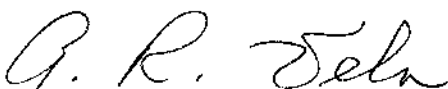A SCREENING OF FUNGI FOR METABOLITES INHIBITORY

TO THE GROWTH OF BLOOM-FORMING BLUE-GREEN ALGAE

APPROVED:
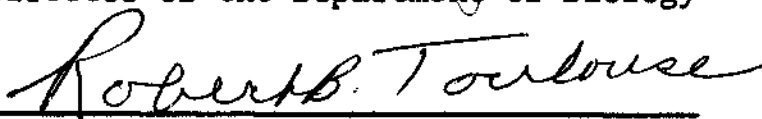
A. S. Kester
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Hardcastle, Ronald V., A Screening of Fungi for Metabolites Inhibitory to the Growth of Bloom-Forming Blue-Green Algae. Master of Science (Microbiology), December, 1970, 48 pp., 3 tables, bibliography, 33 titles.

Sixty-five fungal isolates representing thirty genera were grown for seven days in a mineral salts, yeast extract medium containing three per cent glucose. The filtrates from these fermentations were screened for an inhibitory effect on the growth of three bloom-forming algae, Anabaena flos-aquae, Aphanizomenon flos-aquae, and Microcystis aeruginosa, and a non-blooming strain of Anabaena flos-aquae. Preliminary screening was carried out by placing small amounts of the filtrates into shake flask or test tube cultures of each of the four algae and incubating them at room temperature. When screened under these conditions, eleven of the sixty-five fungal filtrates appeared to inhibit the growth of an alga. When these eleven filtrates were screened under controlled environmental conditions, none of them caused a noticeable inhibition of algal growth.

A SCREENING OF FUNGI FOR METABOLITES INHIBITORY

TO THE GROWTH OF BLOOM-FORMING BLUE-GREEN ALGAE

THESIS

Presented to the Graduate Council of the

North Texas State University in Partial

Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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Denton, Texas

December, 1970

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION

Currently, technology and expertise from many fields of endeavor are being directed, with an increased sense of urgency, to the task of controlling explosive growths of certain algae in man's water supplies. Ironically, the enterprises of man himself are rendering his lakes and reservoirs more susceptible to these algal "blooms". Industrial and domestic wastes contribute to the problem, and as Ohle (1953, 1955) noted, fertilized fields can yield nutrient-laden runoffs which enhance the growth of many kinds of algae, most noticably the bloom-forming kinds. (The term "nutrients" in this sense generally means nitrogen, phosphorous, and trace metals).

This investigation is confined to the bloom-forming Cyanophycean (blue-green) algae Microcystis aeruginosa, Anabaena flos-aquae, and Aphanizomenon flos-aquae. These organisms have proven to be the most frequent offenders in forming freshwater blooms. There are, however, other blue-green genera and indeed other divisions of algae (Chrysophyta, Chlorophyta) which contain organisms that grow to bloom proportions; as examples there are Oscillatoria rubescens (Pavoni, 1963),

Gleotrichia echinulata (Phinney and Peek, 1961), and Aster-
ionella formosa (Lund, 1959).

Many investigations of the various manifestations of
blue-green algal blooms have been made. Sylvester and Anderson
(1964) found Aphanizomenon flos-aquae and Microcystis aerugi-
nosa in so heavy a bloom that recreational facilities were coat-
ed and fouled beyond utility. Gerloff et al.(1950) recorded
aesthetically obnoxious blooms with these same two organisms.
Microcystis aeruginosa has been identified by Sawyer (1947),
Wohlschlag and Hesler (1951), and Singh (1953) as the pri-
mary organism in heavy blooms affecting the taste and odor of
drinking water. Sawyer et al.(1943) and Benoit and Curry
(1961) documented very odorous and foul tasting water result-
ing from blooms of Microcystis aeruginosa and Anabaena flos-
aquae. Aphanizomenon flos-aquae was also found comprising a
double bloom with Anabaena flos-aquae , as reported by Doma-
gella (1933). It was also noted in this investigation that
after eleven years of treatment with CuSO$_4$, the Aphanizomenon
had developed a resistance to the chemical which necessitated
a 400 per cent increase in dosage for effective treatment.

Objectionable odor and taste, and unsightliness are not
the only serious problems resulting from the blooms of blue-
green algae. Mackenthun et al.(1948) reported heavy fish mort-
ality resulting from the anaerobic conditions following the

decay of cells of an Aphanizomenon flos-aquae bloom, and

Prescott (1948) found Aphanizomenon flos-aquae, Microcystis

aeruginosa, and Anabaena flos-aquae to be the producers of

toxins which killed fish and cattle.

Control of blue-green algal blooms and their many attend-
ant complications can be approached from two avenues. The

first involves the elimination of excess nutrients from waters

bound for watersheds (such as effluents from sewage treatment

plants) or from the watersheds themselves. Aside from common-
sense practices like minimizing the runoff from farmlands and

planning adequate sewage treatment facilities, several tech-
niques have been proposed to eliminate excess nutrients. Mc-
Cauley (1963) proposed large-scale distillation of certain

types of effluents bound for watersheds to reduce the overall

level of bloom nutrients in those watersheds. Various chemical

agents such as $FeSO_4$, Al_2SO_43, Ca^{++} , and Mg^{++} were studied

by Rohlich (1961) with regard to their ability to coagulate

and flocculate phosphates. Henrickson (1963) proposed the

use of large-scale ion exchange devices for the removal of

nitrogen and phosphorous.

Sewage treatment plants accomplish the removal of much

NO_3 and PO_4 from waste water. These ions are first removed

in a settling tank by being caught in and on settling partic-
ulate material. Those ions remaining in the water are then

utilized by the growing microbial populations of trickling filters, anaerobic tanks, or oxidation ponds.

In some situations, removal of nutrients already in the watershed can be accomplished by sediment dredging, since, as elucidated by Holden (1961), a layer of lake bottom mud one centimeter deep may accumulate most of the phosphates in ten meters of water.

Possibly all of these techniques will find increased application in years to come. The extent to which this is true depends on economic and ecological factors. At this time, economic considerations prove prohibitive in most cases for techniques like distillation, dredging, and ion exchange methods. As for the use of metallic sulfates and ionic metals as flocculants and coagulants, very little is known about the environmental effects of these agents. Sewage treatment plants remove a portion of bloom-enhancing nutrients, but this is really incidental to the primary purpose of eliminating carbonaceous material; waste waters without a sufficient carbon source can not be treated in this way.

It thus appears that nutrient removal, while certainly a fecund area of research and development, is not likely to be the complete answer to blue-green algal blooms, if ever. The ultimate answer may be the propagation of some organism which

can be introduced to feed on algal cells. Until this happens, chemical agents are needed to deal with extant pre-bloom and post-bloom algal populations; this is a second avenue of approach for controlling algal blooms.

Treating a thick bloom with chemicals for the purpose of killing the cells is not always the most desirable approach since the decomposition of algal biomass can deplete dissolved oxygen and once again release inorganic nutrients for another, subsequent bloom. For those instances in which cell destruction would not necessarily precipitate renewed growth conditions (such as in a very light bloom) there are chemical agents which could be used to good advantage. Fitzgerald and Skoog (1954) successfully used the toxin 2, 3 dichloronaphthoquinone on blooms, with little discernible harm to the environment. Ullman et al. (1961) used arsenate with similar results.

