena* as the primary algal test species. The decision was based on several factors, but principally upon the extreme difficulty encountered in trying to grow cultures of *Microcystis* in the open atmosphere at the selected field test site, without contamination by the phagocytic organism *Ochromonas ovalis*, which was found to be omnipresent in the area. Previous experience had consistently revealed *Anabaena* to be the most resistant species to algaecidal action, and any compounds which exhibited

acceptable toxic properties against *Anabaena* proved to be even more toxic to *Microcystis*. Cultures of *Microcystis* were maintained, however, for research on biological-chemical control systems and also for use in compound persistance studies where greater sensitivity at lower chemical concentrations was desirable.

Gorham's medium was used for culturing all algae utilized in the laboratory screening tests. The composition of this medium (in milligrams per liter) was:

$NaNO_3$	496
$MgSO_4 \cdot 7\ H_2O$	49
$CaCl_2 \cdot H_2O$	36
Na_2Co_3	20
$NaSiO_3$	58
ferric citrate	6
EDTA	1

(Hughes et al, 1958) The pH was adjusted to 7.8-8.2 (Kuentzel, 1969).

Anabaena stock cultures showed a better growth pattern under 200 foot-candles of cool-white fluorescent light than at lower intensities. *Microcystis* cultures were cultured at about 100 foot-candles.

The laboratory screening tests were conducted in much the same fashion as those in Phase I (Dow, 1971) utilizing 30 ml algal culture in 125 ml test flasks. These were continuously agitated at 80 excursions per minute in 24°C water bath shakers under a constant cool-white fluorescent illumination of 100 foot-candles. The light was furnished by

four 48-inch, 25-watt cool-white fluorescent bulbs, diffused and attenuated by layers of cheese cloth interposed between the bulbs and the shaker assembly.

Culture growths were monitored by cell counts and by relative intensity readings taken with an AMINCO fluoromicrophotometer equipped with a blue mercury-vapor fluorescent lamp. Light from the lamp was filtered through a 5230 band-pass filter before entering the sample. Due to the extreme sensitivity of this instrument to fluorescing chlorophyll molecules it was possible to take these readings without going through chlorophyll extraction procedures.

Six new compounds were tested for algaecidal activity using the normal three-day laboratory screening procedures. These compounds were selected separately from the analog structure search on the basis of their compositions and structures, as compared to other known algaecides.

Accelerated Screening

After the initial rapid screening tests were completed at Dow's Western Division and the compounds to be put through the secondary screening tests were selected, a stepped-up testing schedule was adopted. In the new schedule the remaining test compounds were formulated by attempting a water solution at first and then trying successively, acetone, alcohol, toluene, xylene and finally methylene dichloride. In most cases where water or acetone proved to be inadequate, xylene was used and balanced to a specific gravity of 1.00 with the addition of about 19 percent perchloroethylene. The test compounds were then laboratory tested at 0.5 ppm for three days against both target algal species, *Microcystis aeruginosa* and *Anabaena flos-aquae*.

All other testing conditions were the same as for the fine screening tests. Cell growth was monitored daily by taking relative intensity readings with the fluoro-microphotometer. Initial and final cell counts were taken also, in the case of *Microcystis*.

The tests were terminated if less than 70 percent or greater than 95 percent activity was achieved against *Anabaena*. If an activity between 70 and 95 percent was obtained, the test was continued for another three days.

Culture Clean-up Procedures

It was discovered that field cultures of *Anabaena* which had become contaminated with *Ochromonas* could be freed from these organisms by a quick-freezing process. Due to the more fragile nature of *Ochromonas* it was found that by subjecting the mixed culture in a sealed container to a dry ice-acetone bath for five minutes and then allowing to warm to room temperature, the *Ochromonas* cells were ruptured by the freezing process whereas most of the *Anabaena* cells survived. After centrifuging and washing the "cleaned" cultures several times a healthy culture of *Anabaena*, free of *Ochromonal* contaminants, could be obtained.

Synergism

Several compounds were tested in combination to determine whether the combined algaecidal activity would be greater than either one alone. Equal quantities of each compound were formulated and added to established culture of *Microcystis* and *Anabaena* for total test chemical concentrations ranging from 0.8 to 0.05 ppm. After seven days the tests were terminated and cell growth monitored by cell counts and relative intensity readings.

Compound Persistence

A test series was planned to determine the toxicity persistence of the prime candidate compounds as functions of time and environmental conditions, using bioassay tests to determine residual toxicities. The experimental design included using "World Health Organization" (WHO) standard hard water, well water with PW-4 medium and deionized water with Gorham's medium. One ppm of the test compound was added to each type of water in 4-liter flotation bags in the field and similarly in 500 ml flasks in the laboratory to serve as controls. Monitoring of the compound degradation was accomplished by bioassays utlizing *Anabaena flos-aquae* cultured in the various waters initially, and subsequently at three-day progressive intervals. After a three-day growth period for each progressive monitoring test, final readings were taken on each culture and the results were compared to controls which were grown under the same conditions with no test compound added.

Formulation

During Phase I an acetone emulsifier, containing two surfactants, had been used successfully in formulating nearly all of the compounds tested. Although this system and the formulation procedure used had proven fairly successful, it was deemed important that the best formulation possible be developed in order that the maximum algaecidal activity efficiency could be achieved.

Standard formulation optimization procedures were utilized in determining whether a non-ionic, anionic emulsifier should be used in establishing the hydrophilic-lipophilic balance (HLB). Generally speaking, the better "HLB" which can be attained, the smaller the emulsified

particles, thus causing more surface contact area and a higher rate of algaecidal activity

An 18-hole formulation board was made and the necessary graduated centrifuge tubes and automatic syringes obtained for a full scale formulation optimization study using the three most probable solvents: toluene, xylene and perchloroethylene, together with matched anionic and nonionic pairs of Atlox, Tumulz and Sponto emulsifiers.

The Atlox emulsifier pair was tested with a 9-hole, 100:100 expansion (Figure 1) in which all of the emulsifier in the solvent contained only anionic emulsifier on one end of the board and only nonionic emulsifier on the other end. By progressive blending of equal portions, seven other emulsifier ratio combinations were obtained. When 1.0 ml of each of these was added to 100 ml water in graduated centrifuge tubes, shaken well and left standing for three days, the most stable emulsion could be easily detected by noting the tube with the least amount of settling. For an even more precise detection another series was run using a 50:100 expansion starting with a 1:1 emulsifier ratio on the left end of the board and all nonionic emulsifier in the solvent on the right end. The solvent blend was 81 percent xylene and 19 percent perchloroethylene to give a specific density as near that of water as possible. For the Atlox pair tested, the optimum blend was determined to be 10 percent emulsifier at the ratio of 18.75 anionic to 81.25 nonionic in the primary concentrate. In practice this ratio was often changed when the test chemical was dissolved in the primary solvent. In those cases additional formulation tests were necessary to determine the optimum "HLB" under various test compound loadings.

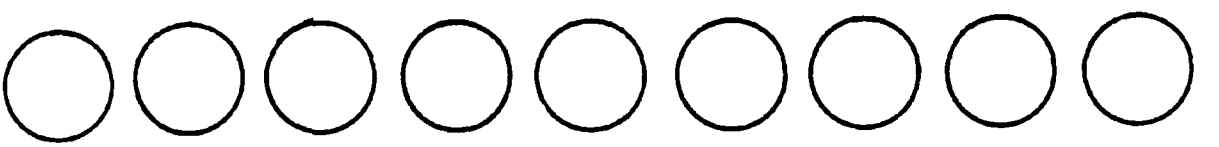
Figure 1

FORMULATION EFFICACY TESTS

Date 9-22-71
Solvent System 81% Xylene; 19% Perchloroethylene
Emulsifiers: Left Atlox 3404F Right Atlox 3425F
Test Compound None

100:100 Expansion

Use Concentrates



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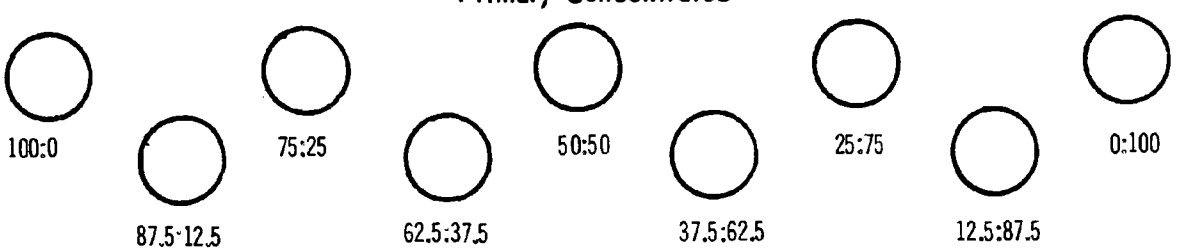
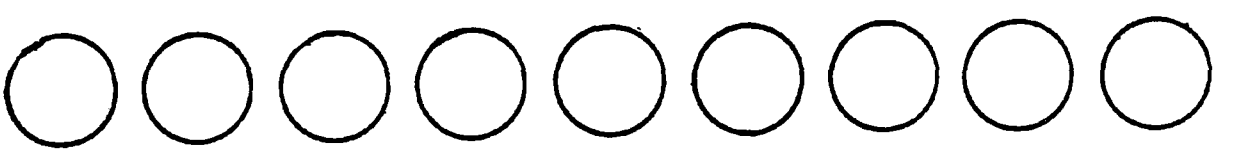


50:100 Expansion

Use Concentrates



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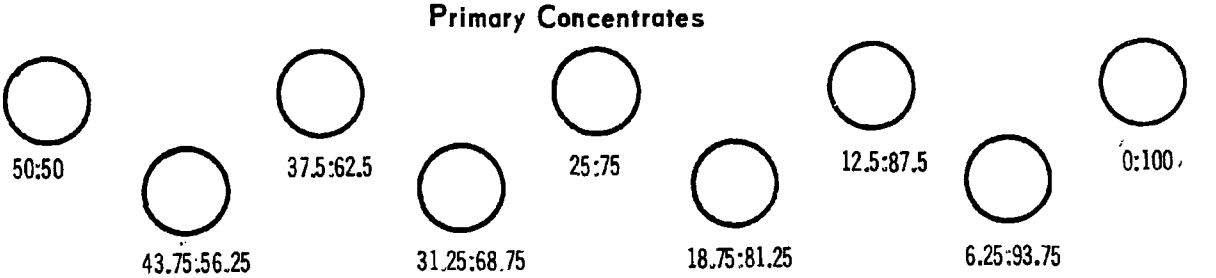


Once a theoretical optimum had been determined for a particular compound, it was then necessary to verify its algaecidal activity when reduced to actual practice. This was done by bench-scale testing in the laboratory following a procedure similar to the secondary screening test. This task had to be completed for each compound recommended for open field testing before actual testing of the compound could begin.

Toxicology Tests

Sixty grams of Compound No. 23 in the crystalline form, 99 percent pure, was sent to Dow's Biomedical Research lab at Midland, Michigan, where the compound was tested for primary skin irritation on rabbits, acute oral lethality using rats and eye irritation on rabbits. A 10 percent w/v primary concentrate formulation of this same compound was also sent to Midland for similar toxicological evaluation. This formulation was made up with a solvent-emulsifier system composed of 81 percent xylene and 19 percent perchloroethylene, to which was added 10 percent Atlox emulsifier with an anionic:nonionic ratio of 25:75.

Because of the NO_2 in the molecular composition of Compound No. 23, the possibility of handling and storing hazards existed and the compound was thus submitted for independent drop weight and differential thermal analysis tests to determine whether any mechanical shock or ordinary reactive chemical handling hazards existed.

Field Tests

The following criteria were used in choosing a site where the small-scale open-environment tests were to be conducted.

It was felt that the site should be:

1. Remote from any chemical plants or areas where agricultural chemicals were to be used.

2. Free from cattle and other domestic animal disturbances.

3. Remote from open waters and trees.

4. Easily accessible by an all-weather road.

5. Located where 110-volt power and an acceptable supply of water could be made available at a reasonable cost.

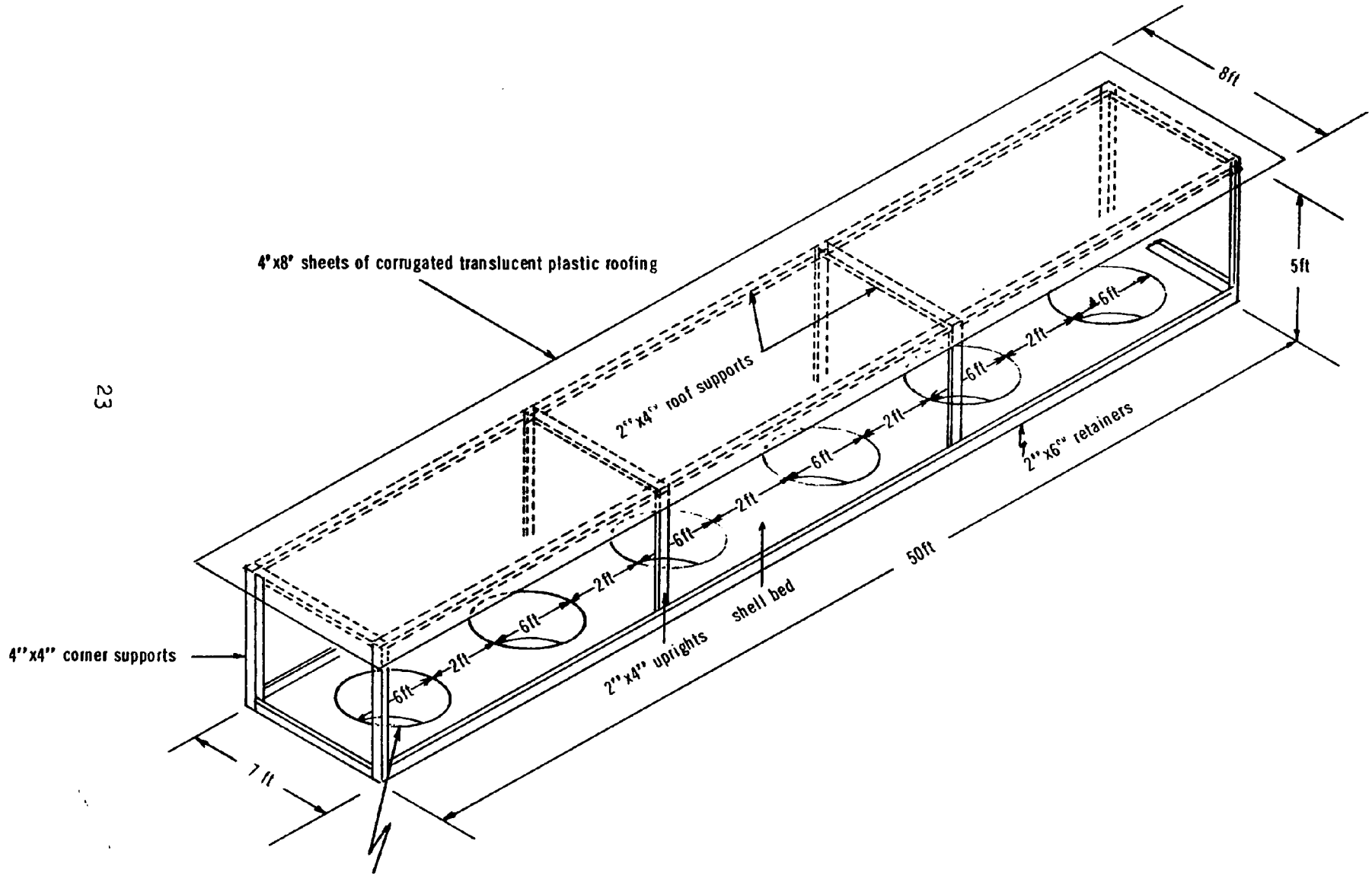
With the above criteria in mind a site was selected near Dow's Ag-Organic Department research facilities at Lake Jackson, Texas.

Test Equipment

A shading roof structure of 4' x 8' sheets of corrugated transluscent plastic was built (Figure 2). Six plastic wading pools, six feet in diameter and 14 inches deep, with enameled, corrugated metal sides were set up under the shading roof. They were then lined with 6 mil guage polyethylene film and filled with 200 gallons of well water piped 300 feet from well number 4 to the test site. A 110-volt supply of electricity was also made available at the test site.

Aside from the decision to use only *Anabaena* for the outdoor algal test species, a few other modifications in experimental design were necessary. The sides of the shading roof structure were covered with a layer of saran mesh screening to help attenuate the wind in an attempt to modify conditions

Figure 2
OPEN ENVIRONMENT TEST DESIGN
(Shading Roof and Support)



sufficiently to allow for continued operation during the winter months. Also, 300-watt heat lamps were suspended above the pools to keep the water temperature from dropping too low. None of these modifications, however, changed the ambient conditions sufficiently to yield significant results from the field tests conducted during cold weather.

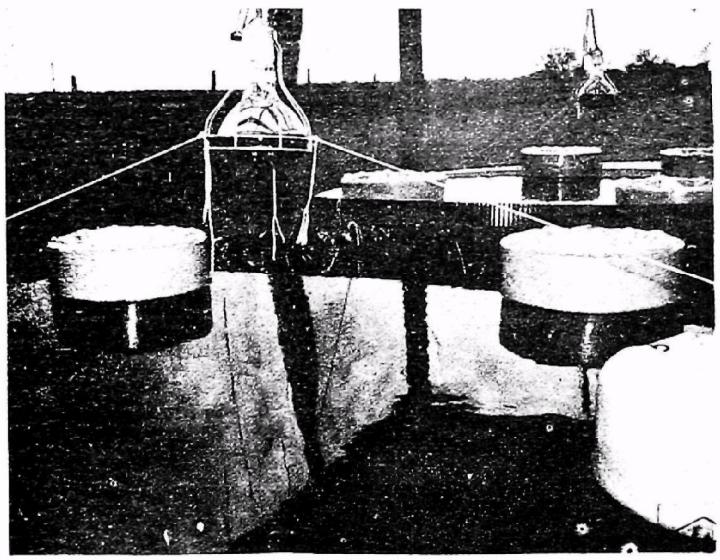
Test Procedures

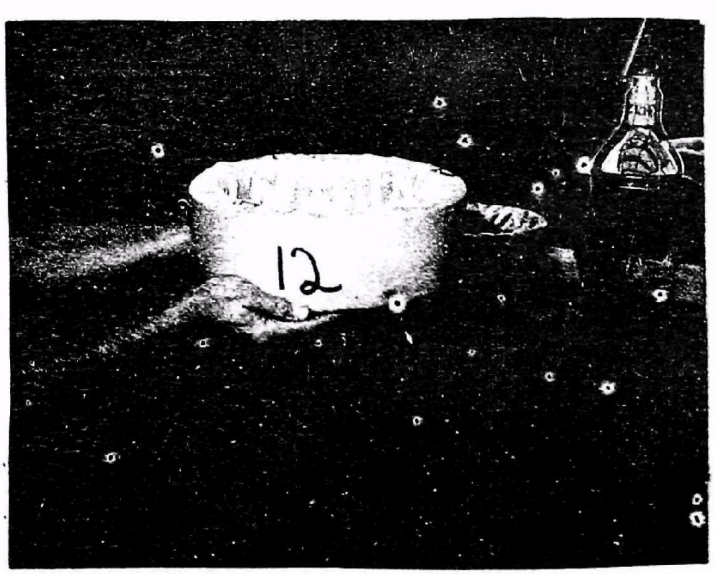
Sixteen-liter polyethylene bags, supported by polyurethane flotation collars were used as test chambers and control vessels in the earlier field tests (Figure 3). Later, field test data on the two final candidate compounds were obtained using 100 gallons of PW-4 media in each of three test pools. After *Anabaena* cultures had become acclimated in the pools and had grown to a concentration giving a relative intensity reading of 0.10, the pools were then inoculated with 1.0, 0.3 and 0.1 ppm of the test compound respectively. Controls were contained in floating 16-liter polyethylene bags in each large pool. The cell growth, or decline, was monitored daily by taking fluoro-microphotometer readings. It was found that more consistent and uniform readings were obtainable if a 200 ml sample, taken from a well-agitated pool, was blended in a Waring blender for 15 seconds and was then added immediately to the cuvette before settling could take place.

Originally, laboratory screening tests were conducted on both *Microcystis aeruginosa* and *Anabaena flos-aquae*. But, early attempts to culture *Microcystis* in the field always resulted in contamination by a phagocytic organism identified as *Ochromonas ovalis*. A series of comprehensive tests were conducted at the selected open-environment test site to determine the source and a possible means of controlling

Figure 3

Test Vessles with Polyurethane Flotation Collars





contamination by the organism. Extreme precautionary sanitation and handling procedures were used. Open-topped, sterile, 5-gallon polyethylene carboys were placed under the shading canopy at the test site and cultures of *Microcystis* were allowed to incubate under ambient conditions. The cultures were prepared with sterile deionized water and Gorham's medium. At five days elapsed time, the *Microcystis* cultures progressed to their log phase of growth, but *Ochramonas* infestation was observed and soon thereafter had taken a heavy toll.

Other *Microcystis* cultures were started in a similar manner, using PW-4 media and well water piped to the test site. All of these cultures, including outdoor and laboratory controls showed the presence of *Ochromonas* within two days. It was concluded, and later confirmed, that the well water itself contained *Ochromonas*. Culture medium which contained water pumped directly from the well, rather than from the storage tank was found to contain *Ochromonas* also, although it took four or five days to detect their presence, indicating many fewer organisms. Additional precautionary measures included disinfecting the shed, grounds and water line from the well to the test area, using 5.0 ppm Clorox solution. It was found that *Ochromonas* was highly susceptible to chlorine at this concentration. Subsequent tests included culturing *Microcystis* in sterilized well water with PW-4 medium and also with sterile Gorham's medium in sterilized wading pools and open battery jars at the test site, with capped controls incubated in the same vicinity as well as under laboratory conditions. Sterilized mosquito netting was placed over one of the wading pool tests to avoid the possibility of insect contact as being the vectors of *Ochromonas* contamination. In spite of all these precautions, all open vessels showed the presence of *Ochromonas* within

five days or less, whereas laboratory and outdoor capped controls remained uncontaminated.

From this study it was concluded that the phagocytic organism *Ochromonas ovalis*, was ubiquitous in this area, and due to its destructive action against *Microcystis*, it was not possible to use *Microcystis* as an algal test species for this portion of the study. Consequently, permission was sought, and granted, by the Federal Project Officer to use *Anabaena* as the sole test species. In view of the fact that past experience had proven *Anabaena* to be consistently the most resistant to previously tested compounds it was felt that no vital information would be lost.

New Culture Medium

With the relatively large volumes of water required in the field tests it was impractical to continue using deionized water and Gorham's medium, as in the laboratory screening tests. An analysis of water from well number 4 at the test site was obtained (Table 1) and a new medium entitled "PW-4 medium" was formulated (Table 2). The new medium was tested for efficacy with the well water, and it was found that lowering the pH of the "10x" concentrate to about 2.8 and then readjusting with sodium hydroxide to a pH of 7.8-8.5, proved to be an acceptable procedure for facilitating the solution of the various nutrient medium additives.