Therefore, given excess nutrients, which should continue to be a problem for many years, and given a blue-green alga which under the right conditions could develop into an odiferous and foul-tasting bloom, it appears that a partial solution would lie in the development of a chemical which would specifically prevent the initial algal cells from multiplying, i. e., keep the bloom from developing. Thus, algal cell biomass would not be a problem.

Copper sulfate has been used as both a bloom toxin and a
bloom preventative for many years (Prescott, 1948). Nichols
(1946) demonstrated that CuSO4 could easily accumulate in
lake muds, and Hesler (1949) focused on ecological dangers
inherent in prolonged use of this salt for controlling blooms.

Thus, the need for a bloom-preventing chemical agent
with few or no undesirable effects provided the impetus for
this investigation; its direction was a search for a fungal
metabolite that would do this job. The rationale for choosing
this direction is based on two biological facts. First, fungi
as a whole elaborate metabolic products of many kinds, often
in great excess. As examples there are the commercially pro-
duced antibiotics penecillin and fusidic acid. The fungi also
produce alkaloids (such as ergot by Claviceps prupurea, 1-
ephedrine by Ephedra sinica, and the deadly poison of Aman-
ita mushrooms), vitamins, organic acids (citric, lactic, ita-
conic, kojic, and over 40 others), aldehydes, esters, nitro-
genous compounds, chlorine and sulfur compounds, and several
pigments (such as b-carotene by Blakeslea trispora) (Gray,
1958). Therefore it seems reasonable to assume that the bio-
synthesis of an antialgal metabolite would lie within the
capabilities of the fungi.

The second justification for screening fungi for anti-

algal metabolites involves the antibiotic phenomenon. Many microorganisms produce antibiotics, and the possibility exists that fungi growing in an aqueous environment could produce such an agent in response to a thickening concentration of algal cells. Indeed, in eutrophic waters containing high concentrations of carbonaceous and inorganic nutrients (which support populations of fungi and blue-green algae, respectively), such a condition could arise.

CHAPTER II

METHODS AND MATERIALS

All fungal cultures were isolated from samples of raw sewage, lake and pond water, shore mud, lake and pond bottom mud, and dry soil. These samples came from several watersheds and surrounding areas in Denton County, Texas.

Water samples were spread undiluted in triplicate on Petri plates of Sabouraud's agar (Difco) to which had been added 0.035 per cent of rose bengal dye and 0.035 per cent of tetracycline to inhibit the growth of rapid-spreading fungi and bacteria, respectively. Samples of sewage, dry soil, and mud were serially diluted and spread in triplicate on plates of this modified Sabouraud's agar.

The plates were incubated at room temperature for three or four days, after which time discrete colonies could be seen. Selection of isolates was made on the basis of differences in colonial color and texture, of the color of metabolites in the agar, and on microscopic structures. Fungal colonies chosen for isolation were transferred to plates of peptone-glucose-acid agar (PGA), (Difco) repeatedly until they appeared to be in axenic culture. The isolates were then inoculated onto

duplicate slants of PGA agar in 20 mm x 160 mm screw-cap test tubes and stored at room temperature. After these slant cultures had sporulated they were prepared to serve as an inoculum for fermentation shake flasks.

Fungal cultures were prepared by inoculating each isolate into 50 ml of medium consisting of 97 per cent L-salts solution (Leadbetter and Foster, 1958), 3.0 per cent glucose, and 0.05 per cent yeast extract, in a cotton-stoppered 250 ml Erlenmeyer flask. Transfer involved introducing 10 ml of sterile 3 per cent saline solution into the stock slant, dispersal of spores and/or mycelial particles into suspension with a sterile inoculating needle, and then pipetting 2 ml of this suspension into the flasks of complex fermentation medium. The inoculated flasks were placed on a rotary shaker (Eberbach) set at 145 rpm. Fermentation was carried out for seven days at room temperature, disregarding any light effects which might be caused by normal use of the ceiling lamps. The mycelium of each flask was then filtered off with a Buchner funnel, using no. 3 Whatman filter discs, and the filtrates placed in labelled screw-cap test tubes and stored in the freezer of a refrigerator until used for screening for the presence of an antialgal metabolite.

The blue-green algae _Microcystis aeruginosa_ and _Aphanizomenon flos-aquae_ were obtained in unialgal culture (pure) from

J. T. Wyatt, Biology Dept., North Texas State University, as
isolates from lakes in north central Texas, and have been
identified as bloom-forming strains by Wyatt (1969). Also
supplied by Wyatt were unialgal cultures of a bloom-forming
Anabaena flos-aquae, NRC no. 44 (Gorham et al., 1964), and a
nonblooming Anabaena flos-aquae from the Indiana University
Culture Collection, labelled IUCC no. 1444 (Starr, 1964).
(Henceforth this A. flos-aquae will be referred to simply as
" 1444" to distinguish it from the NRC Anabaena flos-aquae).
While 1444 is not a bloom former, it has been the subject of
several studies on algal physiology (Tischer, 1965; Davis
and Tischer, 1966) and was included in this investigation to
tie in any results to existing data and conclusions.

ASM-1 medium of Gorham (1961) was used for all algal
growth experiments.

Stock cultures of each alga were kept in 50 ml of ASM-1
medium in cotton-stoppered 250 ml Erlenmeyer flasks, on a
shelf in the laboratory where light was subdued.

Since all experiments required relatively dense algal
inocula, ASM-1 flasks of each of the four algae were kept
in the "log phase" of growth in a growth chamber at all times;
in this manner, an inoculum of a desired concentration could
be obtained by proper dilution from a higher concentration.

In order to determine the optimum age (measured as O. D.) of inoculum for all algal growth curve experiments, triplicate shake flasks were prepared for each of the four algae. Each flask, containing 48 ml of ASM-1 medium, was inoculated with 2 ml of the proper stock culture on hand. (No regard was given to the cell concentration of this preliminary inoculation, as variations in cell concentration would affect only the speed of peak growth attainment, not the characteristic peak itself.) Cultures incubated until they had reached stationary growth phase. (At the end of 14 days, all four algae had done so.)

In determining the best percentages of inocula for all subsequent algal growth curves, algal cell suspensions of the optimal optical densities were introduced into duplicate shake flasks as 1 per cent, 3 per cent, and 10 per cent of the total 50 ml. Incubation proceeded for 12 days. Growth was measured daily.

For determining the effects of raw fungal medium on the growth curves of the algae, three levels (5, 3, and 1 ml) of three dilutions (1/10, 1/20, and 1/50) of the raw fungal medium were introduced into duplicate, freshly inoculated algal shake flask cultures. Incubation of these cultures proceeded until their respective stationary growth phases were reached, or until chlorosis occurred. After 12 days, all experiments were ended. (All algae had reached stationary growth at the end of this time.)

The growth chamber for all algal experiments requiring controlled environmental conditions was a "reach-in" incubator (Hythermco, Pennsauken, N. J.), with inside measurements of 2' x 4' x 6', which had been equipped with six cool-white 40-watt fluorescent lamps mounted vertically, two on each lateral wall and two on the rear wall, and with a light-dark cycle timer. A reciprocal shaker (Eberbach), set at 110 complete strokes per minute with four inches of stroke travel, was placed on the floor of the chamber. At the level of the shaker platform, when unloaded, uniform light meter readings of 275 f. c. were made.