Large Volume Algal Inoculum Cultures

In order to facilitate outdoor testing, test chambers consisting of floating polyethylene bags were used. This resulted in each test being scaled up by a factor in excess of 500. For the final testing of the prime candidate compounds the wading pools contained 15,000 times as much water

TABLE 1

ANALYSIS OF WATER FROM WELL NO. 4

Component	Parts Per Million
HCO_3	430
CO_3	0
OH	0
Total Cl	580
Ca	29
Mg	16
Na	440
Fe	<0.1
SiO_2	16.7
SO_4	5
TDS	1,545
TSS	7
Turbidity	14
Total nitrogen	0.03

pH = 8.4

TABLE 2

PW-4 CULTURE MEDIUM
("1X"-Solution)

Component	Mg/l (Well Water)
$NaNO_3$	240
K_2HPO_4	40
$MgSO_4$	32
$CaCO_3$	15
Ferric Citrate	6
Citric Acid	6
EDTA	1

as in the laboratory screening test flasks. It was apparent that a much larger quantity of algal innoculum would have to be grown to accomodate such tests. A new bank of cool-white fluorescent lights was prepared to give a light intensity of 200 ft-c. Large batches of *Anabaena* were grown in 4-liter flasks and in 5-gallon polyethylene carboys with the tops cut off and then covered with Saran Wrap to protect them from dust and foreign matter. The cultures were agi- by hand daily and their growth patterns monitored by fluorometric readings.

Resynthesis of Compound No. 23

Compound No. 23 (2,5-Dichloro-3,4-dinitrothiophene), selected as the prime candidate compound was resynthesized to obtain larger quantities than those available from the compound library. Of the 100 grams obtained, 60 grams were formulated and sent to Dow's toxicology lab at Midland, Michigan for Class I toxicology tests. The remainder was retained for further formulation studies and field confirmation tests.

Cell Growth Monitoring Procedures

It was found that even *Anabaena* cultures grown freely in the outdoors soon picked up *Ochromonas*, although the presence of the *Ochromonas* did not seem to affect the growth of the *Anabaena*. Since fluorometry had been the principal means of monitoring cell growth of this species, the presence of fluorescing *Ochromonas* cells in the test chambers introduced a considerable amount of error in the readings. Utilizing previously obtained knowledge of the fragile nature of *Ochromonas*, a reliable analytical method was developed. Six milliliters of algal culture were measured in a graduated centrifuge tube, capped tightly and then quick-frozen in a

dry ice-acetone bath. The freezing process ruptured *Ochromonas* cells but left *Anabaena* relatively unharmed. After washing, centrifuging and resuspending to the same volume, reliable relative intensity readings were then obtained.

Biological-Chemical Systems

Further investigations concerning the phagotrophy of the algal species, *Ochromonas ovalis*, and other phagocytic organisms, were conducted in an attempt to improve on a biological algal control system. In cooperation with Dr. M. J. Wynne, consultant from the University of Texas, cultures of several other species of *Ochromonas* were obtained and tested with various compound additives, under varying conditions, to determine optimum activity parameters. Tests were conducted both in the laboratory and in the open atmosphere.

Algal cultures, after becoming well established in the laboratory, were purposely infected with *Ochromonas* in various stages of growth and in combination with other chemicals and nutrient additives. Thirty milliliter quantities of *Microcystis* culture were started in 125 ml Erlenmeyer flasks in "10x" Gorham's medium. The initial cell count was about 1.0×10^6 cells/ml. Various concentrations of test compounds and other micronutrients were added to duplicate sets of test flasks, with one additional flask serving as a control. The flasks were agitated in water bath shakers at 80 excursions per minute under cool-white fluorescent lights of about 120 foot-candles intensity. When the cell counts had reached 3.0×10^6 cells/ml, the test cultures were infected with an *Ochromonas* inoculum of sufficient concentration to give an initial cell count of 1.0×10^3 *Ochromonas* cells per ml.

Cultures were monitored daily by microscopic examination. Those combinations which exhibited the greatest phagocytic activity enhancement were taken to the open environment and tested in a fresh water canal with both open and closure-capped flasks. The flasks were supported in a fresh water canal by a polyurethane flotation device.

The viability of cultures of *Ochromonas* was also studied as a function of time and storage conditions. This included investigations of various types of dispersing media, such as powdered polyurethane, or other types of imbibing materials, and their effects on longevity and viability.

Other types of phagocytic organisms were obtained from the University of Texas Botany Department collection and were cultured and tested in a similar manner. The modes of ingestion and the ultramicro-structure of *Ochromonas* and activity rate comparisons between species were studied by Dr. M. J. Wynne and Dr. Gary Cole of the University of Texas at Austin.

SECTION V

DISCUSSION

By means of a computer search of some 80,000 compounds during Phase I of this effort, thirty-three compounds were selected and tested for algaecidal activity by primary and secondary laboratory screening techniques. Four of these compounds were found to be sufficiently active against the two target blue-green algal species, *Anabaena flos-aquae* and *Microcystis aeruginosa* to be selected as candidates for Phase II testing.

The objective of Phase II was to obtain information on the algal control efficacy of the selected candidate compounds and their active structural analogs, and on combined biological-chemical systems, in a controlled open environment. Specific efforts were directed toward further defining an effective chemical or biological-chemical system which would control the growth of blue-green algae economically and yet be safe to applicators, be non-persistent in the environment and exhibit a high degree of specificity.

Structure Search

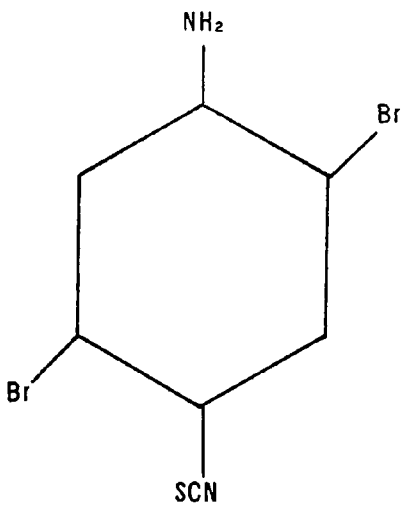
The structures of the four successful Phase I compounds are given in Figure 4 . Since closely related compounds often exhibit similar biochemical properties it was felt that the analogs of these compounds should be investigated to give the broadest possible coverage in a search for compounds having superior algaecidal properties.

A structure search of Dow's library of compounds was undertaken at Dow's computation Research Laboratory in Midland,

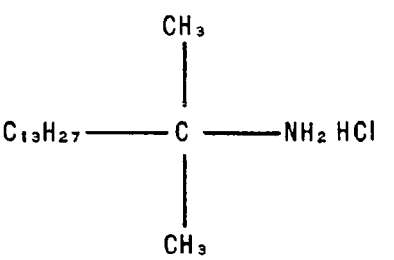
Figure 4

PHASE I - Prime Candidate Algaecides

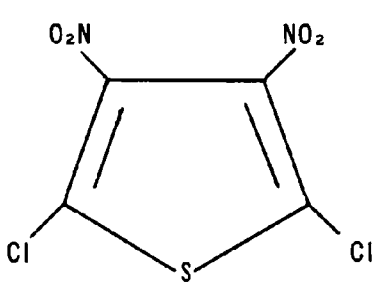
4-Amino-2,5-dibromophenyl
-thiocyanic acid ester
$C_7H_4Br_2N_2S$



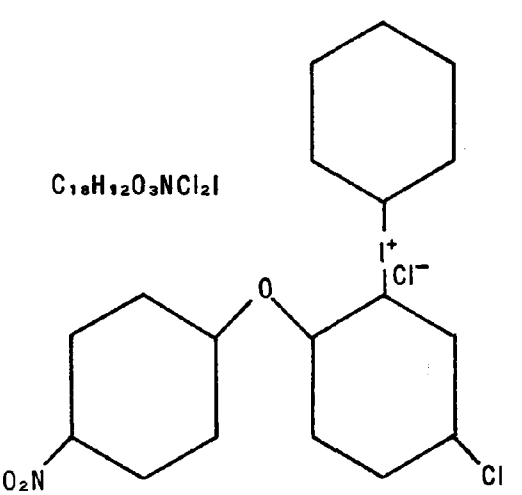
1,1-Dimethyltetradecylamine
-Hydrochloride
$C_{16}H_{35}N\ HCl$



2,5-Dichloro-3,4 dinitrothiophene
$C_4Cl_2N_2O_4S$



[5-Chloro-2-(p-nitrophenoxy)phenyl]
-phenyl Iodonium chloride
$C_{18}H_{12}O_3NCl_2I$



Michigan. From this effort 1309 analogs were identified. A breakdown of the number of analogs of each of the parent compounds is given in Table 3 . One hundred ten of the analogs were common to both compound No. 23 and No. 15.

Rapid Screening Tests

The printout containing the selected analogs was sent to Dow's Western Division Agricultural Research Center at Walnut Creek, California, to be put through a rapid coarse screening test at the rate of about 200 compounds per week. Initially the screening was at a concentration of 5.0 ppm of the test compound on nutrient agar plates. Because of the large number of compounds which were showing up as "positives" at the 5.0 ppm level the test concentration was reduced to 1.0 ppm, with active compounds being also tested at 0.20 ppm and 0.04 ppm. Run downs to the point where the compounds were less than 50 percent active were only undertaken on those compounds which proved to be 100 percent active at the 1.0 ppm level.

Of the two test algae, *Anabaena* consistently proved to be more resistant than *Microcystis* to the analogs of the Phase I compounds tested. The decision was therefore made to use only *Anabaena* in the rapid screening. This proved satisfactory for giving the necessary initial screening information on compound efficacy.

As a result of this testing program 41 compounds were chosen to be tested through the laboratory fine screening program used previously in Phase I.

The majority of the analogs were laboratory tested against both test species of algae at 0.5 ppm for 3 days. Some of

TABLE 3

RESULTS OF COMPUTERIZED ANALOG STRUCTURE SEARCH

Compound Serial Number	Structure Search Criteria	No. of Analogs
8	aromatic thiocyanates	434
15	iodoniums	332
23	thiophenes	497
24	alkylamines	46
	TOTAL	1309

the compounds, before incorporation of an accelerated test schedule, were tested at three levels (1.0 ppm, 0.3 ppm and 0.1 ppm) for seven-day periods. The modified three-day testing program proved to be suitable for identifying compounds worthy of further investigation.

Nearly all of the compounds tested proved to be more toxic to *Microcystis* than to *Anabaena*. Of the 74 compounds tested 15 were at least 90 percent active against *Microcystis* at 0.5 ppm (Table 4). Four of these compounds (No. 50, 54, 55 and 72) proved to have activities of 90 percent or greater against *Microcystis* at concentrations down to 0.1 ppm. Only one compound, No. 61, showed a comparable activity against *Anabaena* within the same test period (Table 4 and 5). This compound, however, was eliminated from the prime candidate list early in the program due to its heavy metal component.

Only five compounds (No. 23, 50, 70, 72 and 73) showed activites of 90 percent or better at 0.8 ppm against *Anabaena* (Table 5). Compounds No. 23 and 73 both exhibited 100 percent activity at 0.8 ppm but showed definate "break points" in the 0.8-0.4 ppm range.

A number of the more promising Phase I compounds were retested, using new formulation procedures, but no better performance was found.

In addition to the Phase I compounds which were retested and the 42 analogs selected for laboratory screening, six other compounds (Nos. 98-103) were tested because, on the basis of the structure and composition, good algaecidal properties could be expected. There were, however, no good algaecides among this group. A series of sixteen additional compounds were tested because of indicated algaecidal activi-

TABLE 4

LABORATORY SCREENING DATA

TEST COMPOUNDS VERSUS *Microcystis aeruginosa*

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm)							
		1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
1	3,3',4',5-Tetrachlorosalicylanilide				0				
2	4-Hydroxy-1-natphythiocyanic acid				0				
3	5,5'-Dichloro-2,2'-dihydroxybenzophenone				<10				
6	1,1-Dimethyl-2-(α,α,α-trifluoro-p-toly)urea				0				
7	2-Benzyl-5,6-dimethylbenzimedizole				78				
15	(5-Chloro-2-(p-nitrophenoxy)-phenyl)phenyliodoniumchloride				100				
21	2,2'-Thiobis(4,6-dichlorophenol)				44				
23	2,5-Dichloro-3,4-dinitrothiophene	100		100	100	100		100	85
24	1,1-Dimethyltetradecylamine, hydrochloride	100	100	100*	100	100*		100*	53*
25	1,1-Dimethyl-9-octadecenylamine, hydrochloride				86				
28	Thio-m-(trifluoromethyl)carbonilic acid, s-p-nitrophenylester				<10				

TABLE 4 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm) 1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
31	n-Dodecyl-n,n-dimethyl-1,3-propanediamine, hydrobromide				54				
33	Phenyltrichloromethylsulfide				74				
34	1,2-Di-o-tolyhydrazine				26				
50	Di-2-thienyliodoniumchloride		100*		99	100*		100*	98*
51	Phenyl-2-thienyliodonium-bromide		95*				95*		49*
52	Phenyl-2-thienyliodonium-chloride		99*				97*		71*
53	(p-Chlorophenyl)(2-thienyl)-iodoniumbromide		93*				75*		0*
54	(p-Chlorophenyl)(2-thienyl)-iodoniumtrifluoroacetate		100*		100		98*		92*
55	(p-Fluorophenyl)-2-thionyl-iodoniumtrifluoroacetate		100*				96*		97*
56	3-Pyridyl-2-thienyliodonium-chloride, hydrochloride		30*				23*		<10*
57	Diphenyliodoniumtrifluoro-acetate		100*				79*		46*
58	Di-2-thienyliodonium-fluoborate				64				

TABLE 4 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm) 1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
59	3-Pyridyl-2-thienyliodonium-trifluoroaceate				64				
60	Phenyl-2-thienyliodonium-trifluoroacetate				57				
61	Tributylisothiocyantotin				83				
62	3-Pyridyl-2-thienyliodonium-bromide				52				
70	2-Iodo-3,5-dinitrothiophene		100*	100*		100*	100*	100*	65*
71	2-Thienyl-m-tolyliodonium-chloride				86				
72	(m-Chlorophenyl)-2-thienyl-iodoniumchloride			100*	100*	100*		100*	93*
73	(p-Chlorophenyl)-2-thienyl-iodoniumchloride		100	100*	100	100*	60	94*	60*
74	(p-Bromophenyl)-2-thienyl-ioconiumchloride				100				
75	2-Thienyl-(α,α,α-trifluoro m-toly)icdoniumchloride		34				25		33
76	2-Thienyl-2,4-xylyliodonium-chloride				60				

TABLE 4 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm)							
		1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
77	2-Thienyl-(α,α,α-trifluoro-m-tolyl)iodoniumtetrafluoroborate				81				
78	2-Thienyl-p-tolyliodonium-chloride				54				
79	2-Biphenlyl-2-thienyl-iodoniumchloride				100				
80	Isothiocyanatotriphenyltin				76				
81	Diphenyliodoniumbromide				24				
82	(o-Chlorophenyl)-2-thienyl-iodoniumchloride			100*	100	100*		71*	58*
83	2-Iodo-5-nitrothiophene				75				
84	(p-Bromophenyl)-2-thienyltri-fluoroacetate				76				
85	(p-Fluorophenyl)-2-thienyl-iodoniumbromide				48				
86	Pyrazol-4yl-2-thienyliodonium-bromide				54				
87	(2-Thienyl)(p-tolyl)iodonium-nitrate				50				

TABLE 4 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm)							
		1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
88	(p-Chlorophenyl)-2-thienyl-p-toluenesulfonate				100				
89	2,4-Dinitrothiophene				87				
91	2-(Chloromethyl)-5-nitrothiophene				82				
92	n-Allyl-5-bromo-3,4-dinitro-2-thiophene				48				
93	2-Bromo-5-nitrothiophene				75				
94	3-thiophenalanine			97*	100	97*		92*	<20*
95	5-Nitro-2-thiophenecarbonitrile				76				
96	2,5-Dibromo-3,4-dinitrothiophene				88				
97	5-Nitro-2-thiophenecarboxamide				0				
98	Tetrachlorothiophene			0		0		0	
99	2,5-Dibromothiophene			0		0		0	
100	1-Chloro-3-iodobenzene			0		0		0	
101	1-Chloro-4-iodobenzene			0		0		0	

TABLE 4 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm) 1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
102	1-Chloro-4-iodosobenzene, di-acetate			0		0		0	
114	n,n-Diethyl-2-(2,4,5-trichloro-phenoxy)ethanamine			44*		50*		57*	24*
115	2-(4-Chlorophenoxy)-n-ethyl-ethanamine								
116	4-Butyl-2-nitrobenzeamine								
117	n-((4-6-Cyanao-2-pyridinyl)oxy) phenyl)-n,n-dimethylurea			56*		52*		36*	0*
118	n-(4-((6-Bromo-2-pyridinyl) oxy)phenyl)acetamide								
119	n'-(4-((6-Bromo-2-pyridinyl) oxy)phenol)-n,n-dimethylurea			82*		75*		64*	52*
$CuSO_4 \cdot 5H_2O$		100	100	91		66		0	
Cutrine		81	17		0				
xylene-perchlor-atlox system at a 43.75:56.25 anionic to nonionic ratio					0				

*7-day tests, all others are 3-day tests

TABLE 5

LABORATORY SCREENING DATA

TEST COMPOUNDS VERSUS Anabaena flos aquae

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm) 1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
1	3,3',4',5-Tetrachlorosalicyl-anilide				<10				
2	4-Hydroxyl-1-napthylthiocyanic acid				17				
3	5,5'-Dichloro-2,2'-dihydroxy-benzophenone				19				
6	1,1-Dimethyl-2-(α,α,α-trifluror-p-tolyl)urea				22				
11	3,4,4',5',6-Pentachloro-2,2'-methylenediphenol				61				
15	(5-Chloro-2-(p-nitrophenoxy) phenyl)phenyliodoniumchloride				0				
21	2,2',-Thiobis-(4,6-dichlorophenol)				0				
23	2,5-Dichloro-3,4-dinitrothio-phene	100		100	57	44		42	44
24	1,1-Dimethyltetradecylamine, hydrochloride			60*	81	<20*		<20*	<20*
25	1,1-Dimethyl-9-octadecenyl-amine, hydrochloride				50				

TABLE 5 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm) 1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
28	Thio-m-(Trifluoro-methyl)carbonic acid, s-p-nitrophenyl ester				0				
31	n-Dodecyl-n,n-diethyl-1,3-propanediamine, hydrobromide				18				
33	Phenyltrichloromethylsulfide				0				
34	1,2-Di-o-tolyhydrazine				0				
50	Di-2-thienyliodoniumchloride			90*		80*		45*	35*
51	Phenyl-2-thienyliodoniumbromide		50*		48		53*		61*
52	Phenyl-2-thienyliodoniumchloride		71*				64*		61*
53	(p-Chlorophenyl)(2-thienyl)iodoniumbromide		<20*				<20*		<20*
54	(p-chlorophenyl)(2-thienyl)iodoniumtrifluoroacetate		94*		33		79*		0*
55	(p-fluorophenyl)-2-thionyliodoniumtrifluoroacetate		74*				60*		0*
56	3-Pyridyl-2-thienyliodonium chloride, hydrochloride		0*				0*		0*
57	Diphenyliodoniumtrifluoroacetate		74*				40*		0*

TABLE 5 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm) 1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
58	Di-2-thienyliodoniumfluoborate				37				
59	3-Pyridyl-2-thienyliodonium-trifluoroacetate				0				
60	Phenyl-2-thienyliodonium-trifluoroacetate				40				
61	Tributylisothiocyantotin				90				
62	3-Pyridyl-2-thienyliodonium-bromide				37				
70	2-Iodo-3,5-dinitrothiophene		100*	96*		25*	0*	0*	0*
71	2-thienyl-m-tolyliodonium-chloride				37				
72	(m-Chlorophenyl)-2-thienyliodonium-chloride			98*	37	81*		40*	<10*
73	(p-Chlorophenyl)-2-thienyliodonium-chloride			100*	42	50*	<10	20*	<20*
74	(p-Bromopheynl)-2-thienyliodonium-chloride				57				
75	2-Thienyl-(α,α,α-trifluoro-m-tolyl)-iodoniumchloride		0				0		0

TABLE 5 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm) 1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
76	2-Thienyl-2,4-xylyliodonium-chloride				31				
77	2-Thienyl-(α,α,α-trifluoro-m-tolyl)-iodoniumtetrafluoroborate				20				
78	2-Thienyl-p-tolyliodonium-chloride				57				
79	2-Biphenylyl-2-thienyliodonium-chloride				40				
80	Isothiocyanatotriphenyltin				40				
81	Diphenyliodoniumbromide				0				
82	(o-Chlorophenyl)-2-thienyliodon-iumchloride			60*	62	22*		0*	0*
83	2-Iodo-5-nitrothiophene				25				
84	(p-Bromophenyl)-2-thienyltri-fluoroacetate				0				
85	(p-Fluorophenyl)-2-thienyliodon-iumbromide				37				
86	Pyrazol-4-yl-2-thienyliodonium-bromide				0				

TABLE 5 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm) 1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
87	(2-Thienyl)(p-tolyl)iodonium-nitrate				0				
88	(p-Chlorophenyl)-2-thienyl-p-toluenesulfonate				25				
89	2,4-Dinitrothiophene				0				
91	2-(Chloromethyl)-5-nitrothiophene				13				
92	n-Allyl-5-bromo-3,4-dinitro-2-thiophene				12				
93	2-Bromo-5-nitrothiophene			41*	56	<20*		<20*	<20*
94	3-Thiophenalanine				11				
95	5-Nitro-2-thiophenecarbonitrile			81	<10				
96	2,5,-Dibromo-3,4-dinitrothiophene			0		0		0	
97	5-Nitro-2-thiophenecarboxamide			0		0		0	
98	Tetrachlorothiophene			0		0		0	
99	2,5-Dibromothiophene			0		0		0	
100	1-Chloro-3-iodobenzene			0		0		0	
101	1-Chloro-4-iodobenzene			0		0		0	

TABLE 5 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm) 1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
102	1-Chloro-4-iodosobenzenediacetate			0		0		0	
104	3,4-Dichlorophenol			52		41		30	
105	3,5-Dichlorophenol			48		39		33	
106	3,5-Dichloro-4-hydroxybenzoic acid			36		33		27	
107	p,p'-Methylenediphenol			27		33		24	
108	Quinizarin			33		27		21	
109	4'-Fluoro-4-biphenylamine			60		55		44	
110	4-(2-Amino-2-oxoethyoxy)benzoic acid, methyl ester			42		42		27	
111	n,n-Dimethyl-n'-(3-methyl-4-nitrophenyl)urea			0		0		0	
112	n,n'-Dimethyl-6(methylthio)-1,3,5-triazine-2,4-diamine			18		0		0	
113	o-(1,2-Dimethylpropyl)-s-carbonodithoic acid, ethyl ester			21		12		21	
114	n,n-Diethyl-2-(2,4,5-trichlorophenoxy)ethanamine								

TABLE 5 continued

Serial Number	Name of Compound	Activity (%) at Various Conc. (ppm) 1.6	1.0	0.8	0.5	0.4	0.3	0.2	0.1
115	2-(4-Chlorophenoxy)-n-ethylethan-amine			<10*		<10*		<10*	
116	4-Butyl-2-nitrobenzeamine			<10*		<10*		<10*	
117	n-((4-6-Cyanao-2-pyridinyl)oxy) phenyl)-n,n-dimethylurea								
118	n-(4-((6-Bromo-2-pyridinyl)oxy) phenyl)acetamide			<10*		<10*		<10*	
119	n'-(4-((6-Bromo-2-pyridinyl)oxy) phenyl)-n,n-dimethylurea								
$CuSO_4 \cdot 5H_2O$			100	92		16		0	
Cutrine		70	0		0				
xylene-perchlor-atlox system at a 43.75:56.25 anionic to nonionic ratio					<10				

*7-day tests, all others are 3-day tests

ty in an unrelated proprietary screening program. None of those tested (Nos. 103-119) (Table 5) showed sufficient algaecidal activity to warrant further testing.

Formulation Study

Each of the test compounds screened through the normal testing procedure was initially prepared by first determining the compound's solubility in water. If the water solubility was adequate no additional additives were used in the preparation. Most compounds however, required prior solution in acetone, xylene or toluene, sometimes with mild heating. Emulsifiers were necessary with xylene or toluene to bring about an emulsion for stabilization. With the aid of a 9-hole formulation board, and equipment assembled for this purpose the optimum formulation of each of the prime candidate compounds was determined.

The optimum ratios of anionic to nonionic emulsifiers for those compounds formulated are given in Table 6. A majority of those compounds tested required no anionic emulsifier (0:100 ratio). Also, it may be noted that Compound No. 23 required a 25:75 emulsifier ratio for optimum conditions when tested with a 10 percent w/v test compound solution, rather than the usual 4800 ppm (or 0.48 percent) laboratory primary concentrate. Compound No. 73 was found to be sufficiently soluble in acetone and water so that once a solution was accomplished in acetone, dilution to testing concentrations was possible without the use of emulsifiers.

Economic Evaluation of Prime Candidate Compounds

On the basis of activity, as determined by the laboratory screening tests, and by elimination of those compounds which contained environmentally unacceptable components, the list

TABLE 6

COMPOUND FORMULATION OPTIMA[1]

Compound Number	Name of Compound	Solvent System	Emulsifiers	Optimum Emulsifier Ratio
3	5,5'-Dichloro-2,2'-dihydroxy-benzophene	81% xylene 19% perchloro-ethylene	Anionic Atlox 3404 + Nonionic Atlox 3495F	0:100
6	1,1'-Dimethyl-2-(α,α,α-tri-fluoro-p-tolyl)urea	" "	" "	0:100
8	4-Amino-2,5-dibromophenyl-thiocyanate	" "	" "	0:100
10	3,4,5-Trichloro-o-creosol	" "	" "	0:100
21	2,2',-Thiobis-(4,6-dichloro-phenol)	" "	" "	25:75
23	2,5-Dichloro-3,4-dinitrothio-phene	" "	" "	31.25:68.75
54	(p-Chlorophenyl)(2-thienyl)-iodoniumtrifluoroacetate	" "	" "	0:100
55	(p-Fluorophenyl)-2-thionyl-iodoniumtrifluoroacetate	" "	" "	0:100
57	Diphenyliodoniumtrifluoro-acetate	" "	" "	0:100
70	2-Iodo-3,5-dinitrothiophene	" "	" "	25:75
80	Isothiocyanatctriphenyltin	" "	" "	0:100
23*	2,5-Dichloro-3,4-dinitrothio-phene	" "	" "	25:75
73	(p-Chlorophenyl)-2-thienylidon-iumchloride	10% Acetone	-- --	---

[1]See Appendix C for detailed data
*For a 10% w/v primary concentrate for large-scale field testing

of compounds under active consideration was narrowed to six prime candidates (Table 7). At this point an economic evaluation of each of these compounds was made in which not only the costs of the basic building materials, but also the reaction hazards and difficultures, were considered. Compound No. 50 was found to be very difficult to synthesize and involved a number of hazardous reaction steps. Compound No. 72, though similar in structure to No. 73, was not only high in component costs but involved more reaction steps and was more difficult to make. Compound No. 24 was eliminated from the prime candidate list on the basis of poor activity against *Anabaena* as revealed in subsequent confirmatory tests. Also, the reactive steps for the production of Compound No. 70 were found to be very difficult; especially the step involving the iodination of thiophene which included the use of mercuric oxide or some other heavy metal.

Compound No. 23 was selected as the best candidate compound remaining after the screening program for intensive investwith Compound No. 73, even though considerably higher in cost, as the alternative choice.

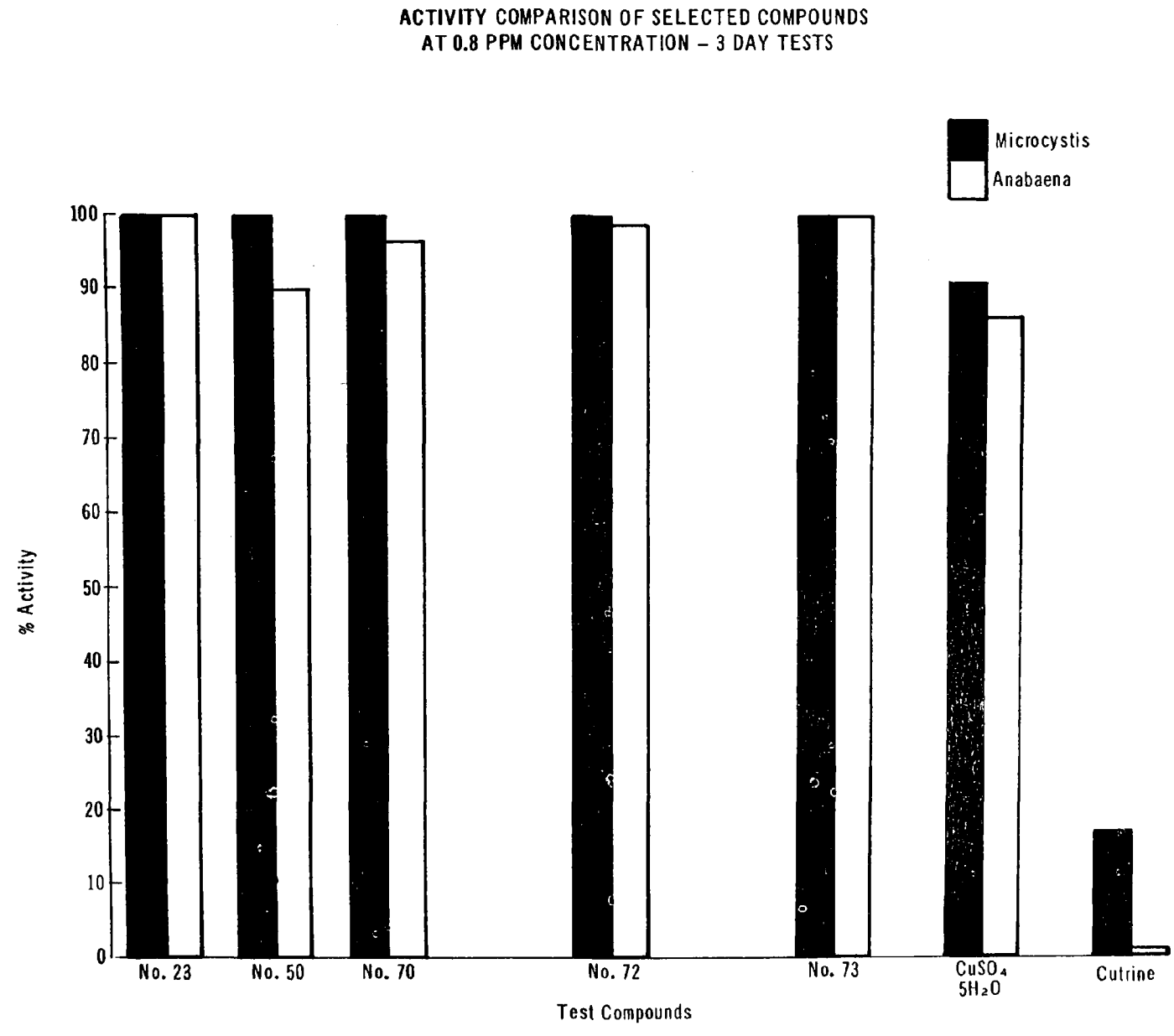
On the basis of compound activity against *Microcystis aeruginosa* compounds No. 23 and 73 compare favorably with $CuSO_4 \cdot 5H_2O$ and are much more active than equal concentrations of Cutrine, a commercial chelated copper compound (Figures 5,6,&7). The cost of treatment for control of *Microcystis* with Compound No. 23 would be approximately 0.512 that of $CuSO_4 \cdot 5H_2O$, taking into consideration the difference in activity and the cost of solvents and emulsifiers necessary in the formulation. The estimated cost of Compound No. 23 alone is 2.02 times that of hydrated copper sulfate. On the same basis, Compound No. 73 and Cutrine would be 3.01 and 15.1 times greater,

TABLE 7

PRIME CANDIDATE COMPOUNDS SELECTED
FOR ECONOMIC EVALUATION

Compound Number	Name of Compound	Descriminating Factors
23	2,5-Dichloro-3,4-dinitrothiophene	use of emulsifiers necessary in formulation
24	1,1-Dimethyltetradecylamine, hydrochloride	insufficient activity against *Anabaena*
50	Di-2-thienyliodoniumchloride	very hazardous reaction steps involved
70	2-Iodo-3,5-dinitrothiophene	iodonation of thiophene is difficult, requires use of mercuric oxide
72	(m-Chlorophenyl)-2-thienyliodoniumchloride	high cost of components and more difficult than No. 73 to make
73	(p-Chlorophenyl)-2-thienyliodoniumchloride	high cost

Figure 5


ACTIVITY COMPARISON OF SELECTED COMPOUNDS
AT 0.8 PPM CONCENTRATION – 3 DAY TESTS
Microcystis
Anabaena
% Activity
100
90
80
70
60
50
40
30
20
10
0
No. 23
No. 50
No. 70
No. 72
No. 73
CuSO4 5H2O
Cutrine
Test Compounds

COMPOUNDS NO. 23 & NO. 73 VERSUS MICROCYSTIS & ANABAENA

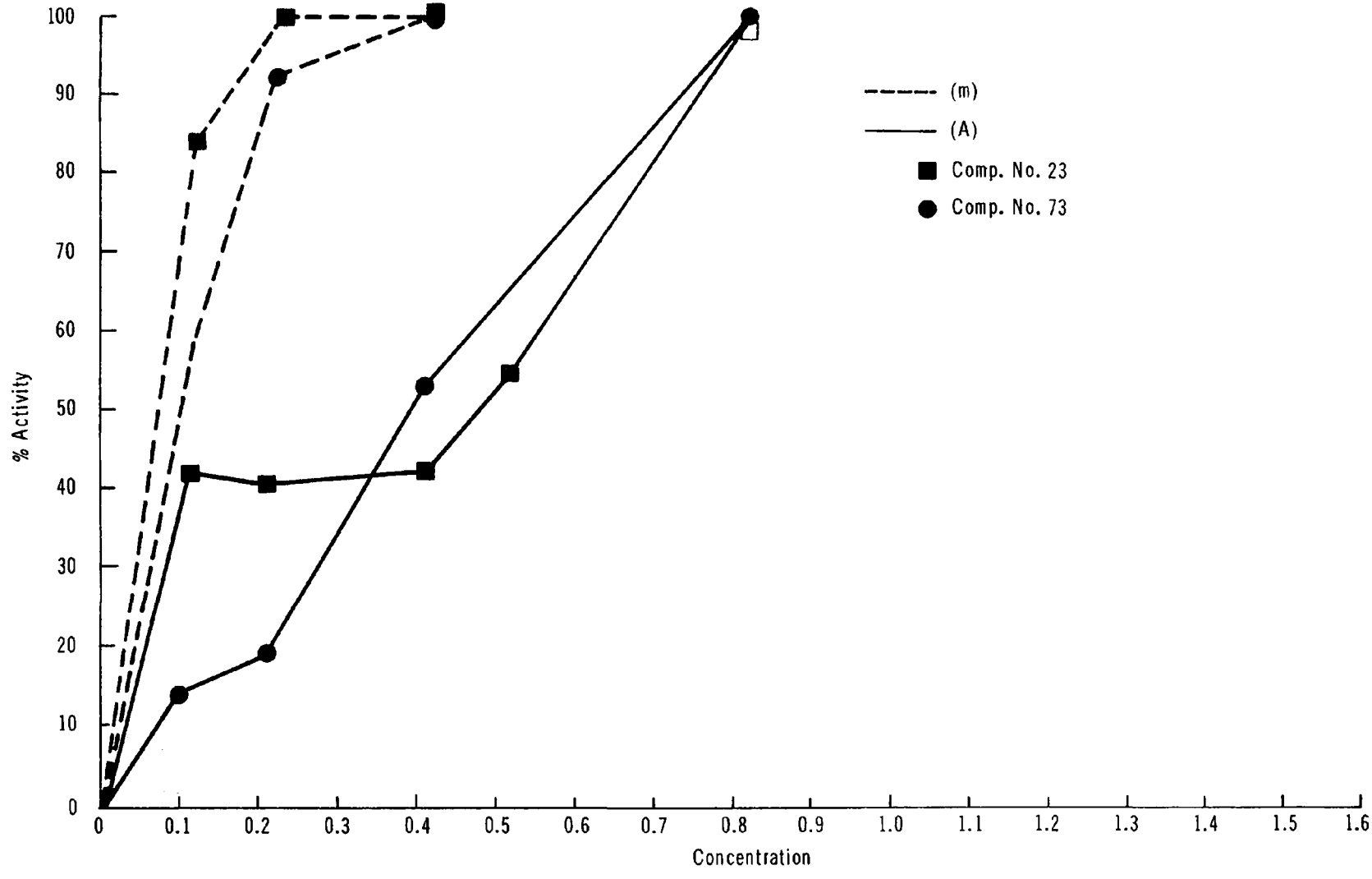
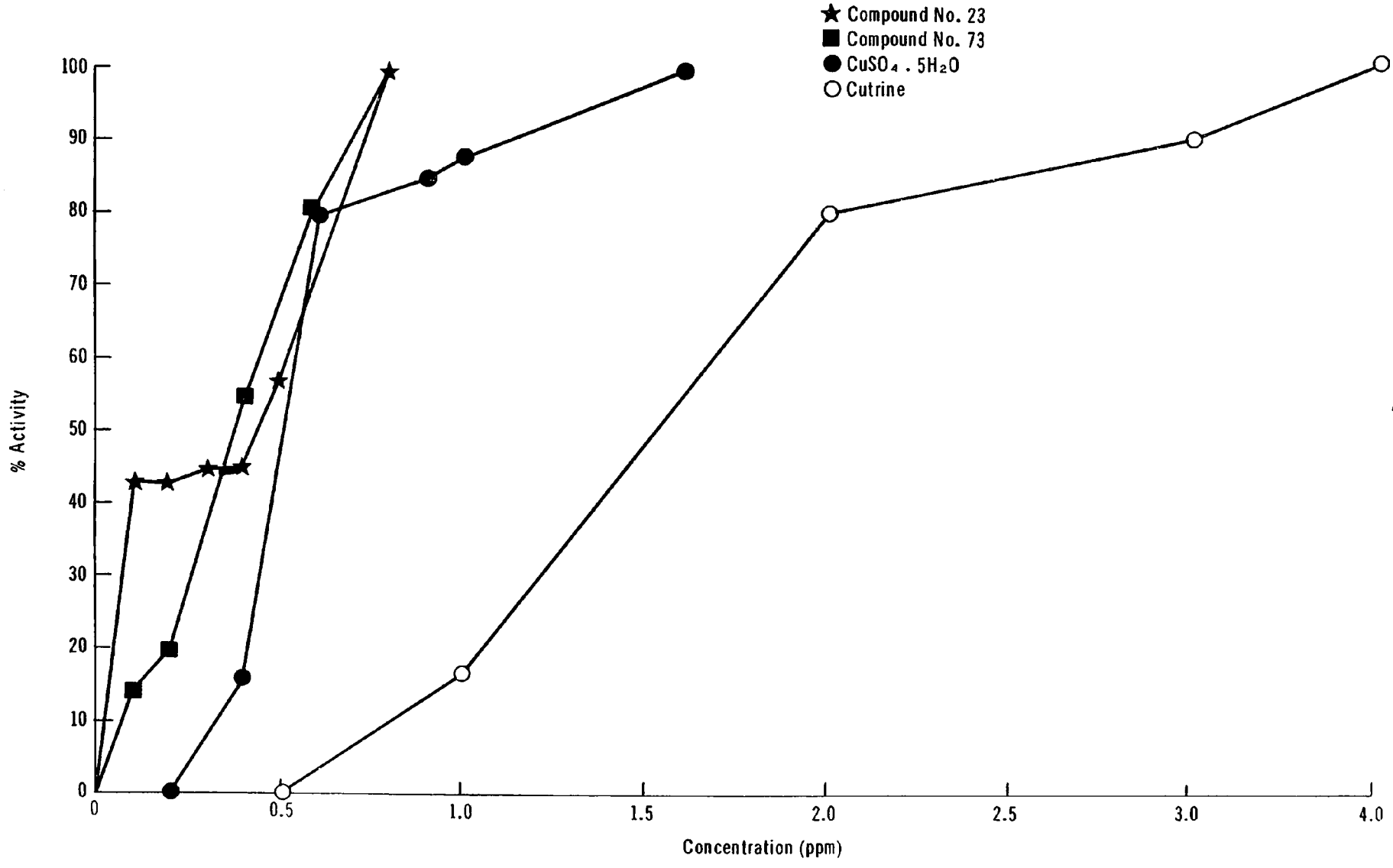


Figure 7

COMPOUND ACTIVITIES VERSUS ANABAENA FLOS AQUAE – 3 DAY TESTS



respectively, than copper sulfate for the same control efficacy (see Table 8).

Compound Re-synthesis and Toxicology Tests

One hundred grams of Compound No. 23 were synthesized since the existing supply was exhausted and larger quantities were needed for the proposed field tests and toxicological studies. The re-synthesis was facilitated by purchasing 2,5-dichlorothiophene and completing the synthesis by reacting with nitric acid in the presence of 30 percent fuming sulfuric acid.

Sixty grams of the newly made compound were sent directly to Dow's Chemical Biology Research Laboratories in Midland, Michigan were Class I toxicology tests were conducted. This included tests for primary skin irritation on rabbits, acute oral lethality using rats and eye irritation tests on rabbits.

The remaining 40 grams of Compound No. 23 were sent to this laboratory where 30 grams were formulated into a 10 percent w/v primary concentrate, using the pre-determined solvent system and optimum emulsifier ratio. This was the concentrate which was diluted 1:100 with water to make a "use" concentrate for the control of blue-green algae, the final concentration at the control level being in the 0.4-0.8 ppm range. The formulated concentrate was returned to Dow's toxicology laboratory in Midland for similar tests on the compound in its formulated state. The crystalline sample submitted for the Class I toxicology tests was 99 percent pure.

TABLE 8

COST COMPARISON OF ALGAECIDAL COMPOUNDS VERSUS Microcystis aeruginosa

Algaecide	Concentration req. for control	lbs/acre ft. req. for control	cost/acre ft.	Comparative cost factor*
I - Test Compound No. 23 (2,5-Dichloro-3,4-dintro-thiophene), including formulation agents	0.2 ppm	0.546	$ 0.415	0.512
II - Test Compound No. 73 (p-Chlorophenyl)-2-thienyl-iodoniumchloride), including formulation agents	0.2 ppm	0.546	$ 2.19	3.01
III - Copper Sulfate ($CuSO_4 \cdot 5H_2O$	1.0 ppm	2.72	$ 0.81	1.00
IV - Cutrine	3.0 ppm	8.16	$12.25	15.1

*Compared to $CuSO_4$ arbitrarily set at 1.00

The toxicology report indicated that the acute oral lethality of the material was moderate and that there was little liklihood of internal injuries resulting from acute ingestion of amounts of the material one might encounter incidental to industrial handling. It was emphasized, however, that serious internal injuries could result from accidental or deliberate ingestion of larger amounts.

Eye contact with the concentrated test material would likely result in moderate pain, severe conjunctival inflammation, moderate iritus and severe corneal injury, with possible impairment of vision if the eyes were not promptly and thoroughly decontaminated. Prolonged skin contact with the test material would likely result in slight redness and moderate swelling. However, if skin contact was repeated or contaminated clothing was worn for several days, slight redness, severe swelling and a slight chemical burn could result. The material may be absorbed through the skin in acutely toxic amounts if contact is prolonged or repeated.

The toxicological report on the formulated sample was very similar to that which resulted from studies of the concentrated compound with no significant differences in recommended precautionary or safe handling procedures.

Shock Sensitivity and DTA Tests

One of the tasks to be completed during Phase II was the determination of production or handling hazards, if any, of the compounds selected as final candidates. Differential thermal analyses (DTA) and shock sensitivity tests conducted by two independent laboratories, both associated with Dow's Reactive Chemicals Team, indicated the existence of no hazards due to ordinary mechanical shock or small-scale pro-

duction of test Compound No. 23. Out of ten drop-weight tests for shock sensitivity no positives were reported. This gave an E_{50}>300 kg-cm, i.e., the energy required to yield positive results from 50 percent of the tests when a 6 kg mass is dropped from a height of 50 cm onto a small quantity of encapsulated sample.

One of the differential thermal analyses (DTA) tests on Compound No. 23 revealed endotherms at 88°C and 327°C but the existance of no exotherms. However, a second test revealed a fairly large exotherm starting at 275-290°C, serving as a warning that a large quantity of the material might produce a fire or explosion hazard. Thus, it is recommended that before multi-pound lots of the material are synthesized, larger samples should be tested for heavy shock sensitivity using blasting caps and tetryl boosters.

Overall, considering the results of the Class I toxicology and Reactive Chemicals tests, it appears that the small-scale production and handling of Compound No. 23 is safe, if the prescribed precautions are properly observed.

After the selection of Compound No. 73 as a prime candidate for further study, it was discovered that previous interest in this compound for another use had resulted in the toxicology and hazardous properties tests having already been conducted. As indicated from the reports on file, this compound also is considered to be relatively free of production and personnel handling hazards.

Field Tests

As the screening tests, economic evaluations and hazardous properties tests progressed, and the list of potential candidate compounds became narrowed to a smaller number, field

tests were undertaken on the prime candidates to determine their behavior as algaecides under conditions more closely simulating those which might be encountered in nature.

Of the four compounds which were tested under field conditions only No. 23 (2,5-Dichloro-3,4-dinitrothiophene) and No. 73 ((p-Chlorophenyl)-2-thienyliodoniumchloride) were sufficiently active to justify their selection as final candidates (Table 9). Compound No. 11 (3,4,4',5,6-Pentachloro-2,3'-methylenediphenol) had held much interest earlier in the screening program due to its rapid action on the algal cell walls, causing an almost immediate initiation in loss of chlorophyll. This compound was later eliminated due to environmental questions regarding its phenyl components and its poor activity at concentrations below 1.0 ppm. Compound No. 70 (2-iodo-3,5-dinitrothiophene) exhibited 96 percent activity against *Anabaena* when screened under laboratory conditions, but in the open atmosphere the activity dropped to 82 percent, indicating probable photodegradation of the compound under those conditions.

The confirmed outdoor activity of Compound No. 23 showed greater algaecidal properties than were indicated during laboratory tests (Tables 4 and 9). The same was true with Compound No. 73, except that due to the onset of cold weather, attempted confirmatory tests were unsuccessful. The relatively high activity at lower concentrations with Compound No. 73 must be accepted with some question due to the fact that control cultures under those conditions did not show a healthy growth, although the growth patterr did return to normal when samples were placed in warmer temperatures under laboratory conditions.

TABLE 9

FIELD TESTS

TEST COMPOUNDS VERSUS Anabaena flos aquae

Compound Number	Name of Compound	Activity (%) at Various Conc. (ppm)							
		1.6	1.0	0.8	0.6	0.4	0.3	0.2	0.1
11	3,4,4',5,6-Pentachloro-2,2'-methylenediphenol			82		87		54	31
23	2,5-Dichloro-3,4-dinitrothiophene		100		100		100		38
70	2-Iodo-3,5-dinitrothiophene	47		17		<10		<10	<10
73	(p-Chlorophenyl)-2-thienyliodoniumchloride		100*		100*				100*

*Colder weather test. Stock cultures before treatment were somewhat below par.