The growth chamber was scheduled for sixteen hours of light and eight hours of darkness. Temperature in the chamber was set at 26° C. The light cycle maintained this temperature, while the temperature during the dark cycle dropped to about 22° C.

Quantitation of all algal growth was done with a Spectrometer 20 spectrophotometer set at 640 mu, with uninoculated ASM-1 medium used as blank.

Screening of all fungal filtrates for an inhibitory effect on the growth of the four algae was done first on a preliminary level; those which indicated a positive test here were screened on a confirmatory level under more controlled conditions.

Although it would have been desirable to perform all screening experiments under the controlled environmental conditions of the growth chamber, the limited capacity of the shaker in the chamber made it necessary to reduce the number of filtrates so screened to those which had demonstrated inhibition of algal growth in a preliminary screening which allowed a large number of filtrates to be tested.

The preliminary screening of fungal filtrates nos. 16 through 65 was accomplished by placing 1 ml of a 1/20 dilution of the filtrates into duplicate cotton-stoppered 250 ml shake flasks containing 45 ml of ASM-1 medium, and 5 ml of algal inoculum of a chosen O. D. These flasks and control flasks, containing no filtrate, were incubated at room temperature on a rotary shaker (Eberbach) set at 130 rpm. Illumination was accomplished with two banks of 40-watt coolwhite fluorescent lamps. Four lamps, two inches apart, were on each bank. A bank was placed 18 inches from each long side of the machine, parallel to that side. Sheets of heavy-duty aluminum foil were shaped to curve up and over the upper level of the double-deck shake platforms, to help equalize the light striking both decks. This apparatus, assembled on the laboratory floor, was scheduled with sixteen hours of light and eight hours of darkness. This schedule was casual. Light meter

readings indicated that the outside rows of the shaker were receiving, unloaded, about 200 f. c. and the center rows were receiving about 180 f. c. Laboratory ceiling lights were used as needed, though generally the flasks did receive the full eight hours of darkness.

Visual examination of test and control flaks were made daily to determine possible effects of fungal filtrates on algal growth.

Fungal filtrates nos. 1 through 15 were preliminarily screened by being introduced into duplicate, freshly inoculated algal cultures in 16 mm x 120 mm screw cap test tubes. All amounts were scaled down to 1/5 the amounts used with the shake flask preliminary screening, i. e., amounts of ASM-1 medium, algal inoculum, and fungal filtrate. Control tubes were prepared with no fungal filtrates. All treatment and control tubes were put in test tube racks and placed in a four-foot-square area on a lab table. A gooseneck lamp with a 75-watt bulb was placed at each corner of this area. These lights stayed on for the duration of the screening, and ceiling lights were used as needed without regard for the experiment. Once a day the tubes were vigorously shaken for aeration, and inspected visually by comparing treatment tubes with control tubes.

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CHAPTER III

RESULTS

The 65 fungal isolates, the fermentation filtrates of which were screened in this investigation, were identified to the genus level according to the keys of Gilman (1957), Barnett (1960), and Cooke (1963). The genera tested are listed in table 1.

TABLE I

GENERA OF FUNGI USED IN SCREENING FOR ANTIALGAL METABOLITES

Genus	Number of Different Strains	Genus	Number of Different Strains
Absidia	1	Clasterosporium	1
Alternaria	1	Cunninghamella	1
Aspergillus	8	Fusidium	1
Brachysporium	1	Fusarium	1
Basidiobotrys	1	Gleocladium	2
Candida	2	Helminthosporium	2
Chaetomium	1	Hirsutella	1
Cladosporium	1	Hormiscium	2

TABLE I --Continued

Genus	Number of Different Strains	Genus	Number of Different Strains
Hormodendrum	2	Phialophora	1
Lunulospora	1	Pullularia	1
Moeszia	1	Sclerotium	1
Monilia	1	Scopulariopsis	1
Mucor	2	Trichoderma	2
Oidium	1	Verticillium	2
Penecillium	13	unidentified	11

In working with the algae, it was first necessary to determine normal growth curves under the controlled conditions of the growth chamber. The first experiment was designed to determine the characteristic point of the stationary phase of growth, or "peak", for each alga, measured as O. D. Finding this characteristic peak under controlled conditions would provide reference points for choosing standard inocula for all subsequent experiments, so that reproducible growth curves could be obtained. Peaks of each organism and inoculum O. D. chosen are given in Table II. These peaks occurred on different days. (As discussed later, each alga peaks out after a characteristic number of days when the inoculum has been standardized.)

TABLE II

CHARACTERISTIC PEAK GROWTH OF ALGAE, AND INOCULUM
CONCENTRATIONS CHOSEN, MEASURED AS O. D.

Organism	Peak Growth O. D. (Replication no.)			Mean Peak Growth O. D.	Inoculum O. D. Chosen
	1	2	3		
"1444"	0.760	0.760	0.760	0.760	0.500
Aphanizomenon flos-aquae	0.310	0.310	0.320	0.314	0.275
Anabaena flos-aquae	0.500	0.510	0.505	0.505	0.400
Microcystis aeruginosa	0.980	0.950	0.970	0.960	0.500

The inoculum O. D. for each organism (see Table II) was chosen in light of the need to have initial cells which were undergoing rapid multiplication in the culture from which they came.

An experiment was next performed to determine the optimum percentage of inoculum of the chosen O. D. the need here was a standard inoculum percentage which could cause the alga to enter the " log phase"of growth almost immediately. (As stated earlier, the best measure against algal blooms would seem to be an agent administered to pre-bloom cells which would prevent them from multiplying. For this reason it was believed that an immediate " log phase" growth level in those cultures

being tested with fungal filtrates would provide the quick-
est way to demonstrate an active substance in the filtrate
which would caused cell multiplication to cease.) In each
case, as seen in Figure 1, the 10 per cent inoculum gave an
immediate and prolonged log phase; for this reason it was
chosen as the percentage of inoculum for each of the algae
in all subsequent experiments.

The next experiment was designed to establish the high-
est level of L-salts-glucose-yeast extract medium which the
four algae could tolerate with no ill effects on the normal
course of their growth curves. Then in screening experiments,
the filtrates from the fungal fermentations were administered
to the algal shake flasks (at the time of inoculation) in
an amount equal to the maximum level of raw medium toward
which the algae had shown tolerance. This approach was taken
in light of the assumption that at least some of the fungal
fermentations would use very little of their L-salts-glucose-
yeast extract medium, and that any inhibition of algal growth
under these circumstances could be due to the fermentation
medium itself and not a substance synthesized by the fungus.