Correlation Analysis - Activity as a Function of Structure

The basic philosophy underlying the initial effort of the Phase II program to "Develop a Selective Algaecide" involved the probability of there being a direct correlation between known active algaecidal compounds and those having related structures. With this in mind a computerized structure search was conducted in which over 100,000 compounds were screened to select analogs of the four surviving compounds from Phase I.

Of the 1309 analogs selected (see Table 3, page 36), 69 came through the rapid agar-plate screening tests as "positives". By comparing the results of the fine-screening tests on these compounds with the related compound structures it was observed that most of the compounds showing good algaecidal properties were either substituted thiophenes or combined iodonium-thiophenes. Of the 69 compounds tested, 40 fitted into these two categories (Table 10).

Outside of the two mercurial compounds (Nos. 40 and 41) and one tin compound (No. 61) there were no compounds with high algaecidal activity among any of the other groups. A classification of the structures of the eleven best compounds, together with the substituent components present in each case, is given in Table 11 . In addition to the thiophene component the most common elements associated with high algaecidal activity was iodine and chlorine, usually, but not always in combination.

A comparison of the compound structures of the single and double thiophenes, together with the combined iodonium thiophenes, listed according to algaecidal ratings is shown in Figure 8. Within this general group of compounds there

TABLE 10

CORRELATION ANALYSIS OF PHASE II TEST COMPOUNDS
ACTIVITY AS A FUNCTION OF COMPOUND STRUCTURE

Benzene Ring Types						5-Membered Ring Thiophenes				Comb: 5&6-Membered Ring Thiophene-Iodonium				Linear Type	
Single		Double		Triple		Single		Double		Two ring		Three ring			
Compound No.	Class	Compound No.	Class	Compound No.	Class	Compound No.	Class	Compound No.	Class	Compound No.	Class	Compound No.	Class	Compound No.	Class
41 (Hg)	I	11	II	80	II	70	I	50	I	51	III	79	II	40 (Hg)	I
100	III	57	II	108	II	23	I	58	III	52	II	88	III	61 (Sn)	I
101	III	81	III			83	III	86	III	53	III	15	II	113 (Thio)	III
102	III	107	III			89	III			54	II				
103	II	109	II			91	III			55	II			24 (amine)	II
105	II	118	III			92	III			56	III				
106	II	119	III			93	III			59	III				
110	II					94	I			60	III				
111	III					95	III			62	III				
114	III					96	I			71	II				
115	III					97	III			72	II				
116	III					98	III			73	I				
8	II					99	III			74	II				
9	III									75	III				
10	III									76	II				
112	III									77	III				
										78	I				
										82	I				
										84	III				
										85	II				
										87	III				

Key: Activity against Anabaena flos aquae

Class I = 95-100% at 1.0 ppm
Class II = 70-95% at 1.0 ppm
Class III = <70% at 1.0 ppm

TABLE 11

ACTIVITY AS A FUNCTION OF STRUCTURE - CLASS I

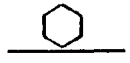
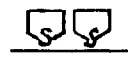
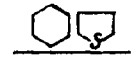
	STRUCTURES						ATTACHMENTS (ELEMENTAL COMPONENTS)							
Compound No.					Linear C-C-C	S	Iodonium	Br	Cl	N	Hg	Sn	R-Group	I
41	x										x			x
70		x				x				x				x
23		x				x			x	x				
94		x				x							x	
96		x				x		x		x				
50			x			x	x		x					
73				x		x	x		x					
78				x		x	x		x				x	
40					x						x			x
61					x	x				x		x		
82				x		x	x		x					

Figure 8

PHASE II TEST COMPOUNDS – STRUCTURE VS ACTIVITY

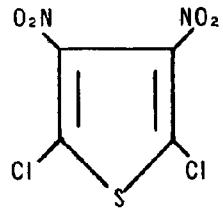
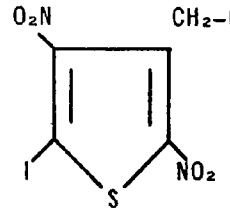
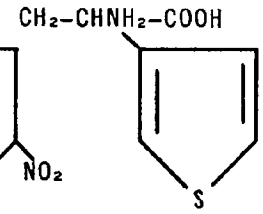
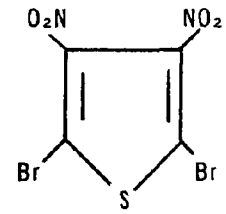
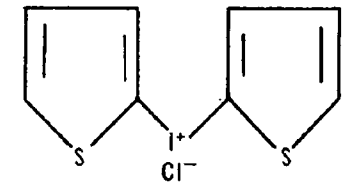
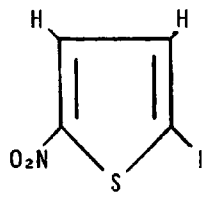
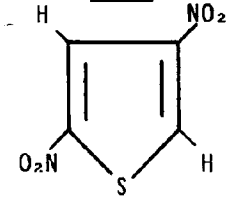
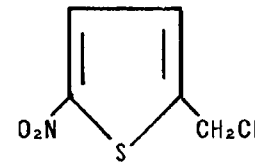
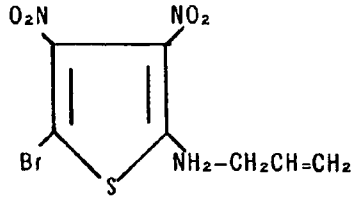
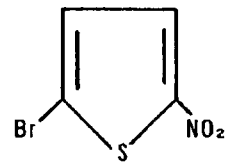
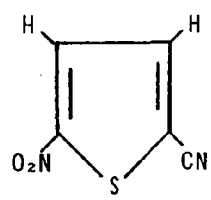
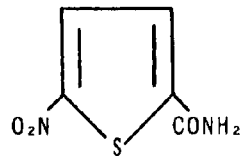
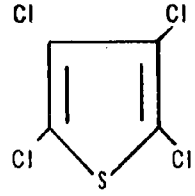
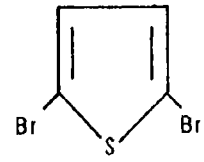
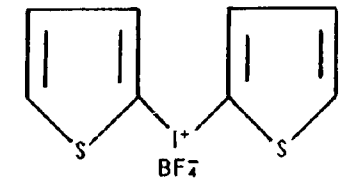
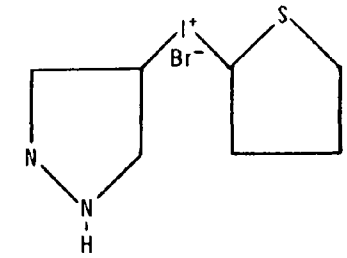
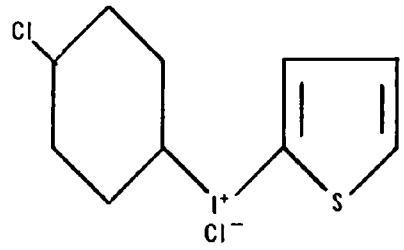
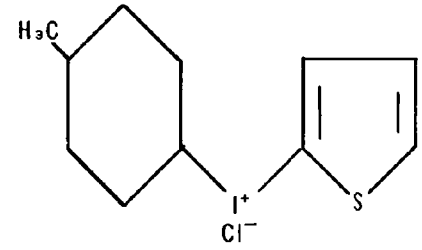
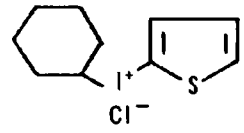
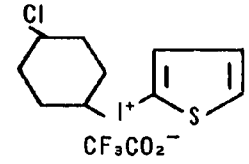
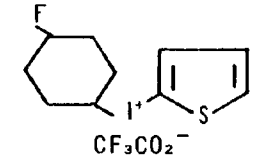
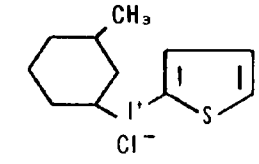
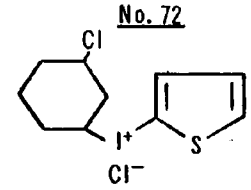
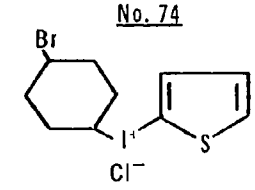
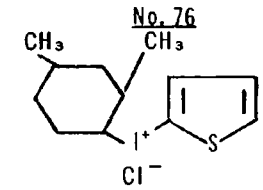
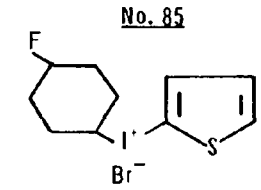
	Single Thiophenes	Double Thiophenes
Class I	No. 23  No. 70  No. 94  No. 96 	No. 50 
Class II	None	None
Class III	No. 83  No. 89  No. 91  No. 92  No. 93  No. 95  No. 97  No. 98  No. 99 	No. 58  No. 86 

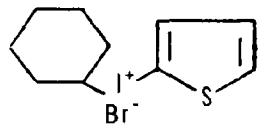
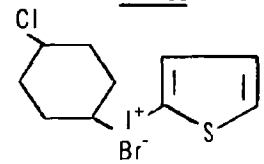
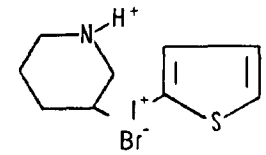
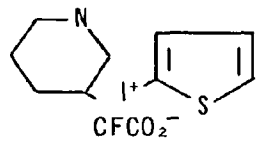
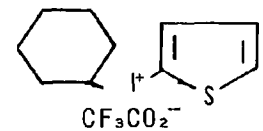
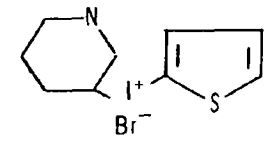
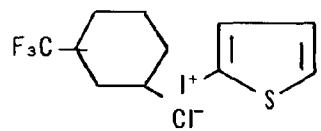
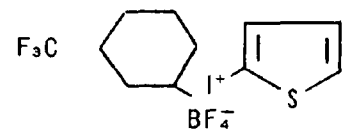
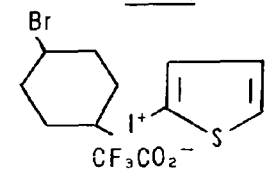
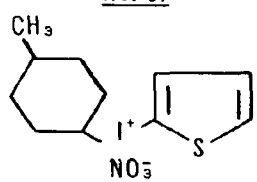
Figure 8 continued

PHASE II TEST COMPOUNDS – STRUCTURE VS ACTIVITY

	Combination: Thiophene – Benzene ring – types
Class I	No. 73  No. 78 
Class II	No. 52  No. 54  No. 55  No. 71  No. 72  No. 74  No. 76  No. 85 

See Table 10 for key to toxicity classes.

Figure 8 continued

	Combination: Thiophene - benzene ring - types
Class III	No. 51  No. 53  No. 56  No. 59  No. 60  No. 62  No. 75  No. 77  No. 84  No. 87 

See Table 10 for key to toxicity classes.

does not seem to be any predominant activity-structure relationship. However, it may be noted that chlorine was always associated with Class I iodonium thiophenes, as well as with the highly active single-thiophene, No. 23. Comparing Class I and III compounds Nos. 23 and 98, and also No. 96 and 99, it would appear that the double NO_2 attachments in the 3 and 4 positions have a definite positive affect on algaecidal action. Also, the increased toxicity of Class I Compound No. 70 over the Class III Compound No. 83 indicates an enhancing effect due to an additional NO_2 group.

The attachment of chlorine in the "meta" position of No. 72 shows a reduction in toxicity as compared to chlorine in the "para" positions of Compound No. 73.

Compound Persistence Tests

A series of tests, planned to determine the toxicity persistence of the two compounds selected as prime candidates, revealed a clear pattern of compound decomposition in the field within three to seven days, but very little breakdown of the compound while stored under laboratory conditions (Table 12). The breakdown under field conditions versus laboratory conditions, as determined by toxicity reduction, is depicted graphically in Figure 9 . It appears probable that the decomposition is due to light action rather than from atmospheric oxidation.

A similar breakdown pattern is shown with respect to Compound No. 73 (Table 13).

TABLE 12

PERSISTENCE STUDY - COMPOUND NO. 23 (2,5-Dichloro-3,4-dinitrothiophene)
at 1.0 PPM VS. *Anabaena flos aquae*
Values in Relative Intensity

Test No.	Components	Conditions	Progressive Monitoring Time						Percent Decomposition
			0 - days		3 days		7 days		
			Initial	Final*	Initial	Final*	Initial	Final*	
I	1 + 4 + T	field	0.07	0.02	0.17	0.57	0.06	0.35	100
I'	1 + 4 + T	lab.	0.07	0.02	0.17	0.46	0.06	0.01	3
I" (control)	1 + 4	lab.	0.07	0.15	0.17	0.54	0.06	0.32	---
II	2 + 4 + T	field	0.07	0.02	0.17	1.34	0.06	0.34	100
II'	2 + 4 + T	lab.	0.07	0.02	0.17	0.03	0.06	0.01	3
II" (control)	2 + 4	lab.	0.07	0.39	0.17	1.50	0.06	0.30	---
III	3 + 5 + T	field	0.07	0.02	0.17	0.70	0.06	0.21	27
III'	3 + 5 + T	lab.	0.07	0.02	0.17	0.02	0.06	0.00	0
III" (control)	3 + 5	lab.	0.07	0.33	0.16	0.36	0.06	0.76	---

Key to Components: 1 = World Health Organization (WHO) water
2 = Well water
3 = Deionized water
4 = PW-4 media
5 = Gorham's media
T = Test Compound at 1.0 ppm

*Final RI's were taken 3 days after initiation of each monitoring culture.

Figure 9

DEGRADATION OF COMPOUND NO. 23
IN WELL WATER AND PW-4 MEDIUM

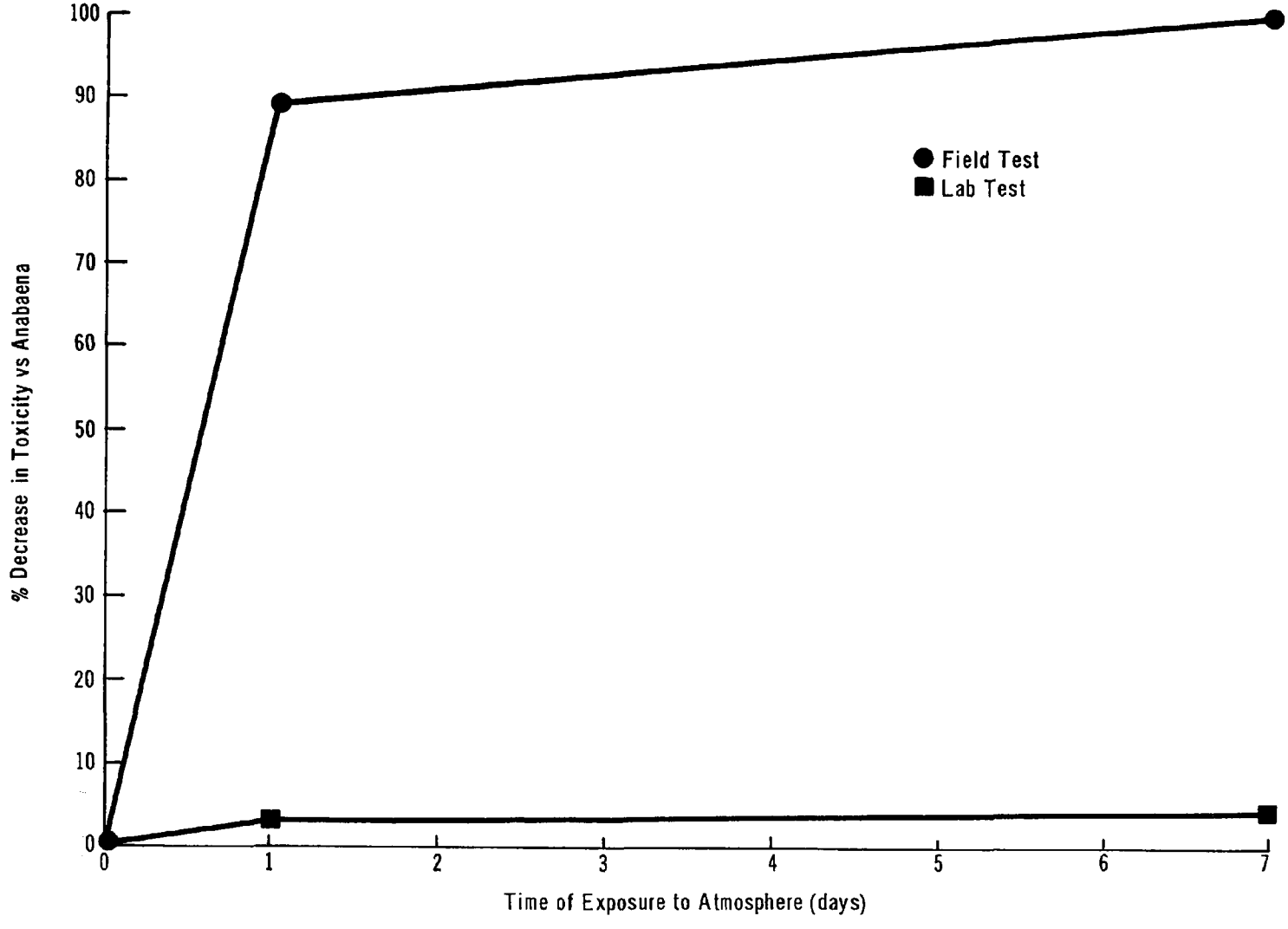


TABLE 13

PERSISTENCE STUDY - COMPOUND NO. 73 AT 1.0 ppm
USING *Anabaena* AS THE TEST SPECIES
(Values in Relative Cell Counts)

Test No.	Components	Conditions	Progressive Monitoring Time						Percent Decomposition
			0 days		3 days		7 days		
			Initial	Final*	Initial	Final*	Initial	Final*	
I	"WHO" water and Compound No. 73 with PW4 media	field	---	---	0.35	0.0	1.13	1.0	45
I'	" "	lab.	1.09	0.0	0.35	0.0	1.13	0.0	0
I"	control	lab.	1.09	1.43	0.35	0.40	1.13	2.3	-
II	well water with PW4 media and Compound No. 73	field	---	---	0.35	0.0	1.13	0.83	36
II'	" "	lab.	1.09	0.0	0.35	0.0	1.13	0.0	0
II"	control	lab.	1.09	1.33	0.35	0.43	1.13	2.3	-
III	Ion water and Compound No. 73	field	---	---	0.35	0.0	1.13	3.11	89
III'	w/Gorham's media	lab.	1.09	0.0	0.35	0.0	1.13	0.0	0
III"	control	lab.	1.09	1.10	0.35	0.51	1.13	3.5	-

*Final cell counts were taken 3 days after initiation of each monitoring culture. Numbers indicate cell counts x 10^6, made after blending in a blender for 15 seconds.

Biological Control Systems

Early in the Phase I study an organism, subsequently identified as *Ochromonas ovalis*, was discovered which was phagocytic to *Microcystis aeruginosa*. Since that time the effort to develop an effective chemical algaecide has been paralleled by an effort to determine the optimum activity parameters of phagocytic organisms.

Attempted field studies on test compounds for the control of *Microcystis* were unsuccessful because of repeated infestation by *Ochromonas*, which soon destroyed the algal test cultures. A seven-day study was conducted to determine the mode of *Ochromonas* infestation. Open-topped and muslin-covered vessels containing *Microcystis* cultures were set out at the test site. All became infected within a few days. The only ones in which *Ochromonas* did not appear were those covered with Saran Wrap (Table 14). From this and other field experiences it was concluded that *Ochromonas* was naturally ubiquitous in this locality and that because of its presence it would not be possible to conduct successful field tests using *Microcystis* as the test species.

An attempt was made to find other organisms which might also serve as biological agents to control the growth of various species of blue-green algae.

Through the cooperation of Dr. M. J. Wynne, of the Department of Botany, University of Texas at Austin, cultures of four other species of *Ochromonas* were obtained. Three of these (*O. danica*, *O. malhamensis* and *O. minuta*) were obtained from the University of Indiana Algal Culture Collection. The fourth strain (*O. bastrop*) was isolated from a pond at Bastrop, Texas and is being maintained as part of a teaching collection at the University of Texas.

TABLE 14

FIELD STUDY TO DETERMINE THE SUSCEPTIBILITY OF *Microcystis* TO OCHRMONAL INFESTATIONS IN WHICH VARIOUS PROTECTIVE COVERINGS ARE UTILIZED AS BARRIERS (A SEVEN DAY STUDY)

Barrier Type	Initial *Microcystis* Cell Count X 10^6	Initial Ochromonas Cell Count X 10^4	Final *Microcystis* Cell Count X 10^6	Final Ochromonas Cell Count X 10^4	Effective Ochromonal Barrier
Muslin Cloth	0.2	0	0.3	1.0	No
Saran Wrap	0.2	0	0.9	0	Yes
Control (open top)	0.2	0	0.08	0.5	No

Early tests conducted by Dr. Wynne at the University of Texas indicated that three of the four *Ochromonal* species did phagocytize *Microcystis* under laboratory conditions. *O. minuta*, a relatively small-sized species did not exhibit this property. Under the laboratory conditions utilized in that study, using a yeast-liver extract medium, *O. bastrop* was reported to be the most voracious feeder, with *O. malhamensis* showing the least activity of the three (see appendix C). Studies conducted at this laboratory using Gorham's medium produced basically the same results (Table 15). Figures 10 and 11 show graphical comparisons of the various activity rates as functions of time. *O. ovalis* showed the highest activity rate of any of the species tested, with nearly complete depletion of *Microcystis* within the first day after inoculation.

A test utilizing the four active species of *Ochromonas* in light and dark conditions produced only slight differences in phagocytic activity as a result of the absence or presence of light (Table 16).