The highest concentration of this fungal medium which
all four algae could tolerate best was 1 ml of a 1/20 dilut-
ion (see Figures 2, 3, 4, and 5). Therefore in screening
experiments, each treatment flask received 1 ml of a 1/20

Figure 1. Growth curves of all four algae, each with inoculum percentages of 10 % (A), 3 % (B), and 1 % (C).
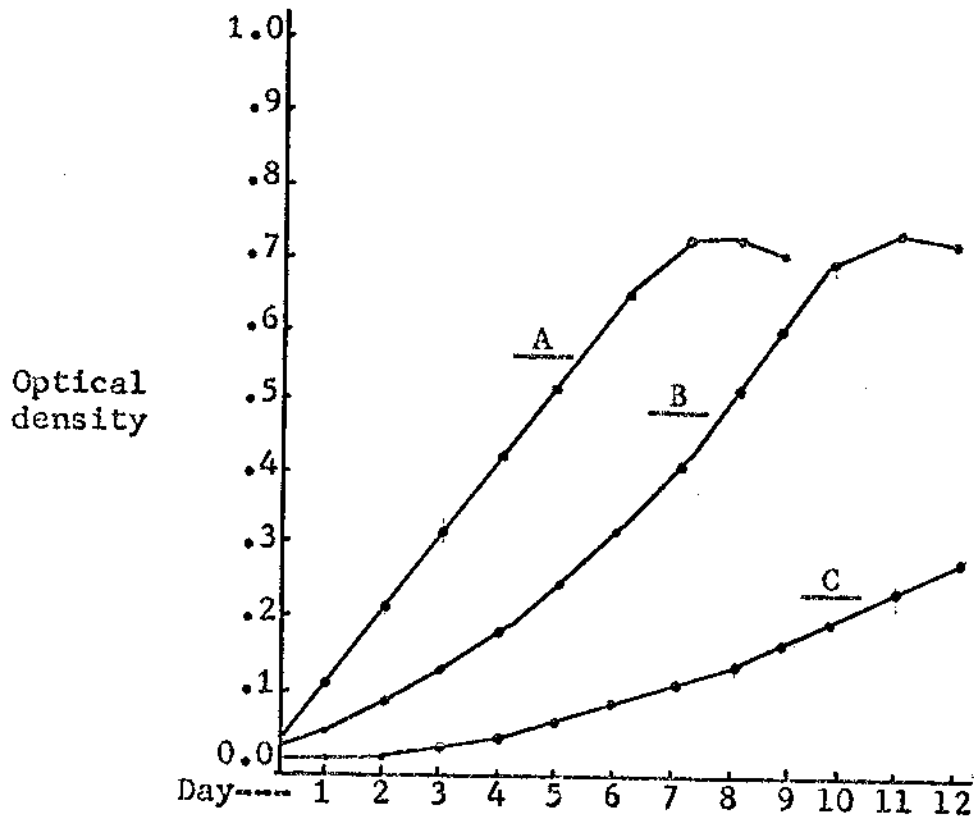
Fig. 1a—Growth curves of "1444".
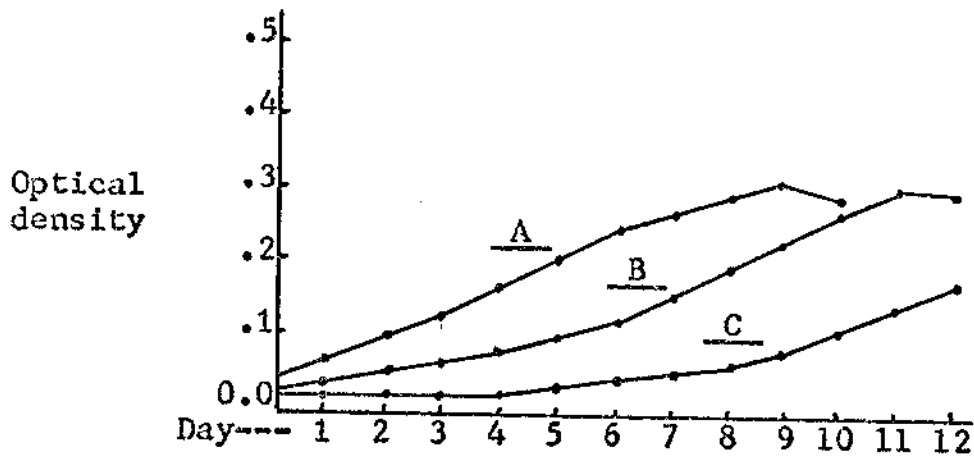
Fig. 1b—Growth curves of Aphanizomenon flos-aquae.
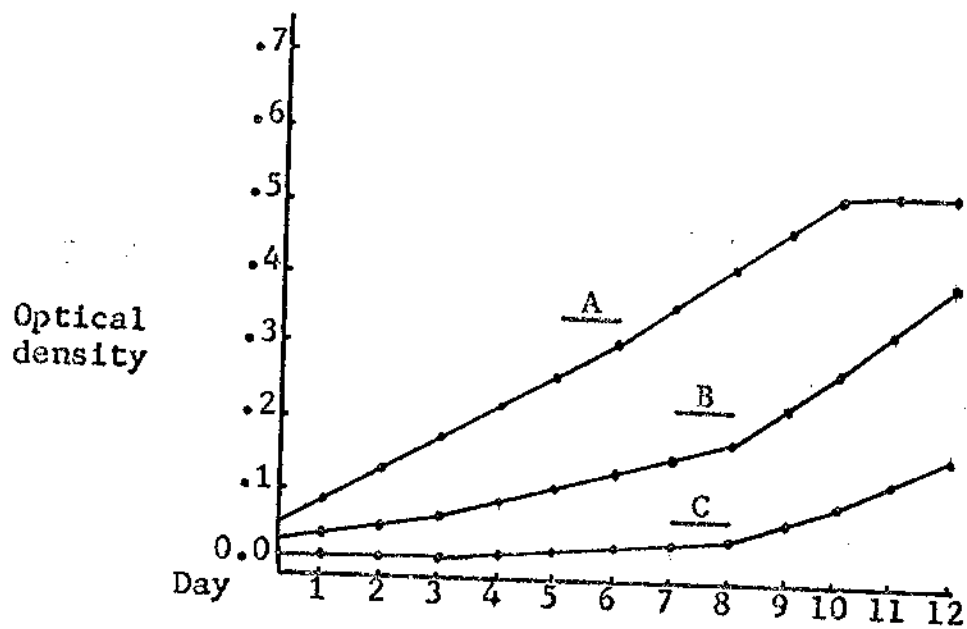
Fig. 1c. Growth curves of Anabaena flos-aquae
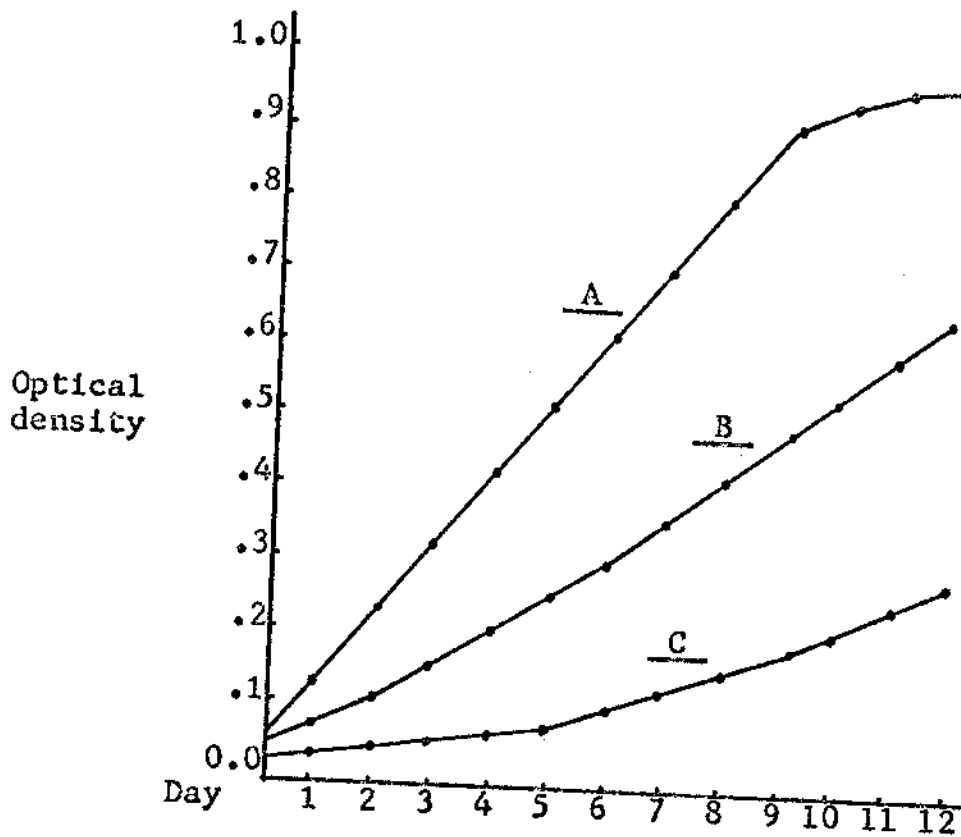
Fig. 1d. Growth curves of Microcystis aeruginosa