Phagocytic Activity Enhancement Systems

A number of experiments were conducted to determine possible conditions which might positively effect the rate of growth of *Ochromonas* or in some way cause an increase in their ability to phagocytize *Microcystis*. A number of the test compounds which were found to have low activities against blue-green algae, proved to be stimulators to the growth and phagocytic activity of *Ochromonas*. For example, in the presence of Compound No. 14 the *Ochromonas* count was 20 x 10^4 cells/ml after two days incubation as compared to only 14 x 10^4 cells/ml in the flasks where no test compound was present (Table 17). Also, in the same tests, at 0 + 3 days

TABLE 15

PHAGOCYTIC ACTIVITY OF FOUR SPECIES OF
Ochromonas ON *Microcystis aeruginosa*

Species	CELL COUNTS								Percent Control
	0 + days		0 + 1 day		0 + 2 days		0 + 6 days		
	(M)x10^6	(OCH)x10^4	(M)x10^6	(OCH)x10^4	(M)x10^6	(OCH)x10^4	(M)x10^6	(OCH)x10^4	
O. bastrop	0.84	1.0	0.63	0.35	0.03	4.5	0.0	12.2	100
O. danica	0.84	1.0	0.42	0.40	0.53	22.60	0.34	2.0	92
O. malhamensis	0.84	1.0	0.90	0.50	1.10	0.45	0.75	11.2	82
O. ovalis	0.84	1.0	0.07	3.5	0.0	8.6	0.0	8.2	100
Controls (avg)									
(M) only	0.84	---	1.18	---	1.13	---	4.30	---	—

Figure 10

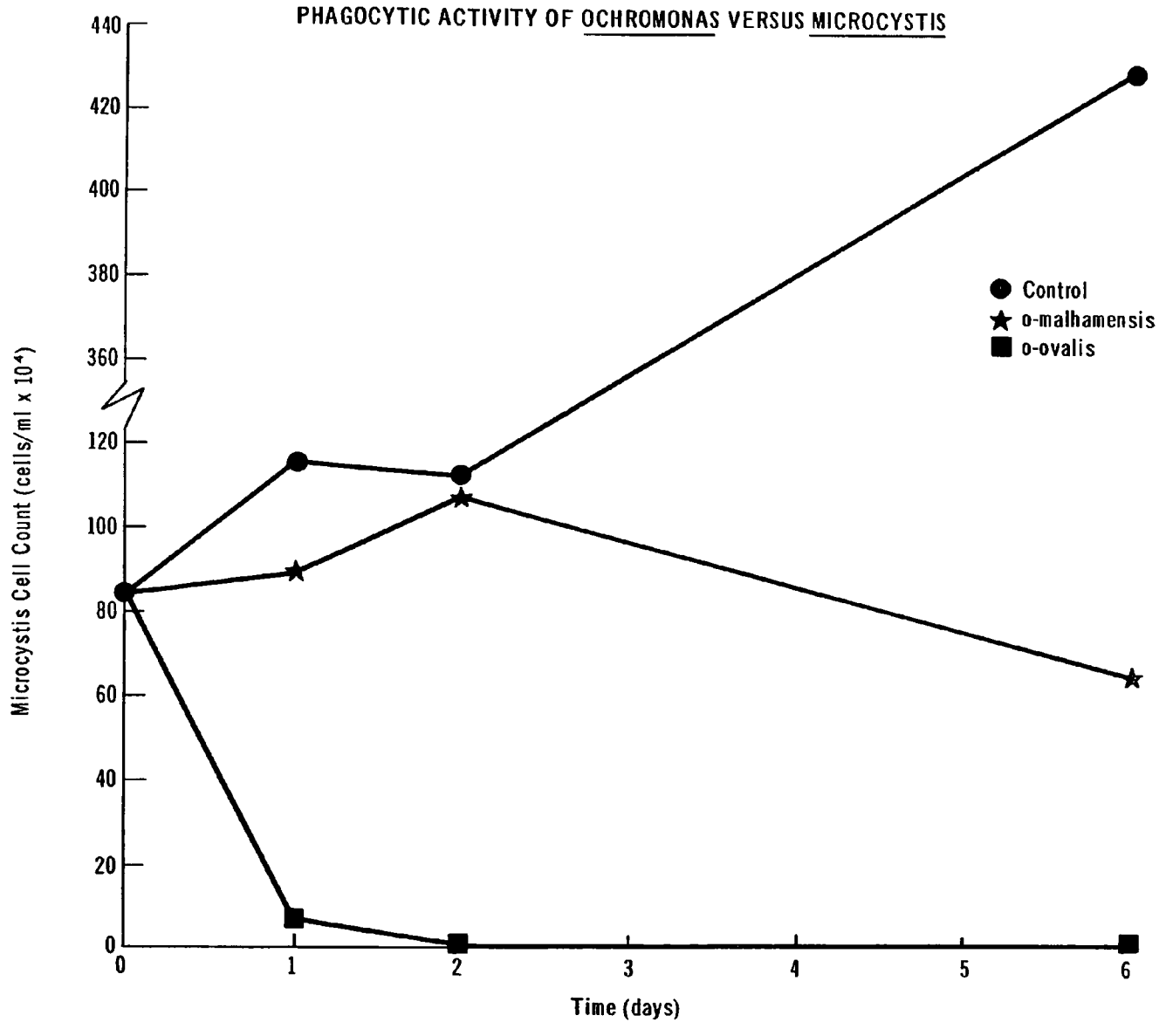

PHAGOCYTIC ACTIVITY OF OCHROMONAS VERSUS MICROCYSTIS
Microcystis Cell Count (cells/ml x 10⁴)
440
420
400
380
360
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (days)
Control
o-malhamensis
o-ovalis

Figure 11

PHAGOCYTIC ACTIVITY OF OCHROMONAS VERSUS MICROCYSTIS

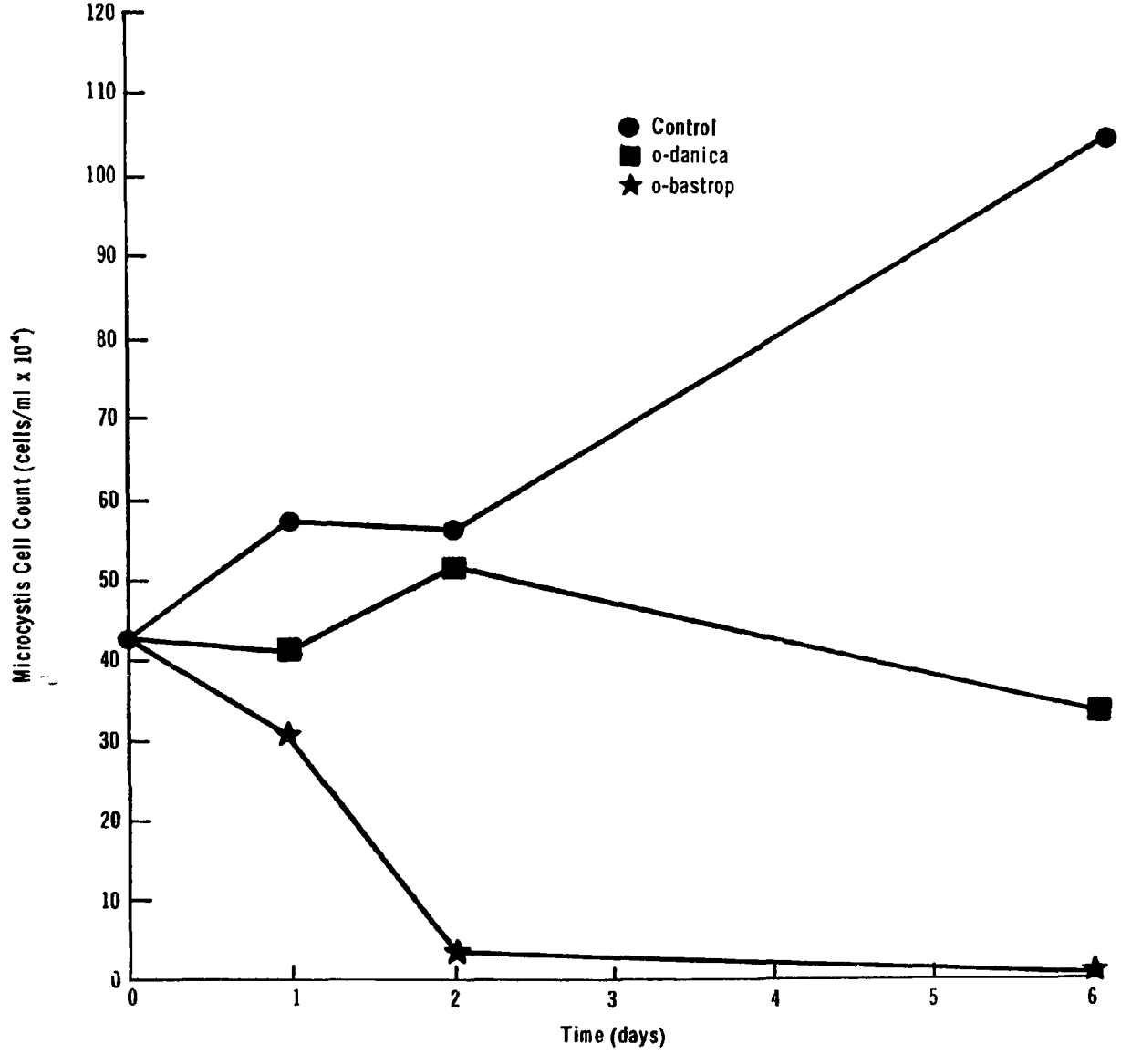


TABLE 16

A FOUR DAY STUDY OF THE RATE OF PHAGOCYTIC ACTIVITY OF VARIOUS SPECIES OF *Ochromonas* AGAINST *Microcystis aeruginosa* IN LIGHT AND DARK CONDITIONS

Ochromonas Species and Lighting Conditions	Initial		Final		Activity
	Microcystis Cell Count X 10^6	*Ochromonas* Cell Count X 10^4	*Microcystis* Cell Count X 10^6	*Ochromonas* Cell Count X 10^4	% Control
Ochromonas malhemensis					
in light	1.0	0.1	0.03	3.0	100
in dark	1.0	0.1	0	3.0	100
Ochromonas danica					
in light	1.0	0.1	0.58	2.0	65
in dark	1.0	0.1	0.56	1.6	46
Ochromonas ovalis					
in light	1.0	0.1	0	3.2	100
in dark	1.0	0.1	0	2.6	100
Ochromonas bastrop					
in light	1.0	0.1	0.01	3.0	100
in dark	1.0	0.1	0	2.6	100
Control - in light	1.0	---	1.65	---	---
Control - in dark	1.0	---	1.02	---	---

TABLE 17

TEST COMPOUND NO. 14 EFFECT ON *Ochromonas* ACTIVITY

Conditions	Average Cell Counts							
	0 - Days		0 + 1 Day		0 + 2 Days		0 + 3 Days	
Components Added	(M) X 10^6	(Och) X 10^4	(M) X 10^6	(Och) X 10^4	(M) X 10^6	(Och) X 10^4	(M) X 10^6	(Och) X 10^4
Test Chem. + (Och)	5.5	0.1	0.8	3.6	0.6	20	0.0	10
Test Chem. only	4.5	0.0	0.7	0.0	1.9	0.0	3.3	0.0
(Och) only	4.0	0.1	0.8	1.2	1.2	14.0	0.8	9.5
No Test Chem. or (Och)	4.5	0.0	4.0	0.0	4.6	0.0	5.2	0.0

(M) = *Microcystis aeruginosa*

(Och) = *Ochromonas ovalis*

the *Microcystis* culture was completely depleted when the test compound was present, but 0.8 x 10^6 cells/ml were left where no test chemical was used. A similar pattern is seen with respect to Compound No. 6 (Table 18), except that the growth of *Ochromonas* in the cultures with no test chemical surpassed the others after the first 24 hours. This relationship is more easily seen in the graphical representation shown in Figure 12. Also, test Compound No. 11 at 0.1 ppm indicated some improvement to the system for control of *Microcystis* (Figure 13).

A comparative study of the phagocytic activity of the four most active species of *Ochromonas*, with respect to three selected test compounds, was made. The rate of *Microcystis* depletion resulting from the activity of *O. bastrop*, *O. danica* and *O. malhamensis*, was greater in the presence of both compounds, No. 117 and 119 at 0.2 ppm (Tables 19 and 20). These compounds, however, did not show any significant effect on the growth or phagocytic activity of *O. ovalis*. Compound No. 114 produced no improvement in activity of any of the four *Ochromonal* species tested (Table 21).

Ochromonal Microstructure and Mode-of-Action Study

Healthy dense cultures of *O. danica* were prepared by Dr. Wynne and his co-worker Dr. Gary Cole, University of Texas, and examined by means of transmission electron microscopy to determine the organelle arrangement within this type of cell. In normal cells of *Ochromonas danica* a single, large vacuole occurs in the posterior end of the cell (Figure 14). The arrangement of the organelles is evident, with the nucleus situated at the anterior end of the cell and usually two fairly large chloroplasts on opposite sides. The density of the vesicles at the anterior end are note-

TABLE 18

ENCHANCEMENT OF OCHROMONAS ACITIVTY
BY TEST COMPOUND NO. 6

Components Added	Cell Density, cells/ml. 0 - Days (M) X 10^6	0 - Days (Och) X 10^4	0 + 1 Day (M) X 10^6	0 + 1 Day (Och) X 10^4	0 + 2 Days (M) X 10^6	0 + 2 Days (Och) X 10^4
Test Chem. + (Och)	2.4	1.4	0.75	15	0.0	18
Test Chem. only	2.4	0.0	3.4	0.0	2.8	0.0
(Och) only	2.4	1.4	1.4	12.7	0.0	35
No Test Chem. or (Och)	2.4	0.0	3.2	0.0	4.1	0.0

(M) = Microcystis aeruginosa

(Och) = Ochromonas ovalis

Figure 12

INFLUENCE OF COMPOUND NO. 6 AT 1.0 PPM ON THE
PHOGOCYTIC ACTIVITY AT OCHROMONAS OVALIS ON MICROCYSTIS AERUGINOSA

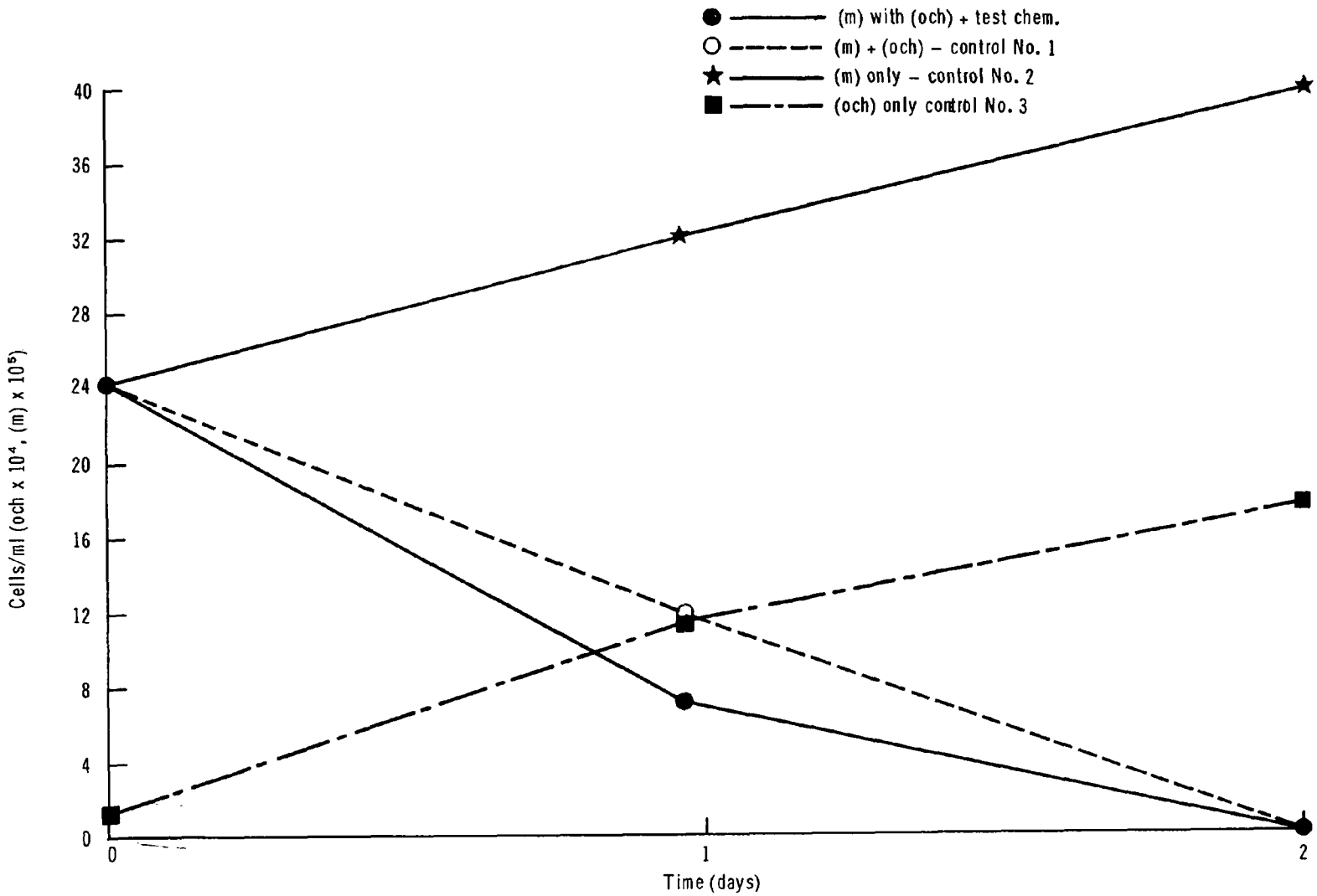


Figure 13

INFLUENCE OF COMPOUND NO. 11 AT 0.1 PPM ON THE
PHAGOCYTIC ACTIVITY OF *OCHROMONAS OVALIS* ON *MICROCYTIS AERUGINOSA*

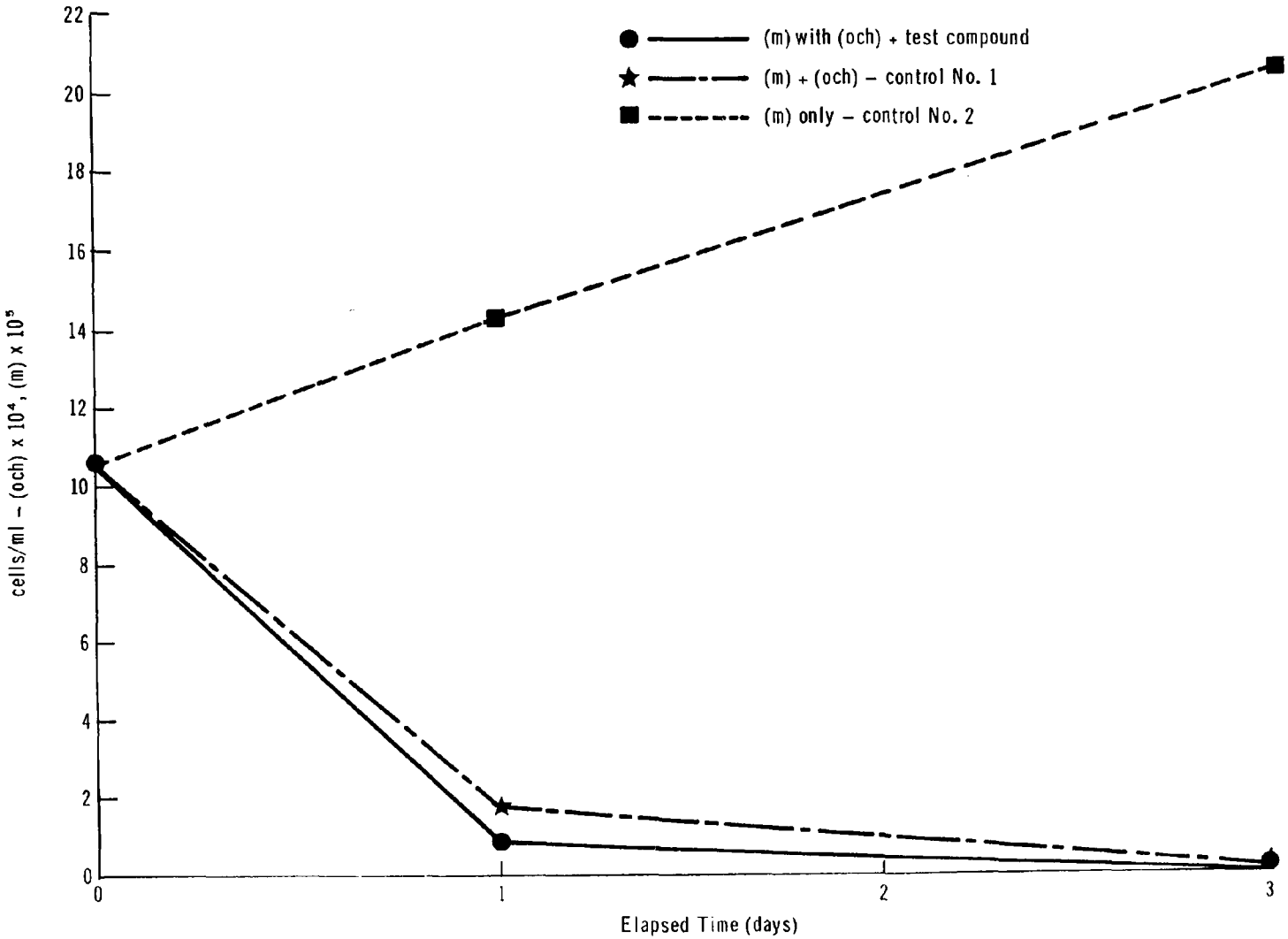


TABLE 19

INFLUENCE OF TEST COMPOUND NO. 117 AT 0.2 PPM ON THE PHAGOCYTIC ACTIVITY OF FOUR SPECIES OF *Ochromonas*

Species	CELL COUNTS						Percent Control
	0 - days		0 + 1 day		0 + 2 days		
	(M)x10^6	(OCH)x10^4	(M)x10^6	(OCH)x10^4	(M)x10^6	(OCH)x10^4	
O. bastrop+(M)+(T)	0.84	1.0	0.47	1.80	0.0	7.3	100
Control*	0.84	1.0	0.63	0.35	0.03	4.5	98
O. danica+(M)+(T)	0.84	1.0	0.73	0.65	0.19	1.9	84
Control*	0.84	1.0	0.42	0.40	0.53	2.60	54
O. malhamensis+(M)+(T)	0.84	1.0	0.98	0.30	0.95	0.70	16
Control*	0.84	1.0	0.90	0.50	1.10	0.45	<10
O. ovalis+(M)+(T)	0.84	1.0	0.07	3.0	0.0	9.1	100
Control*	0.84	1.0	0.07	3.5	0.0	8.6	100
(M) only - Control No. 1	0.84	---	1.20	---	1.20	---	---
(M) only - Control No. 2	0.84	---	1.16	---	1.06	---	---

Key: (M) = *Microcystis aeruginosa*
(OCH) = *Ochromonas sp.*
(T) = Test compound

*Same algal components, but with no test compound

TABLE 20

INFLUENCE OF TEST COMPOUND NO. 119 AT 0.2 PPM ON THE PHAGOCYTIC ACTIVITY OF FOUR SPECIES OF *Ochromonas*

Species	CELL COUNTS						Percent Control
	0 - days		0 + 1 day		0 + 2 days		
	(M)x10^6	(OCH)x10^4	(M)x10^6	(OCH)x10^4	(M)x10^6	(OCH)x10^4	
O. bastrop+(M)+(T)	0.84	1.0	0.48	2.3	0.0	7.0	100
Control*	0.84	1.0	0.63	0.35	0.03	4.5	98
O. danica+(M)+(T)	0.84	1.0	0.61	0.35	0.22	0.80	82
Control*	0.84	1.0	0.42	0.40	0.53	2.60	54
O. malhamensis+(M)+(T)	0.84	1.0	0.74	0.20	0.76	0.80	33
Control*	0.84	1.0	0.90	0.50	1.10	0.45	<10
O. ovalis+(M)+(T)	0.84	1.0	0.04	4.1	0.0	6.0	100
Control*	0.84	1.0	0.07	3.5	0.0	8.6	100
(M) only - Control No. 1	0.84	---	1.20	---	1.20	---	---
(M) only - Control No. 2	0.84	---	1.16	---	1.06	---	---