Figure 2. Growth of "1444" subjected to three levels (5, 3, and 1 ml) of three dilutions (1/10, 1/20, and 1/50) of raw fungal fermentation medium.
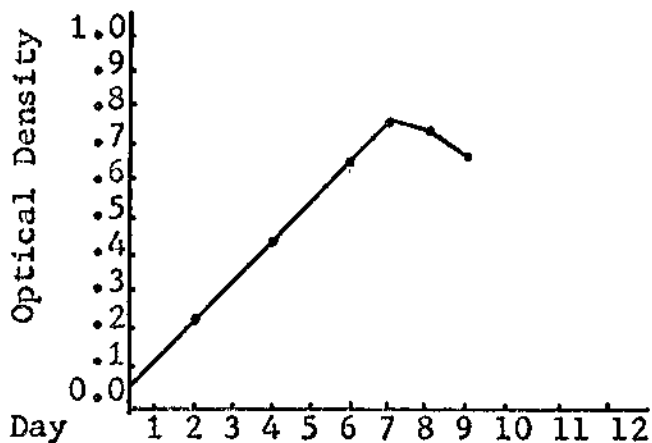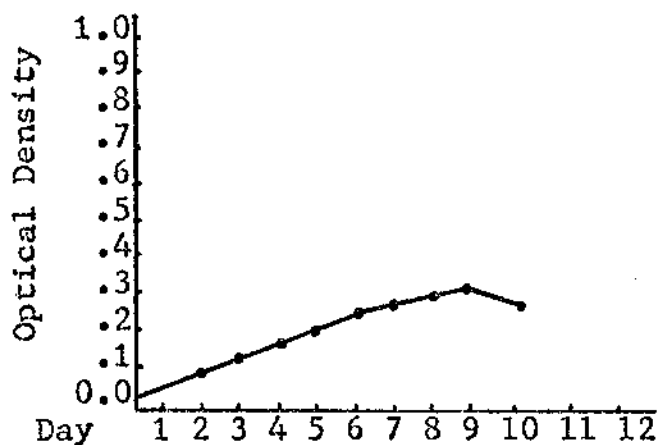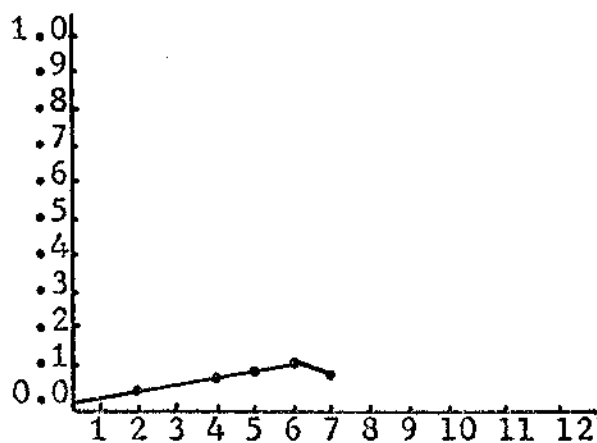

Fig. 2a---Control; no raw fungal medium added.
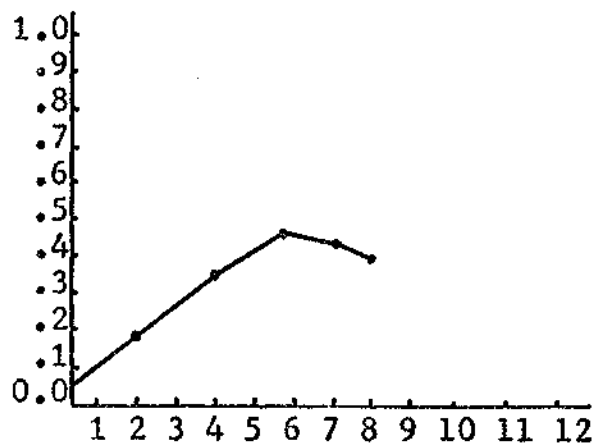
Fig. 2b--Add'n of 5 ml of 1/10 dilution of raw fungal medium.
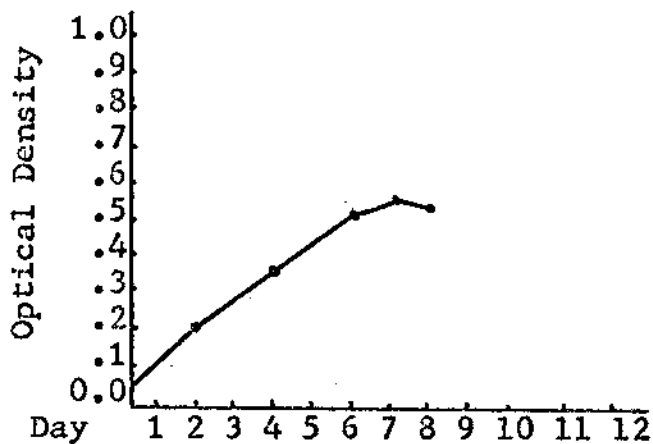
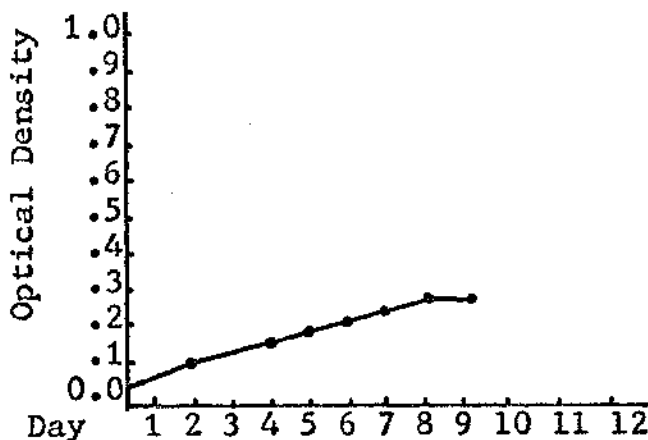Fig. 2c---Add'n of 5 ml of 1/20 dilution of raw fungal medium.
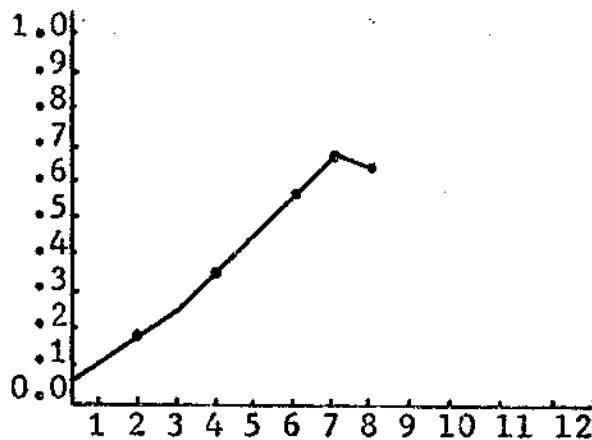
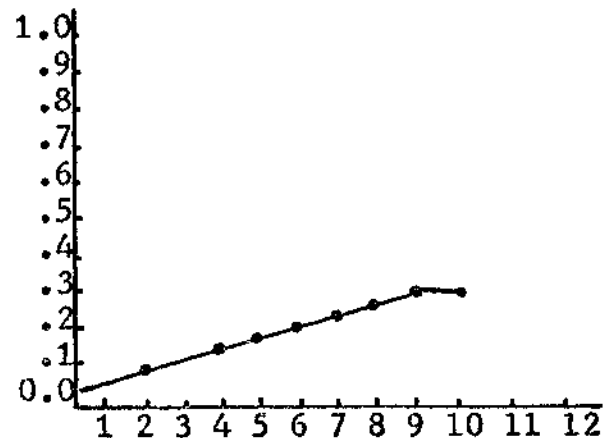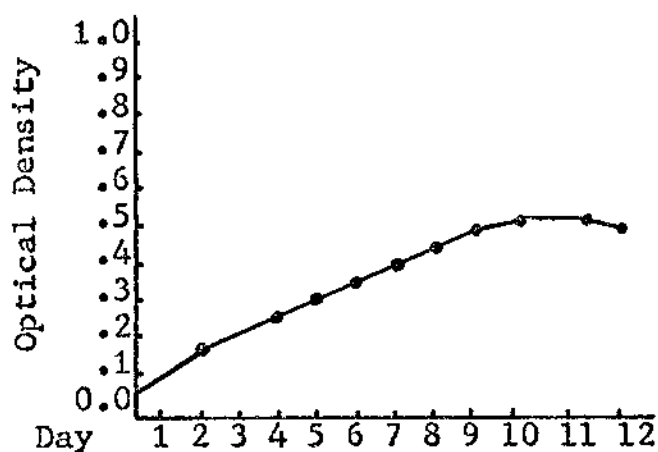Fig. 2d---Add'n of 5 ml of 1/50 dilution of raw fungal medium.
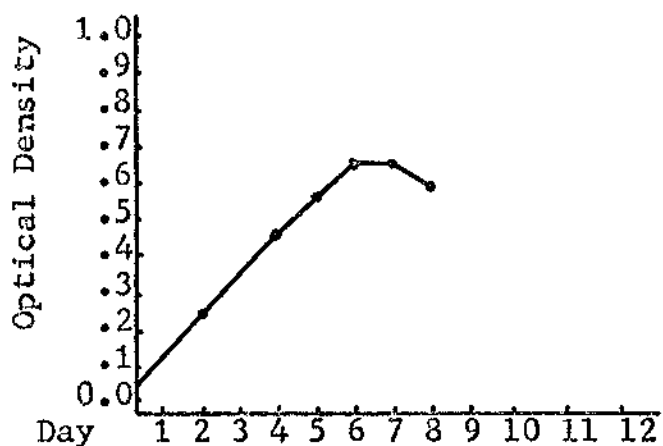
Fig. 2e. Add'n of 3 ml of 1/10 dilution of raw fungal medium.
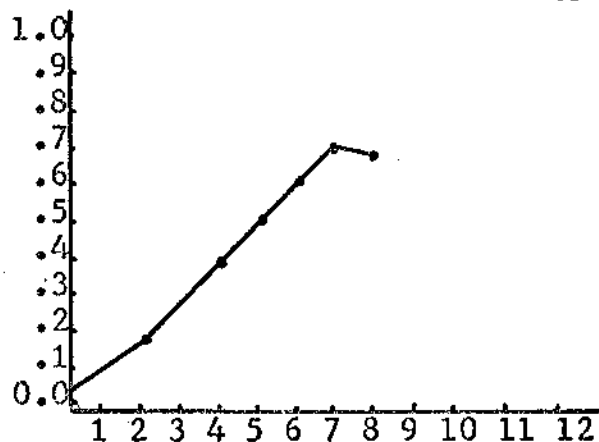
Fig. 2h. Add'n of 1 ml of 1/10 dilution of raw fungal medium.
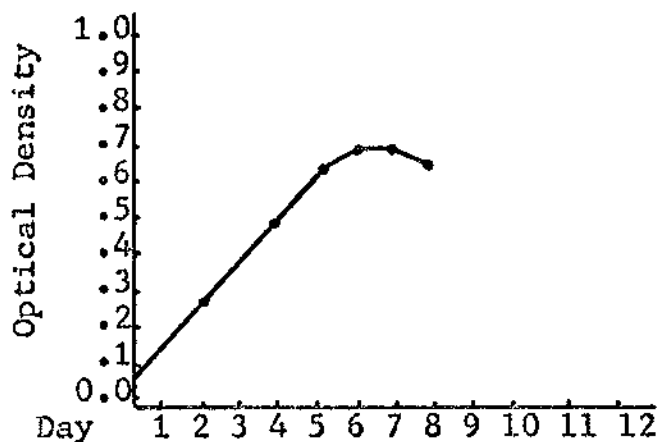
Fig. 2f. Add'n of 3 ml of 1/20 dilution of raw fungal medium.
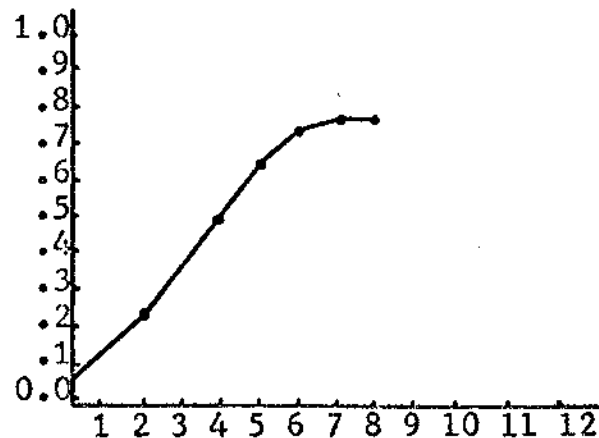
Fig. 2i. Add'n of 1 ml of 1/20 dilution of raw fungal medium.
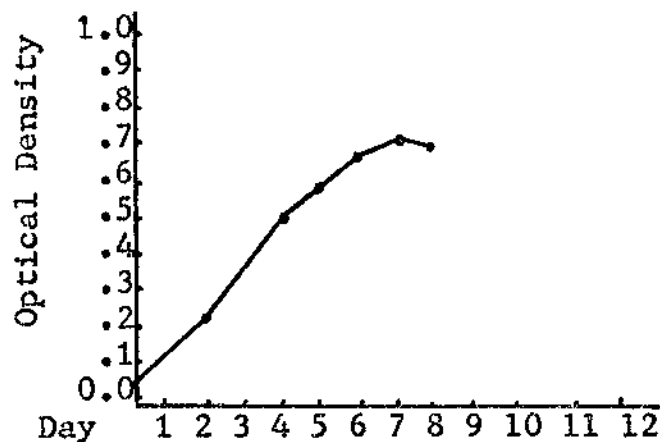
Fig. 2g. Add'n of 3 ml of 1/50 dilution of raw fungal medium.
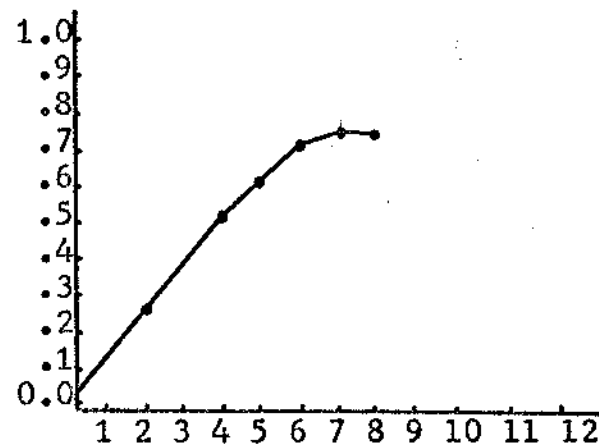
Fig. 2j. Add'n of 1 ml of 1/50 dilution of raw fungal medium.