Key: (M) = *Microcystis aeruginosa*
(OCH) = *Ochromonas sp.*
(T) = Test compound

*Same algal components, but with no test compound

TABLE 21

INFLUENCE OF TEST COMPOUND NO. 114 AT 0.2 PPM ON THE PHAGOCYTIC ACTIVITY OF FOUR SPECIES OF *Ochromonas*

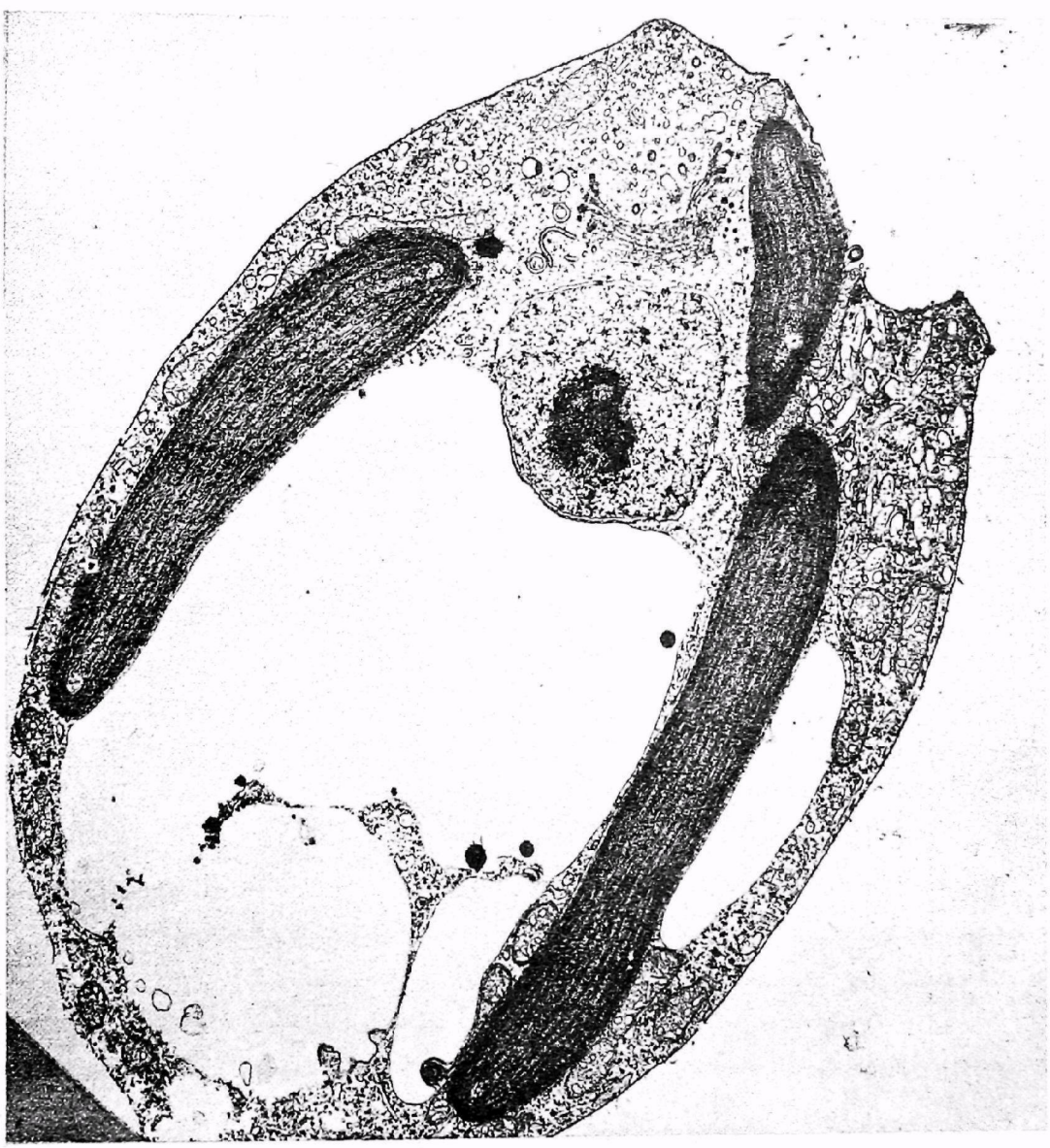
Species	CELL COUNTS						Percent Control
	0 - days		0 + 1 day		0 + 2 days		
	(M)x10^6	(OCH)x10^4	(M)x10^6	(OCH)x10^4	(M)x10^6	(OCH)x10^4	
O. bastrop+(M)+(T)	0.84	1.0	1.08	0.25	1.08	<0.1	<10
Control*	0.84	1.0	0.63	0.35	0.30	4.5	74
O. danica+(M)+(T)	0.84	1.0	1.07	0.20	0.97	<0.1	<10
Control*	0.84	1.0	0.42	0.40	0.53	2.6	54
O. malhamensis+(M)+(T)	0.84	1.0	0.88	0.20	1.13	<0.1	<10
Control*	0.84	1.0	0.90	0.50	1.10	0.45	<10
O. ovalis+(M)+(T)	0.84	1.0	0.91	0.25	1.05	0.10	<10
Control*	0.84	1.0	0.07	3.5	0.0	8.6	100
(M) only - Control No. 1	0.84	---	1.20	---	1.20	---	---
(M) only - Control No. 2	0.84	---	1.16	---	1.06	---	---

Key: (M) = *Microcystis aeruginosa*
(OCH) = *Ochromonas sp.*
(T) = Test compound

*Same algal components, but with no test compound

Figure 14

Ochromonas danica - Organelle Arrangement



worthy, as are the coiled or circular striated cylinders located between the Golgi bodies and the nucleus, also near the anterior end. These striated cylinders are found to often be associated with the contractile vacuole (Figure 15). The mitochondria surrounding the chloroplasts have distinctly dialated cristae (Figure 16). In this same figure the parallel-arranged bundles of microtubules around the outer periphery of the cell are evident. These cytoskeletal structures are thought to serve by affording rigidity and shape to the "naked" cells. A *Microcystis* cell is seen in close proximity at the lower right-hand corner of Figure 16.

In Figure 17 is shown an engulfed *Microcystis* cell within *O. danica*. The food vacuole seems to be distinct, at least initially from the normally present vacuole. We could expect it to be probable for these two vacuoles to be able to fuse. If this should be the case the prey may be captured in individual food vacuoles which secondarily merge into a common reservoir. It appears, from this photomicrograph, that the outer envelope of the ingested *Microcystis* seems to have been enzymatically disintegrated and that the discrete cell wall layer appears to be in the early stages of disintegration at localized regions.

From optical microscopic examination of combined *Ochromonas-Microcystis* cultures it appears that *Microcystis* cells are phagocytized by *Ochromonas* only when fortuitous contact is usually made, rather than by purposeful seeking. When contact is established, a definite adhesion to the gelatinous envelope seems to occur and engulfment is accomplished at that point. It is not uncommon to observe several *Microcystis* cells engulfed within a single *Ochromonas* cell at a time.

Figure 15

Striated Cylinders Associated with Contractile Vacuoles in *O. danica*

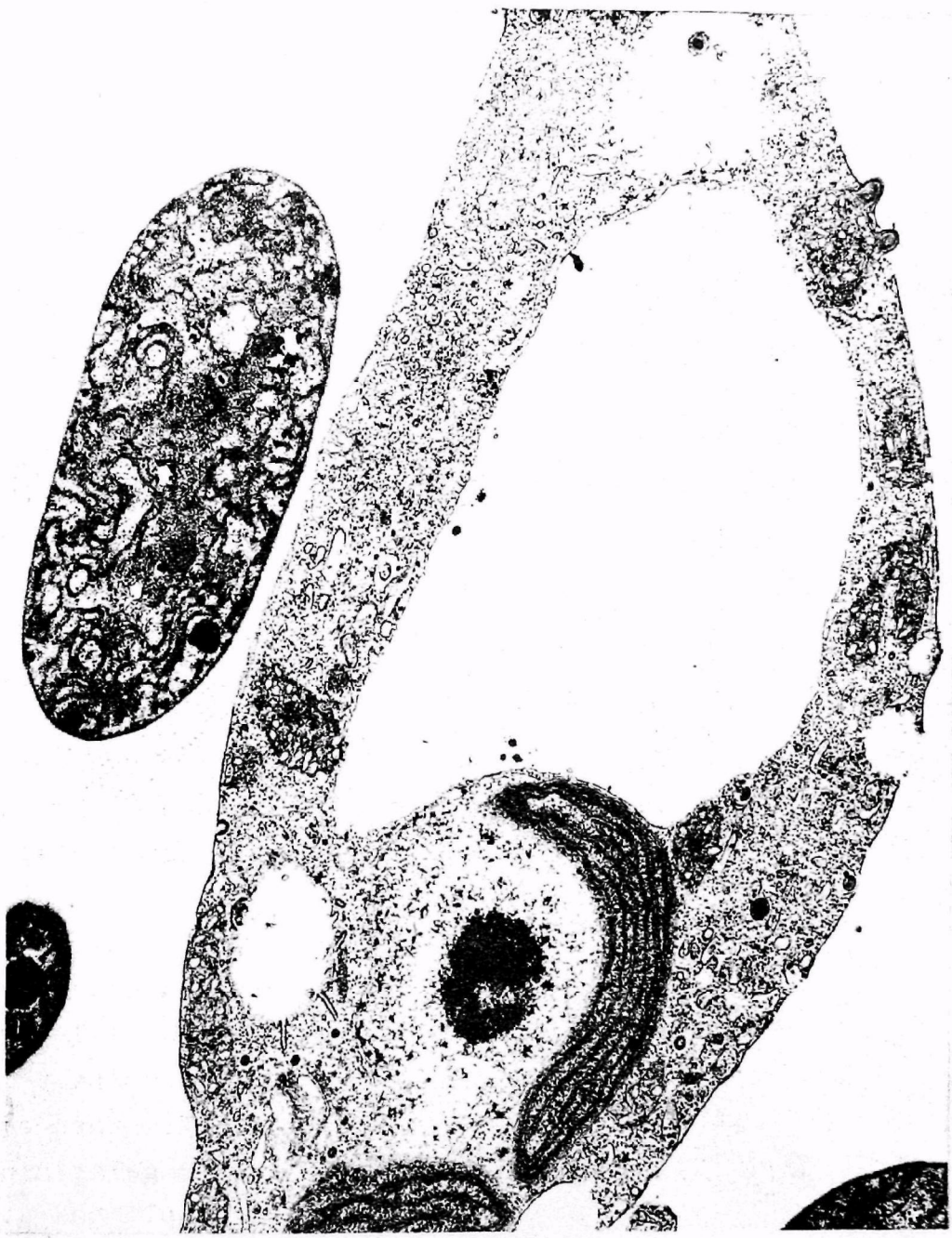


Figure 16

Ochromonas danica - Showing Mitochondria, with Dialated Cristae

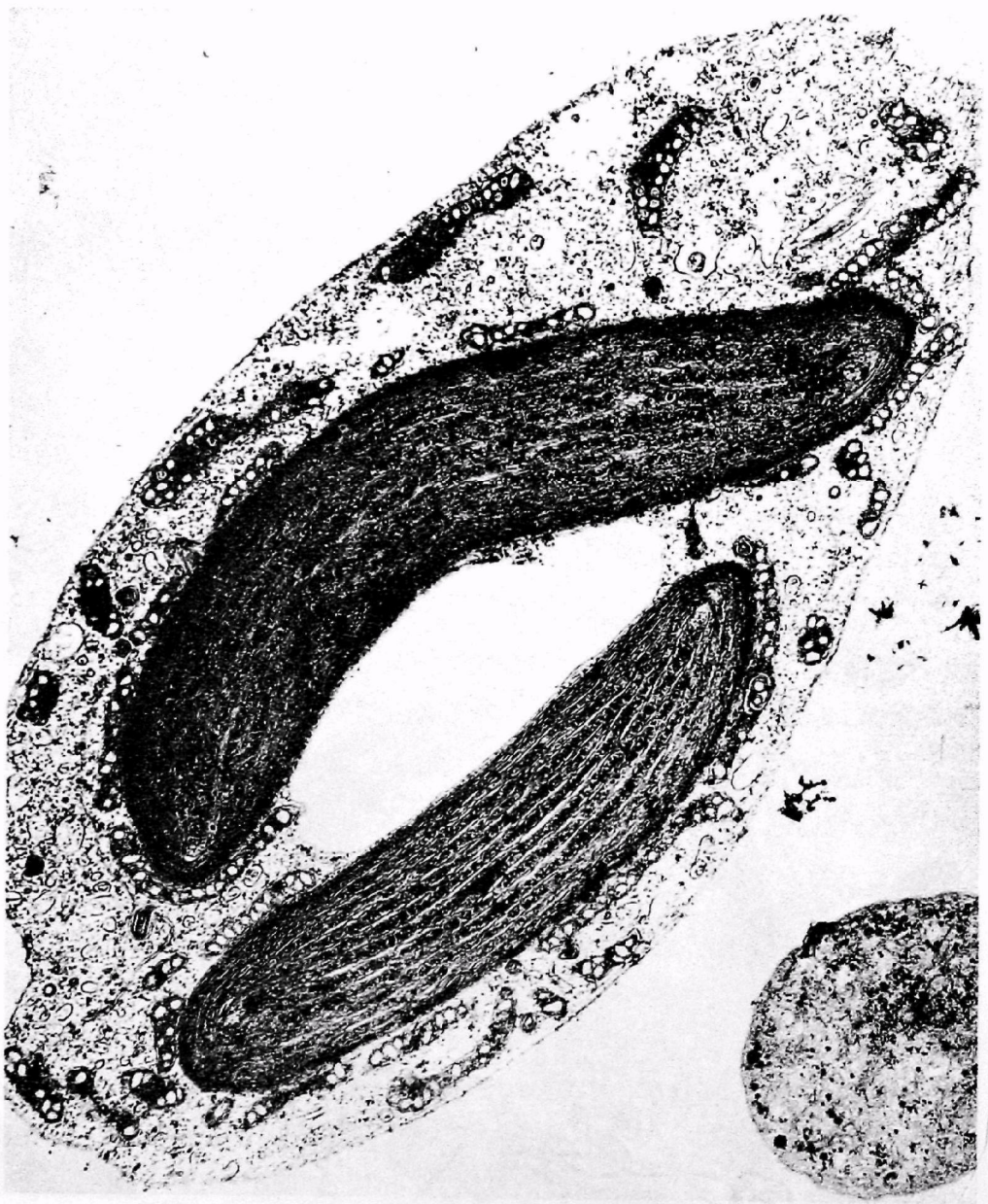
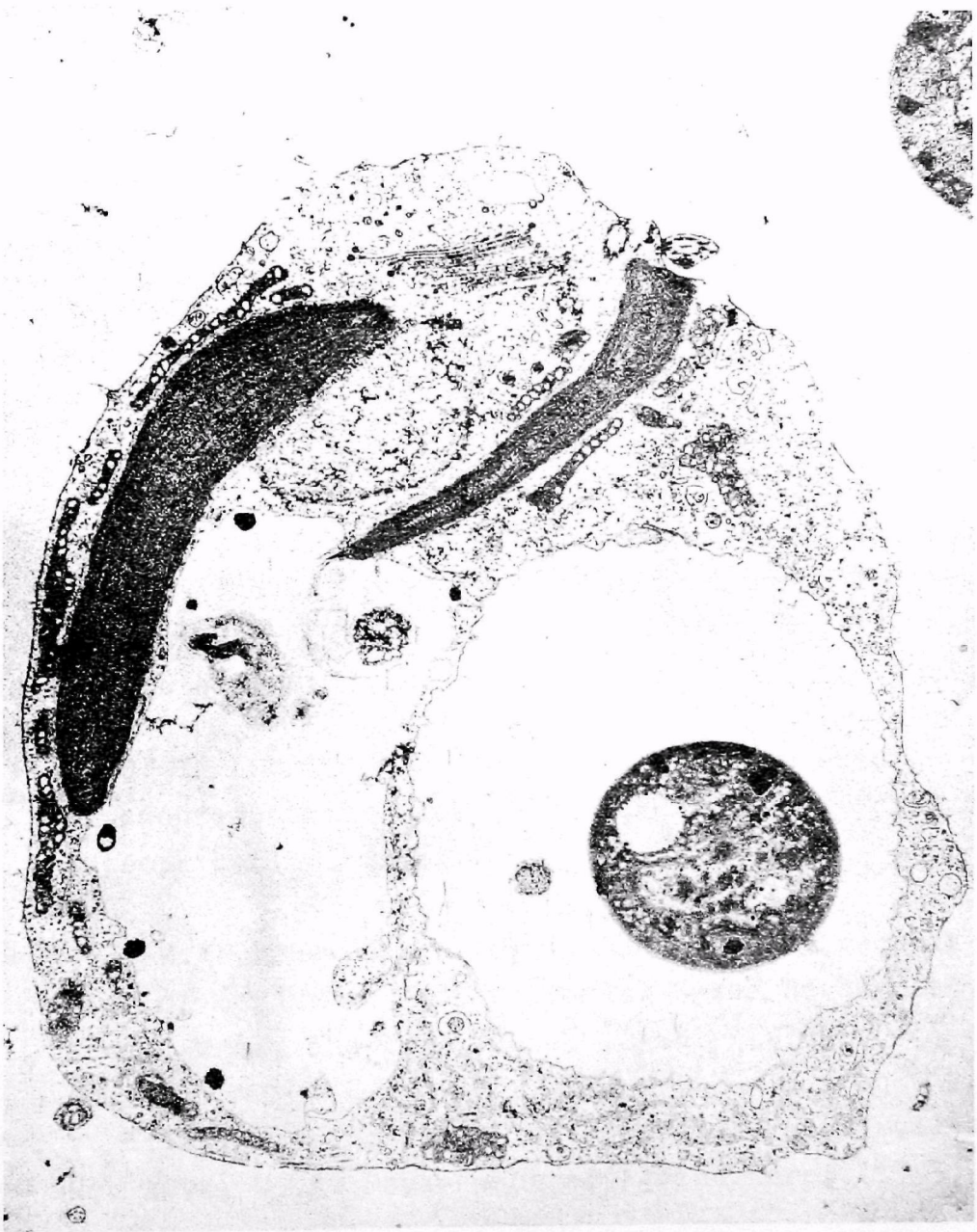


Figure 17

Transmission Electron Micrograph of *Ochromonas danica* Showing Ingested *Microcystis aeruginosa*



SECTION VI

ACKNOWLEDGEMENTS

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Drs. Michael J. Wynne and Gary Cole of The Department of Botany, University of Texas at Austin, served as consultants and aiding investigators on the biological control aspect of the project.

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SECTION VII

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SECTION VIII.

APPENDIX A.

Synergistic Activity Tests

TABLE 22 - LABORATORY SCREENING TESTS

Compounds No. 11 & 72 (equal quantities of each)

3,4,4',5',6-Pentachloro-2,2'-methylenediphenol and (m-Chlorophenyl)-2-thienyliodoniumchloride

	Microcystis aeruginosa			Anabaena flos-aquae		
	Initial Cell Count (Cells/mlx10^6)	Final Cell Count (Cells/mlx10^6)	Compound Activity (% Control)	Initial R.I.	Final R.I.	Compound Activity (% Control)
0.8 ppm						
Flask 1	1.0	0.0	100	0.27	0.12	68
Flask 2	1.0	0.0		0.27	0.12	
0.4 ppm						
Flask 1	1.0	0.0	100	0.27	0.43	0
Flask 2	1.0	0.0		0.27	0.40	
0.2 ppm						
Flask 1	1.0	0.26	75	0.27	0.47	0
Flask 2	1.0	0.40		0.27	0.47	
0.1 ppm						
Flask 1	1.0	0.94	35	0.27	0.32	0
Flask 2	1.0	0.86		0.27	0.32	
0.05 ppm						
Flask 1	---	---	---	---	---	--
Flask 2						
Control						
Control 1	1.0	1.41		0.27	0.38	
Control 2	1.0	1.39		0.27	0.35	

NOTE: R.I. = Relative Intensity

TABLE 23 - LABORATORY SCREENING TESTS

Compounds No. 70 & 73 (equal quantities of each)

2-Iodo-3,5-Dinitrothiophene and (p-Chlorophenyl)-2-thienyliodoniumchloride

	Microcystis aeruginosa			Anabaena flos-aquae		
	Initial Cell Count (Cells/mlx10^6)	Final Cell Count (Cells/mlx10^6)	Compound Activity (% Control)	Initial R.I.	Final R.I.	Compound Activity (% Control)
0.8 ppm						
Flask 1	1.0	0.0	100	0.27	0.28	10
Flask 2	1.0	0.0		0.27	0.38	
0.4 ppm						
Flask 1	1.0	0.0	100	0.27	0.40	0
Flask 2	1.0	0.0		0.27	0.39	
0.2 ppm						
Flask 1	1.0	0.0	100	0.27	0.42	0
Flask 2	1.0	0.0		0.27	0.41	
0.1 ppm						
Flask 1	1.0	0.27	80	0.27	0.35	0
Flask 2	1.0	0.36		0.27	0.36	
0.05 ppm						
Flask 1	---	--	---	---	--	--
Flask 2						
Control						
Control 1	1.0	1.41		0.27	0.38	--
Control 2	1.0	1.39		0.27	0.35	--

NOTE: R.I. = Relative Intensity

TABLE 24

COMPARATIVE ALGAECIDAL PROPERTIES OF VARIOUS FORMULATIONS OF TWO BATCHES OF SYNTHESIZED COMPOUND NO. 23 AGAINST *Anabaena flos aquae* (THREE-DAY TEST)

Components and ppm	Relative Intensity Initial Reading	Relative Intensity Final Reading	% Control
23-A-1.6 ppm[1]	0.24	0.12	100
23-A-0.8 ppm[1]	0.24	0.12	100
23-A-0.4 ppm[1]	0.24	0.25	60
23-X(old)-1.6 ppm[2]	0.24	0.10	100
23-X(old)-0.8 ppm[2]	0.24	0.15	89
23-X(old)-0.4 ppm[2]	0.24	0.21	71
23-X(10%-old)-1.6 ppm[3]	0.24	0.10	100
23-X(10%-old)-0.8 ppm[3]	0.24	0.16	86
23-X(10%-old)-0.4 ppm[3]	0.24	0.22	69
23-X(10%-new)-1.6 ppm[4]	0.24	0.11	100
23-X(10%-new)-0.8 ppm[4]	0.24	0.17	83
23-X(10%-new)-0.4 ppm[4]	0.24	0.23	66
$CuSO_4$*- 1.6 ppm	0.24	0.033	100
$CuSO_4$*- 0.8 ppm	0.24	0.058	100
$CuSO_4$*- 0.4 ppm	0.24	0.22	69
Control (no test chemical)	0.24	0.35	---

[1]Formulated with acetone and newly synthesized compound
[2]Formulated with an old Atlox-xylene-perchlor "use" concentrate
[3]Formulated with a previously synthesized compound (10% by weight in primary concentrate)
[4]Formulated with a newly synthesized compound (10% by weight in primary concentrate)
*$CuSO_4 \cdot 5H_2O$

SECTION VIII.

APPENDIX B.

Phagocytic Activity of *Ochromonas* Against *Microcystis aeruginosa*

TABLE 25

A FOUR DAY STUDY OF THE RATE OF PHAGOCYTIC ACTIVITY OF VARIOUS SPECIES OF *Ochromonas* ON *Microcystis aeruginosa*

Ochromonas Species	Initial		Final		Activity % Control
	Microcystis Cell Count X 10^6	*Ochromonas* Cell Count X 10^4	*Microcystis* Cell Count X 10^6	*Ochromonas* Cell Count X 10^4	
Ochromonas danica	1.5	1.3	0	0	100
Ochromonas malhamensis	1.5	8.0	0	6.4	100
Ochromonas minuta	1.5	7.0	6.1	3.7	0
*Ochromonas sp. (Freeport)	1.5	8.0	0	3.1	100
Control	1.5	---	5.9	---	---

*An unknown species found in the Freeport area

TABLE 26

EFFECT OF TEST COMPOUND ON OCHROMONAS ACTIVITY

Components Added	0 + 5 Days* (M) x 10^6	0 + 5 Days* (Och) x 10^4	0 + 6 Days (M) x 10^6	0 + 6 Days (Och) x 10^4	0 + 7 Days (M) x 10^6	0 + 7 Days (Och) x 10^4	0 + 8 Days (M) x 10^6	0 + 8 Days (Och) x 10^4	0 + 9 Days (M) x 10^6	0 + 9 Days (Och) x 10^4	0 + 12 Days (M) x 10^6	0 + 12 Days (Och) x 10^4
Test chemical + Ochromonas	1.4	0	0.25	0.2	0.09	1.0	0.1	1.0	0.05	5.0	0	2.2
Test chemical only	1.7	0	1.37	0	1.6	0	1.51	0	1.0	0	0.32	0
Ochromonas only	1.6	0	1.23	1.0	0.12	3.5	0.07	5.1	0.02	10.0	0	9.0
Control (Nothing added)	0.7	0	0.5	0	0.32	0	0.13	0	0.18	0	0.5	0

*Cultures of Microcystis were established and left to adapt under ambient conditions for first 5 days and then appropriate test flasks were inoculated with Ochromonas and others with test compound at 0.1 ppm.