Figure 3. Growth of <u>Aphanizomenon flos-aquae</u> subjected to three levels (5, 3, and 1 ml) of three dilutions (1/10, 1/20, and 1/50) of raw fungal fermentation medium.
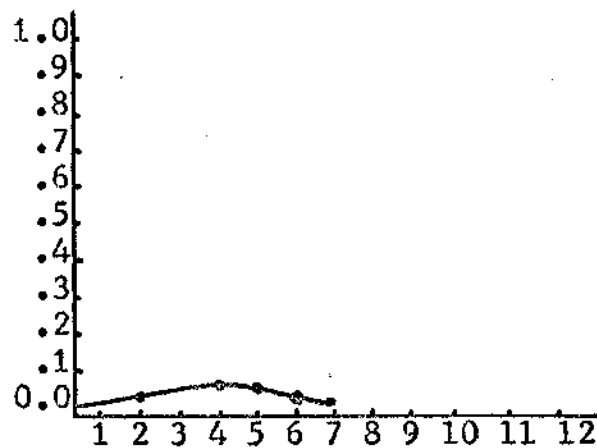

Fig. 3a. Control; no raw fungal medium added.

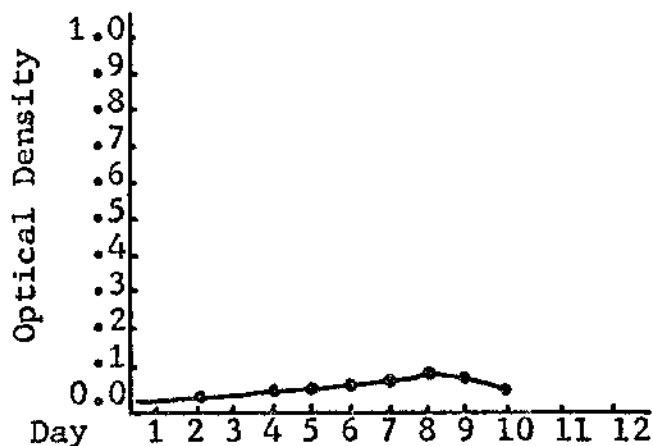Fig. 3b. Add'n of 5 ml of 1/10 dilution of raw fungal medium.
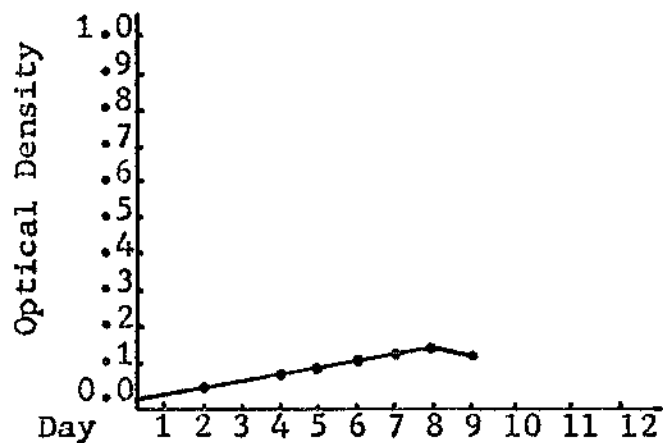
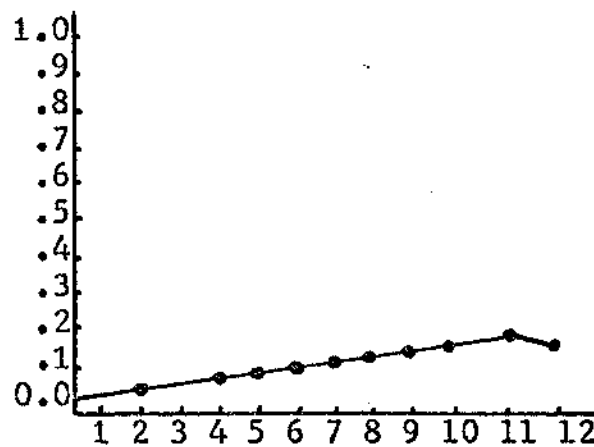Fig. 3c. Add'n of 5 ml of 1/20 dilution of raw fungal medium.
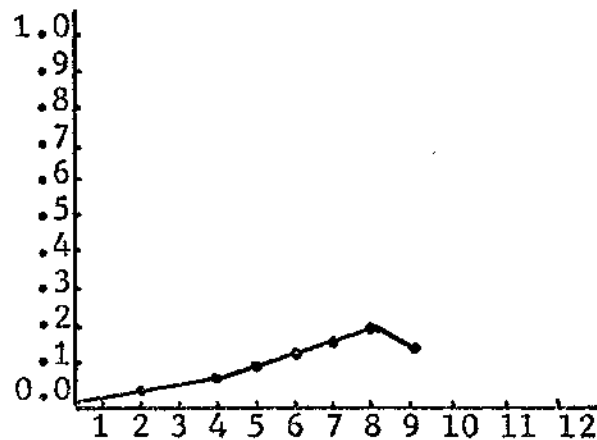
Fig. 3d. Add'n of 5 ml of 1/50 dilution of raw fungal medium.
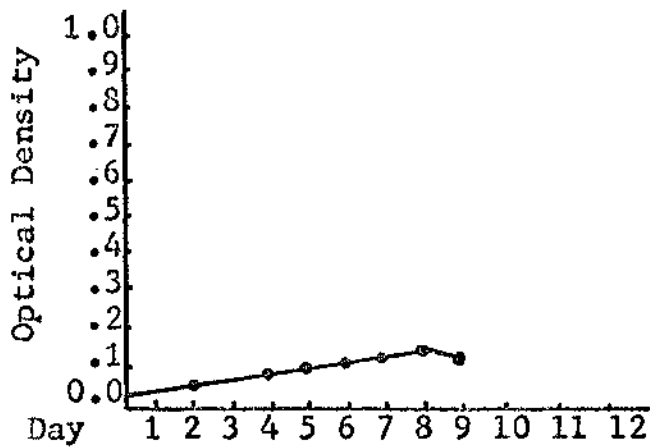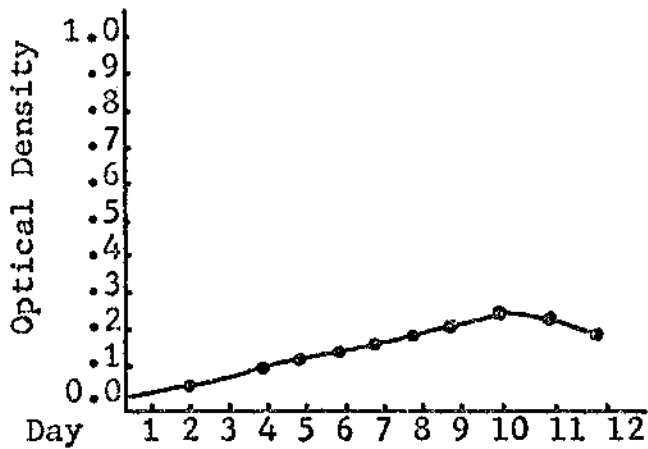