TABLE 27 - OCHROMONAS ACTIVITY ENHANCEMENT TEST AT 0.1 PPM

Compound No. 11 - 3,4,4',5,6-Pentachloro-2,2'-methylenediphenol

Components Present	0 - Days (M) $X10^6$	0 - Days (OCH) $X10^4$	0 + 1 Day (M) $X10^6$	0 + 1 Day (OCH) $X10^4$	0 + 3 Days (M) $X10^6$	0 + 3 Days (OCH) $X10^4$
Test Chemical + (OCH)	1.05	0.55	0.08	5.5	0.0	5.8
Test Chemical	1.05	----	1.47	---	2.02	----
(M) + (OCH)	1.05	0.55	.17	5.3	0.0	6.15
(M) Only	1.05	----	1.43	---	2.06	----

$SIF_{1,3}$ = 1.2

TABLE 28

EFFECT OF TEST COMPOUND No. 21 ON *Ochromonas* ACTIVITY

Components Added	0 - Days		0 + 1 Day		0 + 2 Days		0 + 3 Days		0 + 4 Days		0 + 7 Days	
	(M) x 10^6	(Och) x 10^4	(M) x 10^6	(Och) x 10^4	(M) x 10^6	(Och) x 10^4	(M) x 10^6	(Och) x 10^4	(M) x 10^6	(Och) x 10^4	(M) x 10^6	(Och) x 10^4
Test chemical + Ochromonas	1.4	0	0.25	0.2	0.09	1.0	0.1	1.0	0.05	5.0	0	2.2
Test chemical only	1.7	0	1.37	0	1.6	0	1.51	0	1.0	0	0.32	0
Ochromonas only	1.6	0	1.23	1.0	0.12	3.5	0.07	5.1	0.02	10.0	0	9.0
Control (nothing added)	0.7	0	0.5	0	0.32	0	0.13	0	0.18	0	0.5	0

SECTION VIII.

APPENDIX C.

Studies of *Microcystis-Ochromonas* Interactions

by

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Studies of *Microcystis-Ochromonas* Interactions

One phase of this project was to survey available cultures of *Ochromonas* for phagocytic capabilities of other species besides the original Dow isolate, *O. ovalis*. Three species were obtained from the Indiana Culture Collection, namely, *O. danica*, *O. malhamensis*, and *O. minuta*. Also, a strain isolated from a pond at Bastrop, Texas, (*O. bastrop*) was being maintained in the teaching collection of The University of Texas. This last mentioned *Ochromonas* comes closest to *O. fragilis*, using Huber-Pestalozzi's Key (1941), but differs from that species in having stigmata (eyespots).

The three Indiana University species have been cultured in *Ochromonas* medium: *O. danica* yields excellent growth rapidly, *O. malhamensis* produces moderate growth, *O. minuta* grows very slowly and does not yield large quantities under the conditions utilized. The *Ochromonas* from Bastrop has been grown in 1/3 BBM-soil water, which is not a highly enriched medium in contrasts to the above *Ochromonas* medium.

Of these strains that have been examined besides the *O. ovalis*, only *O. minuta* (a relatively small-sized species) does not seem to phagocytize the *Microcystis*; no cases of ingested blue-green algal cells have been observed. *O. bastrop* proved to be the most voracious "feeder", often with several *Microsystis* cells ingested per *Ochromonas* cell. The cells are larger than those of *O. ovalis*. *O. danica* is also an efficient feeder, but typically only one blue-green algal cell will be ingested at a time. *O. malhamensis* also ingests blue-green algae but seems to be the least effective of the three.

Some difficulties have been encountered in obtaining good growth of the *Microcystis*. The initial results were poor to negligible growth using CG11 and BG10, two media used successively for other blue-green algae. The reason for this lack of good growth of *M. aeruginosa* is uncertain. But lately we have used Gorham (1X) medium, as has been done by Dow, and we are finally achieving better growth. Bubbling in a 3 percent CO_2-air mixture did not enhance growth.

We have hopes of carrying out ultrastructural studies involving the electron microscope, and preliminary results were encouraging in regard to the fixation-images of both the *Microcystis* and the *Ochromonas* (in this case, *O. danica*). We have delayed further fixations until we have achieved denser growth of *Microcystis*, since low concentrations of the blue-green alga would mean that few *Ochromonas* cells will have engulfed blue-greens.

A survey of the literature on the mechanism of phagocytosis (or endocytosis) is underway, this involving ultrastructural investigations. There has been extensive work recently but largely confined to the ciliates (*Tetrahymena* and *Paramecium*) and amoebae. We have come across nothing specifically on phagocytosis in *Ochromonas*, although some workers (as Bouck and Gibbs) have examined the cellular details of *Ochromonas*. So this area seems to be untouched and thus promising. Many ciliates have cytostomes in which a series of food vacuoles will be continually formed. The method in *Ochromonas* is different. Phase-contrast light microscopy demonstrates a very rapid engulfment, i.e., almost immediate engulfment when contact is fortuitously made. These species of *Ochromonas* do have large vacuoles and the blue-green algae seem to be deposited in these large, already present vacuoles rather than in special food vacuoles.

O. bastrop would at present seem to offer the best organism of the group studies on further analyses of interactions with *Microcystis*.

Some additional insight into the relationship between the phagocytic *Ochromonas* (utilizing *O. danica* in this study) and the engulfed *Microcystis aeruginosa* has been gained with transmission electron microscopy (Hitachi HU-11-E). A better understanding of the organelle arrangements within these two types of cells has also been achieved, and it seems worth-while to discuss certain of these aspects in this addendum to the original report submitted June 1, 1972. It might also be pointed out that attempts have also been made at uncovering three-dimensional details of these two cells by means of the "freeze-etch" device accompanied by transmission electron microscopy, but preliminary results are not yet considered to be of sufficient quality to include in this report. Yet that technique should provide us with a different perspective of the two cells, once the problems of fixation and preparation have been solved.

The following is a discussion of observations made on the accompanying electron micrographs (Figures 18-27).

Figures 18 and 19

In normal cells of *Ochromonas danica*, i.e., grown in axenic culture, a single large vacuole occurs in the posterior region of the cell. The arrangement of organelles is evident, with the nucleus situated at the anterior end of the cell and with usually two chloroplasts located on opposite sides of the cell. Note the density of vesicles in the anterior region of the cell, with their probable origin from a Golgi body occupying a conspicuous position at this pole. We are particularly interested in the coiled or circular, striated

Figure 18

Transmission Electron Micrograph of *O. danica* Showing Large Vacuole and Organelle Arrangement

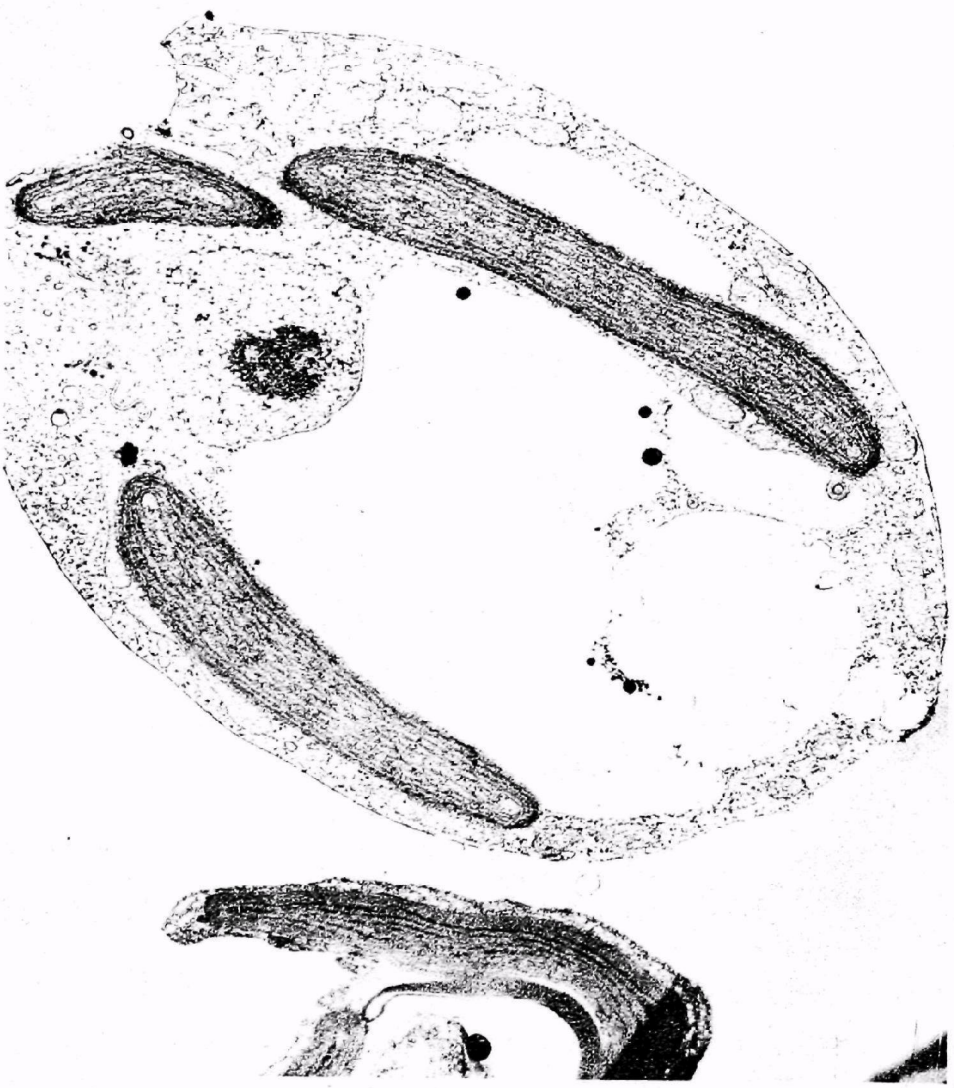
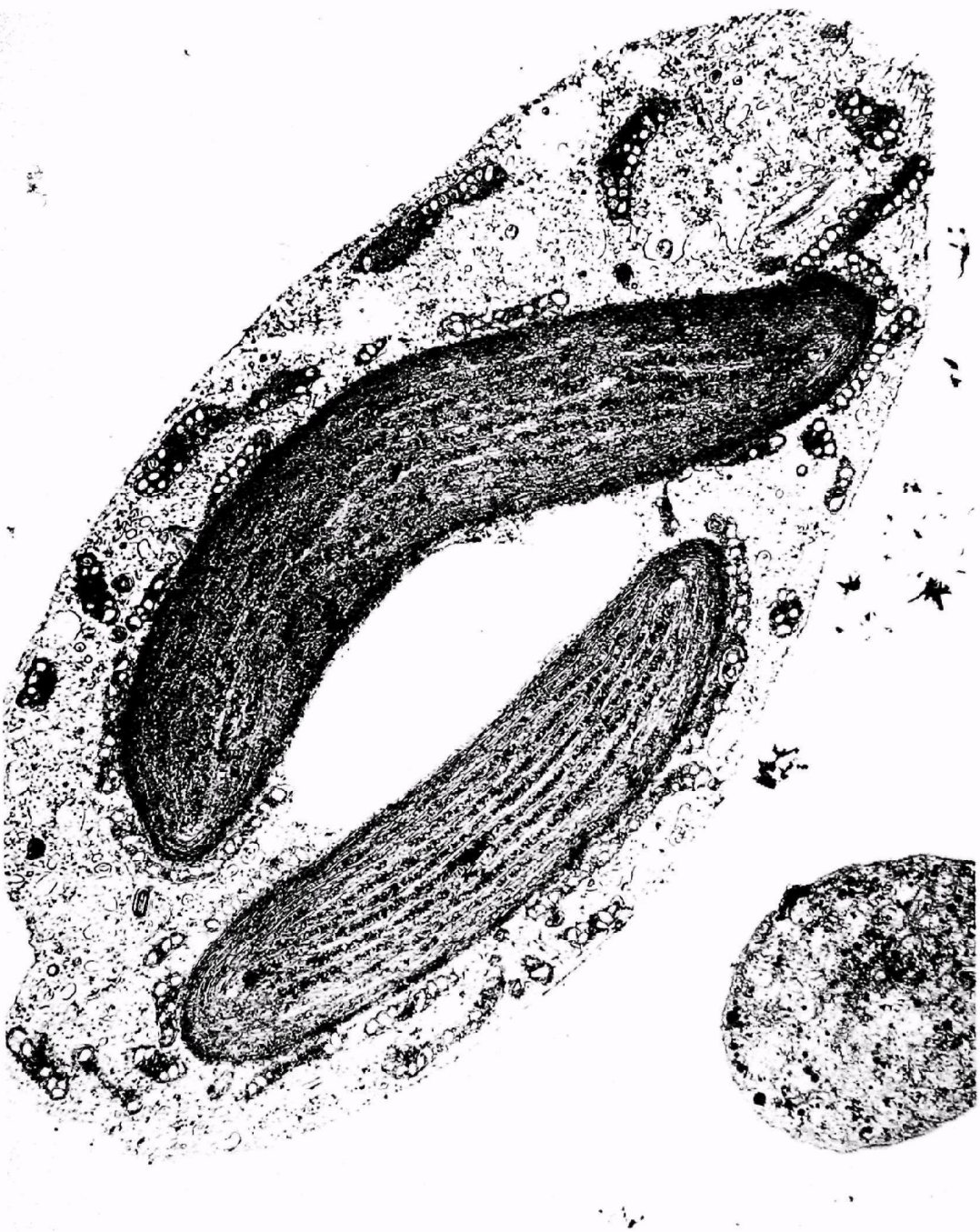


Figure 19

Ochromonas danica - Mitochondria, with
Dialated Cristae and Bundles of Microtubules



cylinders (Figure 18), which are at times associated with contractile vacuoles (Figure 22). The mitochondria (Figure 19) have distinctively dilated cristae, which at first were regarded as artifacts of fixation; however, Gibbs' study of mitosis in the same species (J. Phycol. 8(3), 1972) also portrayed these swollen cristae. This would suggest they are normal. In Figure 19 the parallel-arranged bundles of microtubules are evident. These cytoskeletal elements function to afford a rigidity to the shape of these naked cells. We have consistently observed these microtubules, which are particularly noticeable when they become concentrated at the two poles of the cell. We have not encountered any mention in the literature of their occurrence in these naked chrysomonads.

Figures 20 and 21

Normal cells of *Microcystis aeruginosa* grown in axenic culture. The fibrous nature of the nucopeptide envelope surrounding the thin multi-layered wall proper is faint though detectable. We often note osmiophilic (dark-staining) regions in these cells, which is an aid in their quick recognition, especially when they are later engulfed by the *Ochromonas*. The photosynthetic lamellae are loosely arranged which is characteristic of prokaryotic cells. A region of different density in the central portion may be chromatin.

Figure 22

The size relationship is seen in this figure. It is critical to catch the very earliest stages of contact and food vacuole formation, and this seems at the moment to be a fortuitous event of fixation. The process is rapid but should be possible to visualize also at the EM-level. The two evagination "blebs" on the opposite side of the cell are of uncertain

Figure 20

Microcystis aeruginosa Near Dividing Stage
Showing Mucopeptide Envelope

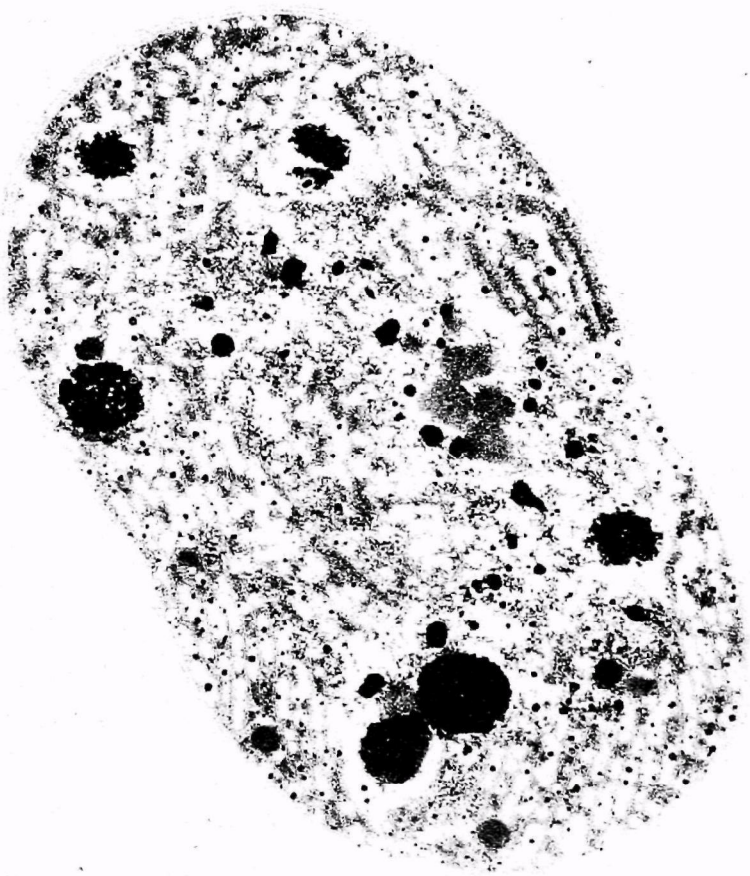


Figure 21

Microcystis aeruginosa - Depicting Photosynthetic Lamellae

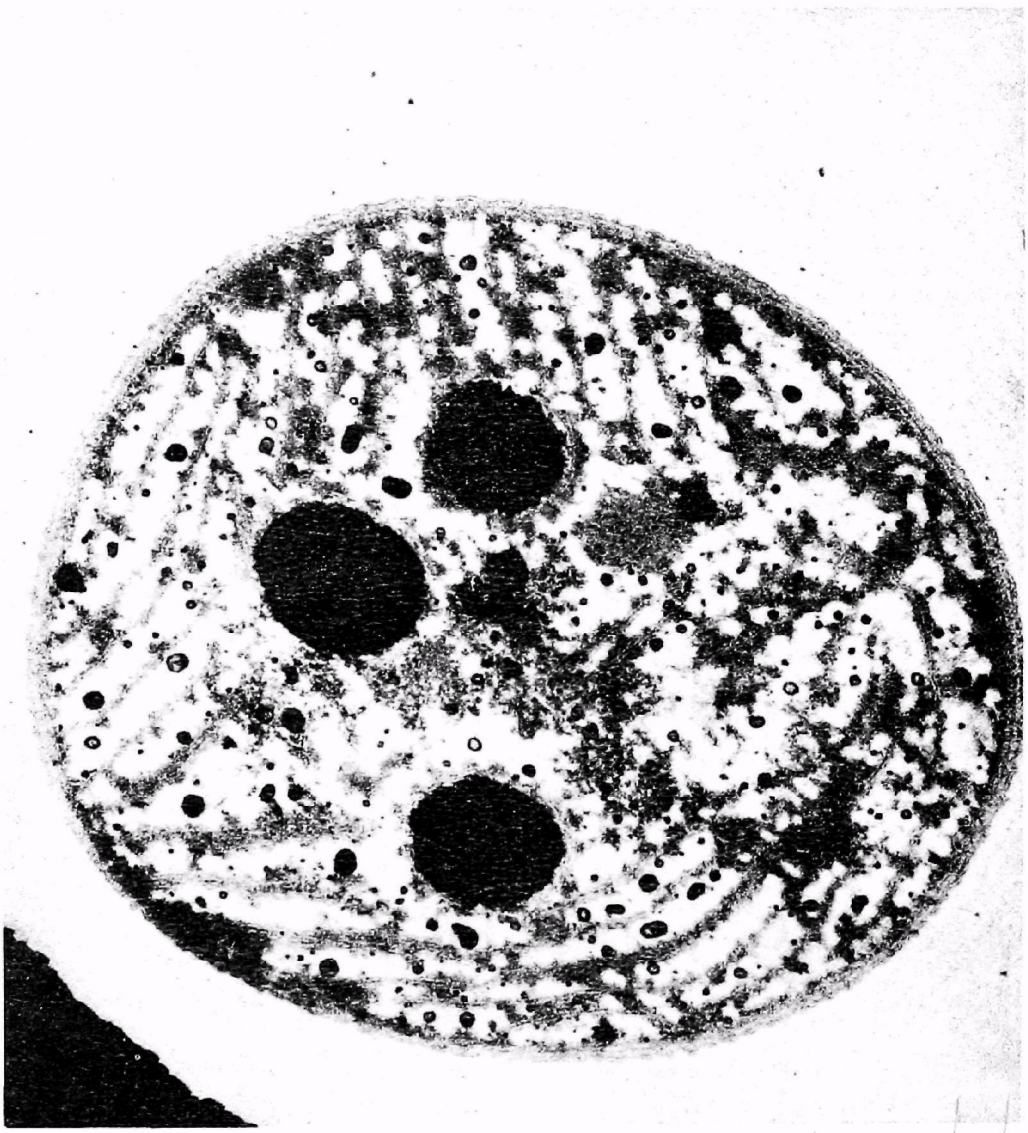
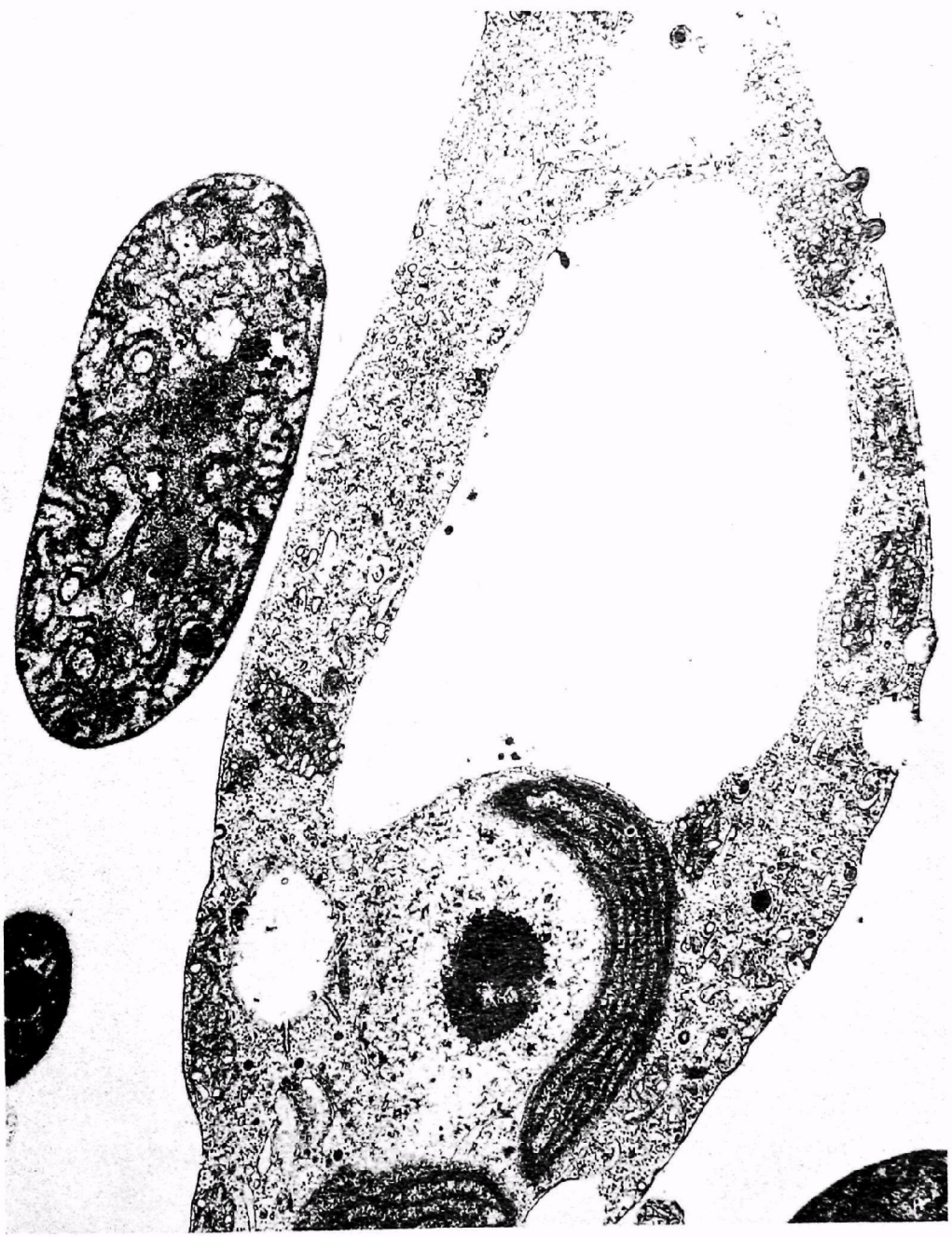


Figure 22

Ochromonas danica - Transmission Electron Micrograph
Showing Striated Cylinders and
Size Relationship to *M. aeruginosa*



function. The narrow striated cylinders seem to be feeding into the contractile vacuole near the nucleus of the *Ochromonas*.

Figure 23

Detailed view of a contractile vacuole of *O. danica*. In addition to numerous fusing cylinders, a large number of circular vesicles are in the immediate vicinity of the contractile vacuole.

Figure 24

Engulfed *Microcystis* within *O. danica*. Our observations indicate that the food vacuole is distinct, at least initially, from the normal present vacuole. This duality is also seen in Figure 27, in which the food vacuole contains bacteria from a contaminated culture. We would expect it probable for these vacuoles to be able to fuse. Perhaps the prey is captured in individual food vacuoles, which secondarily merge into a common reservoir. It is uncertain whether the material in the second vacuole of Figure 24 represents digested products of *Microcystis* or is a part of the *Ochromonas* cytoplasm. It might be mentioned that the envelope of the ingested *Microcystis* is no longer noticeable, and the discrete wall layer appears to be in the early stages of disintegration at localized regions.

Figure 25

Detail of *Ochromonas* cytoplasm. The proliferating Golgi bodies as well as the outer nuclear envelope, which extends out to surround the chloroplast(s), are both portrayed in this electron micrograph.

Figure 23

Ochromonas danica - Transmission Electron Micrograph
Showing Contractile Vacuole at Anterior End

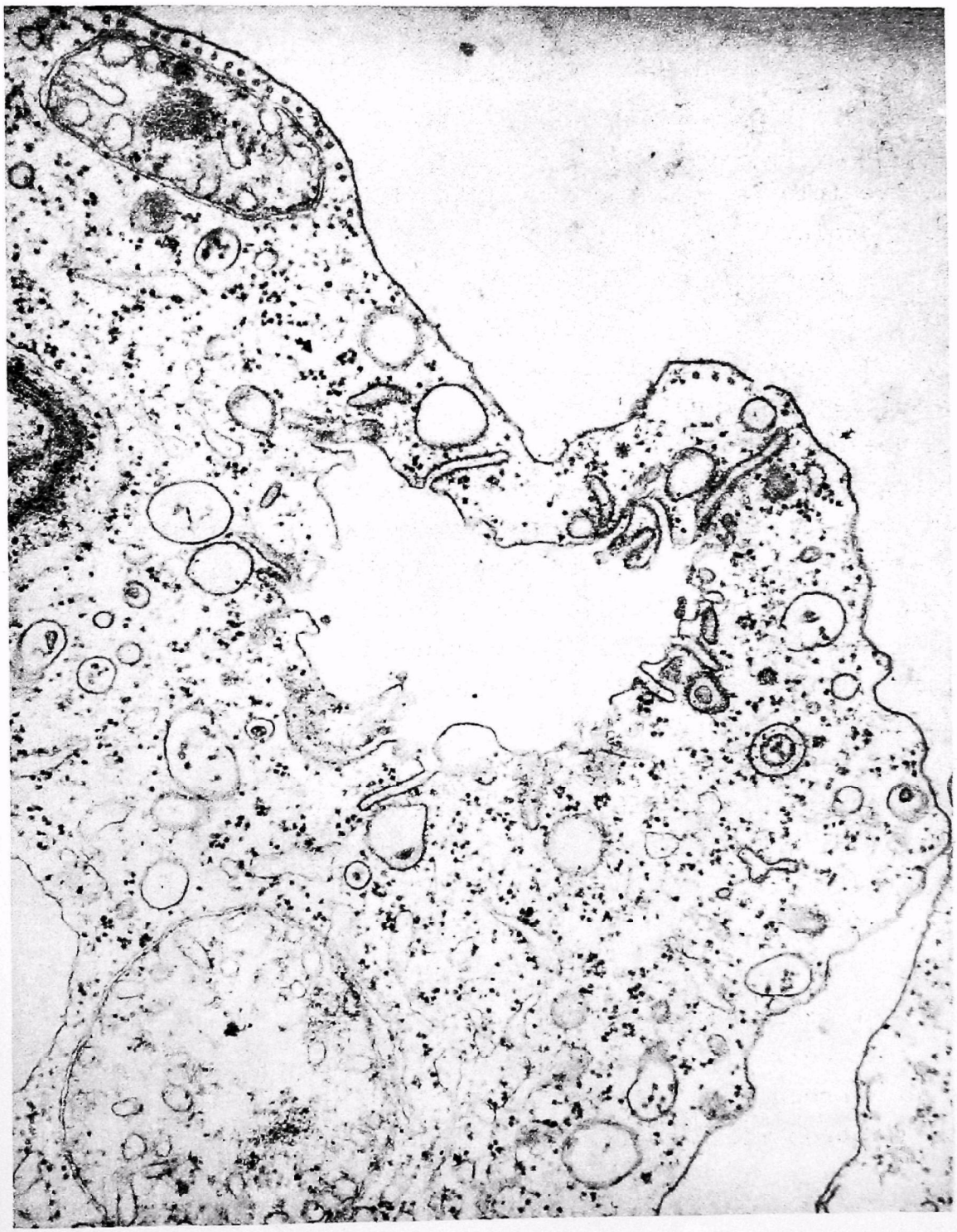


Figure 24

Ochromonas danica with Engulfed *Microcystis*

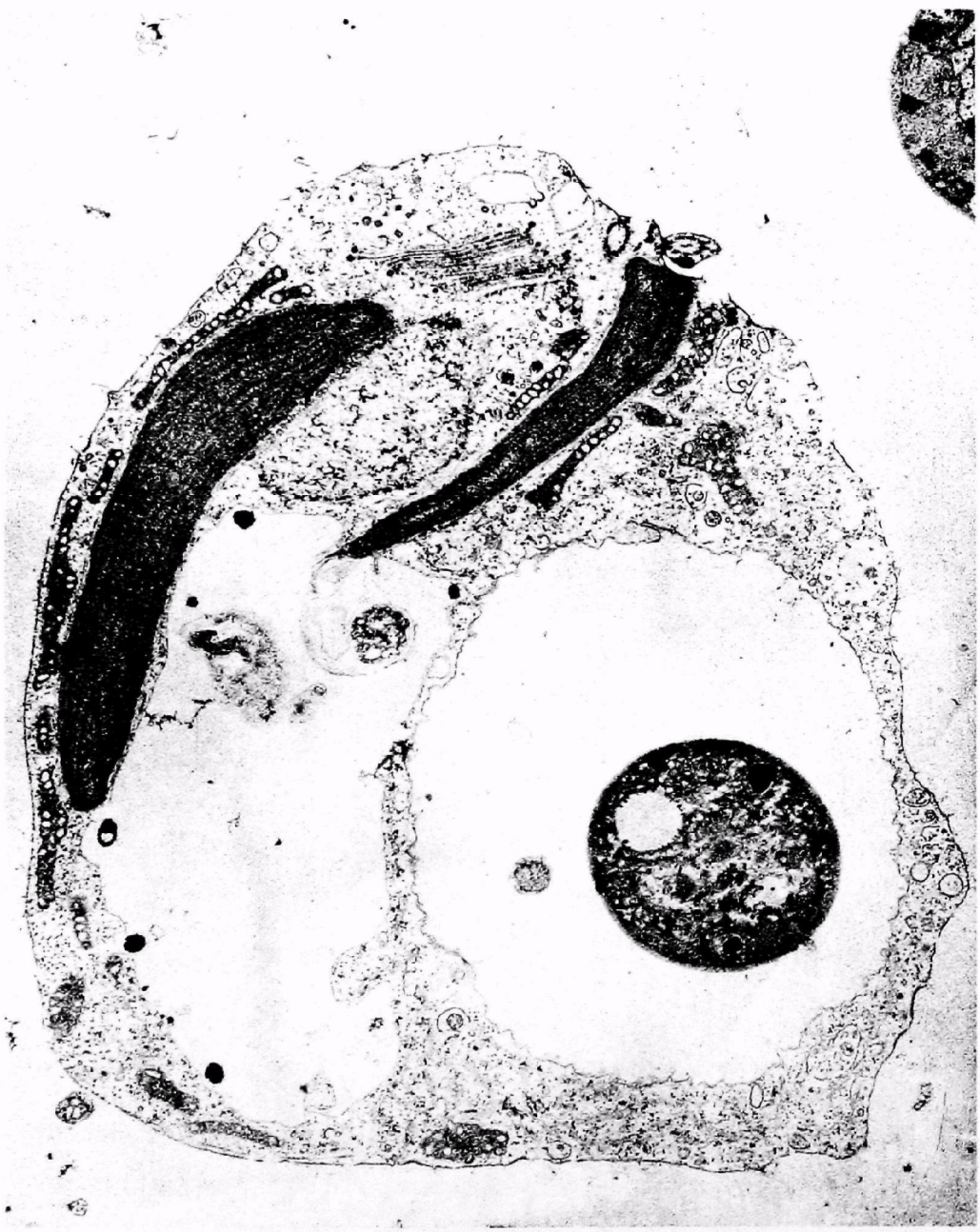


Figure 25

Ochromonas danica - Details of Cytoplasm

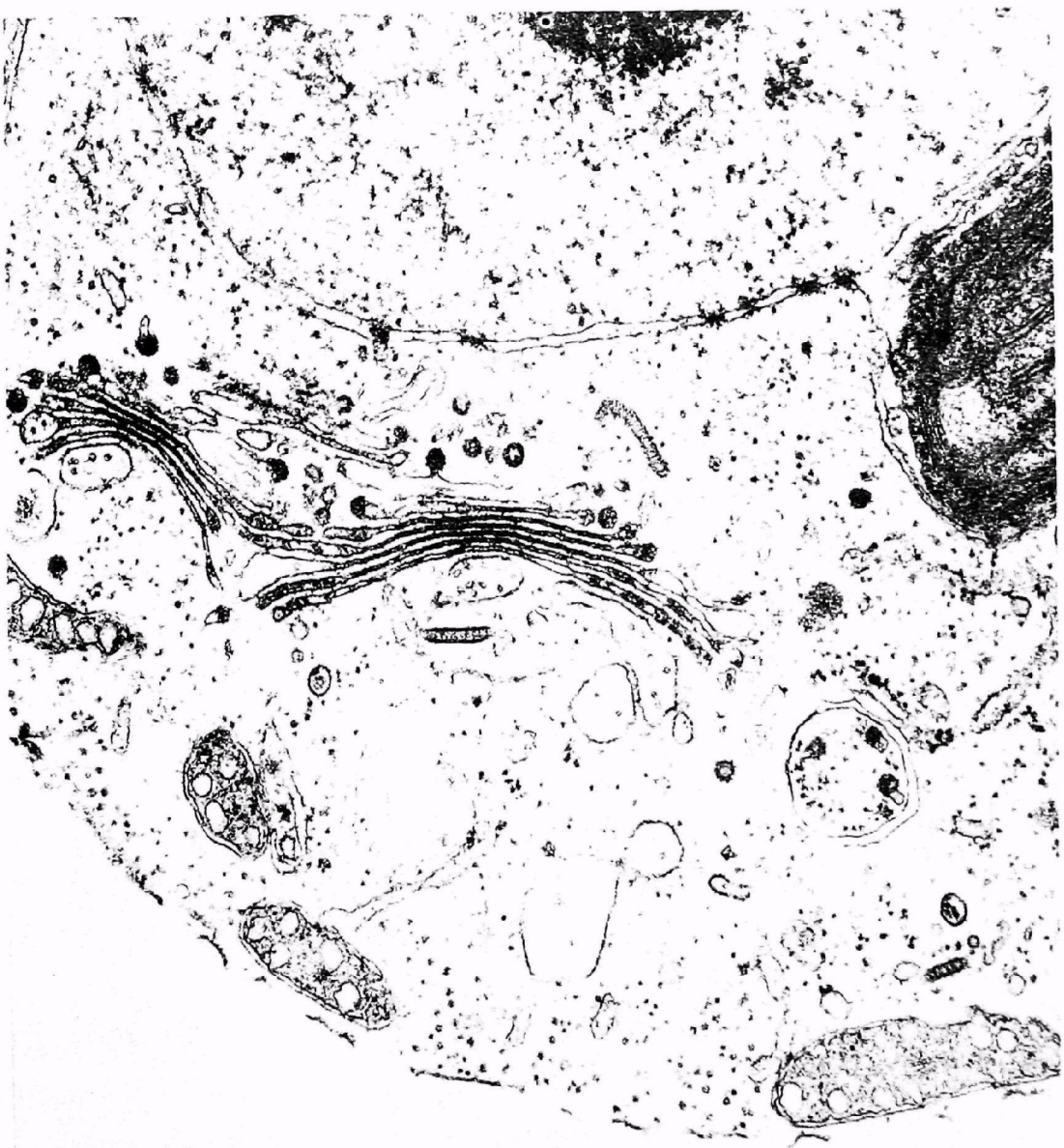


Figure 26

Microcystis contained with *Ochromonas*. The blue-green cell does not show obvious signs of digestion at this point, other than the absence of the fibrous outer envelope. We would expect there to be a production of lysosomes, which function in the digestion process. Some vesicles are present, but it may be too early for their abundance.

Figure 27

O. danica from a bacterized culture, with a food vacuole of bacteria. Two vacuoles are present, one with captured bacteria and the other without. In this particular tube we noted that the *Ochromonas* seemed to prefer the bacteria over the *Microcystis*, which was also added. Note the less dense appearance of the ingested bacteria than that of those on the outside of the cell.

The problem of obtaining dense cultures of *Microcystis* for inoculating into dense cultures of *O. danica* has been solved by simply growing the blue-green under less intense illumination. We can now achieve rapid and dense growth of *Microcystis*.

Figure 26

O. danica Containing Engulfed *Microcystis* with Outer Envelope Pre-digested

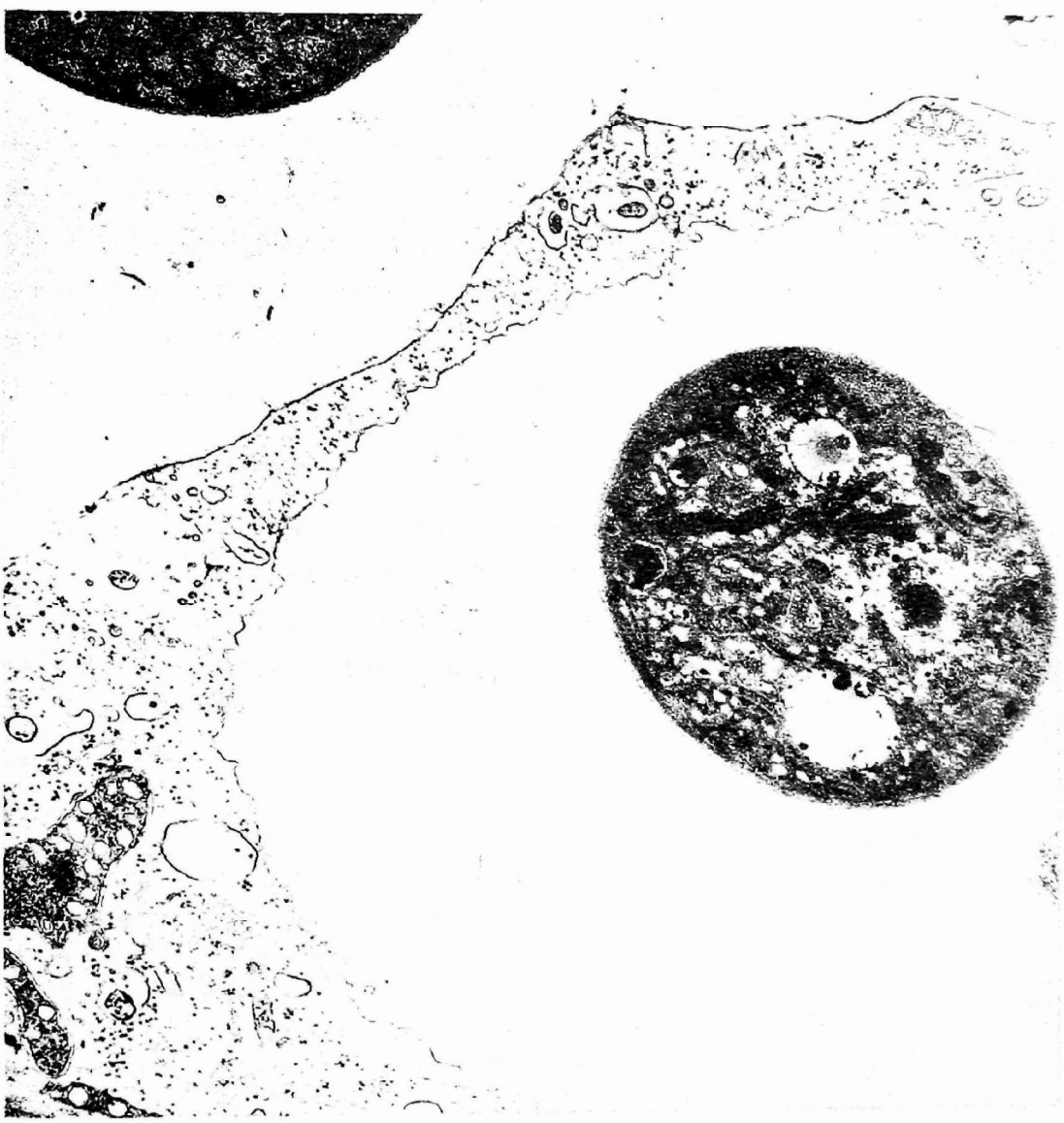
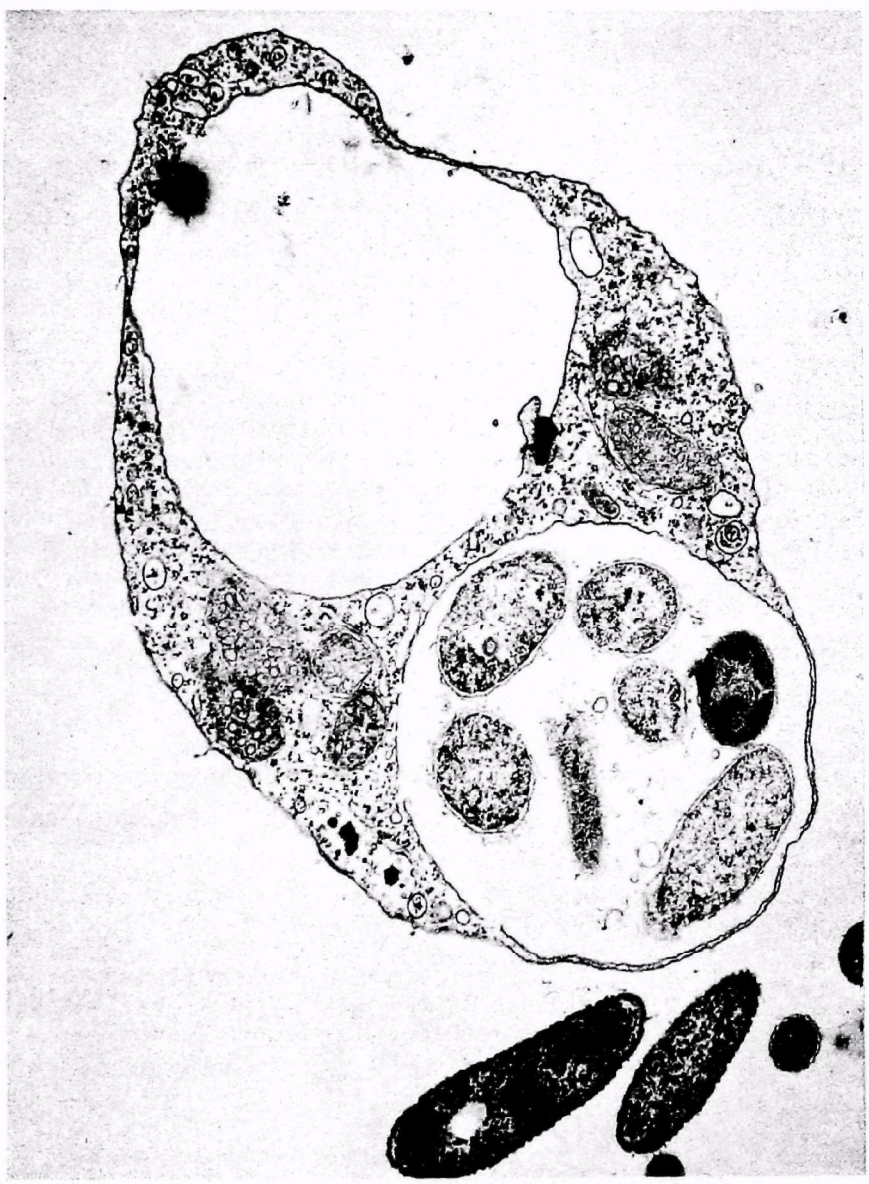


Figure 27

Ochromonas danica with Ingested Bacteria



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16. Abstract

The objective of this project was to develop a compound which would effectively and economically control the growth of nuisance species of blue-green algae with a minimum impact on desirable forms of life in the aquatic environment.

A computerized structure search of more than 100,000 compounds was made to select the analogs of the following four Phase I prime candidates: 2,5-Dichloro-3,4-dinitrothiophene; [5-Chloro-2-(p-nitrophenoxy)phenyl]phenyliodoniumchloride; 4-Amino-2,5-dibromophenylthiocyanate; and 1,1-Dimethyltetradecylamine, hydro-chloride. Through this endeavor 1309 compounds were selected, 41 of which emerged from a rapid, agar-plate screening as candidates for final laboratory screening tests.

A golden-brown flagellate, Ochromonas ovalis, which exhibited phagocytic activity against the blue-green alga, Microcystis aeruginosa, was discovered during Phase I.

Further research and development of biological-chemical control system included studies involving several species of Ochromonas and conditions which would enhance their phagocytic activity against Microcystis aeruginosa, with Ochromonas ovalis proving to be the most voracious feeder.

17a. Descriptors
*algicides, *selective chemical control, *algal control, *nuisance algal control, Cyanophyta, laboratory assay, biocontrol.

17b. Identifiers
*selective algicide, *blue-green algae, *Anabaena, *Microcystis, *analogs, test compound, phagocytic, selective screening, Ochromonas.

17c. COWRR Field & Group 05G

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