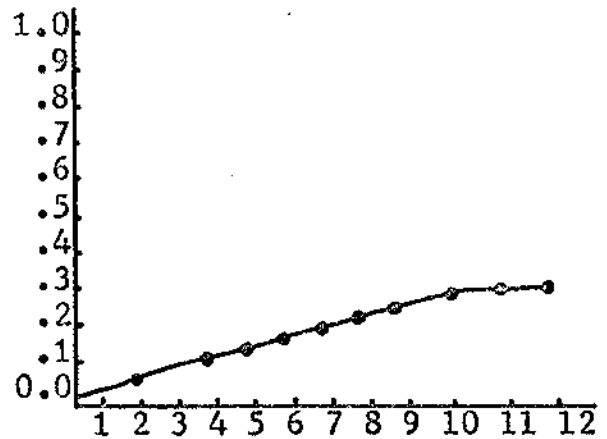Fig. 3e. Add'n of 3 ml of 1/10 dilution of raw fungal medium.
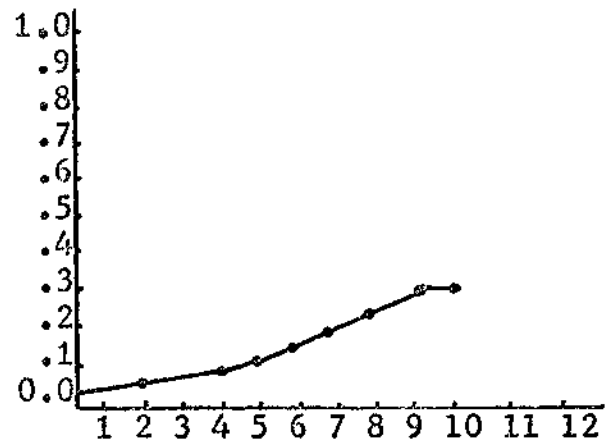
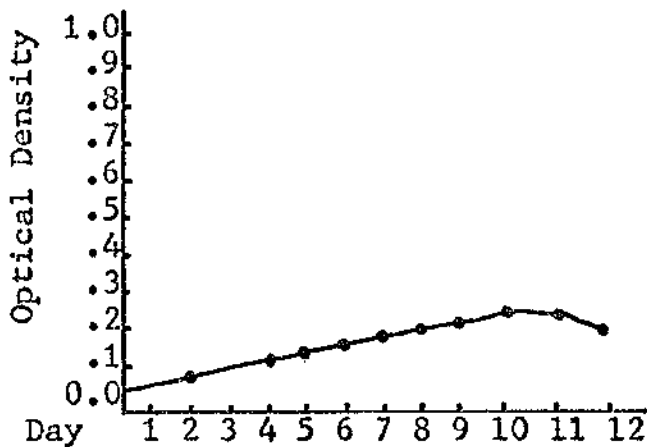Fig. 3h. Add'n of 1 ml of 1/10 dilution of raw fungal medium.
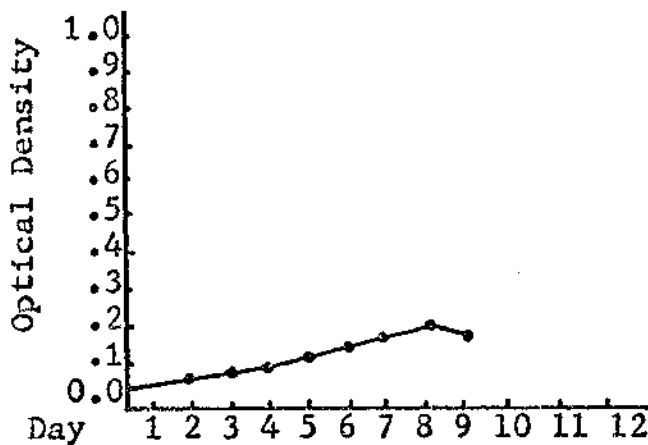
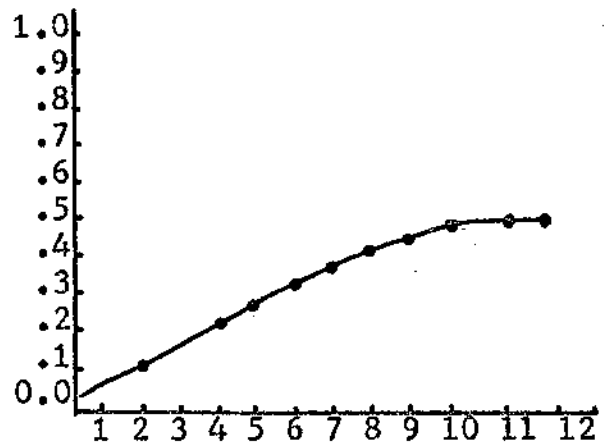Fig. 3f. Add'n of 3 ml of 1/20 dilution of raw fungal medium.
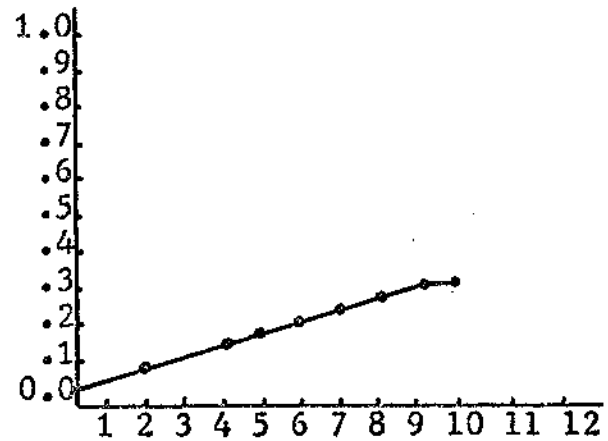
Fig. 3i. Add'n of 1 ml of 1/20 dilution of raw fungal medium.

Fig. 3g. Add'n of 3 ml of 1/50 dilution of raw fungal medium.

Fig. 3j. Add'n of 1 ml of 1/50 dilution of raw fungal medium.

Figure 4. Growth of <u>Anabaena flos-aquae</u> subjected to three
levels (5, 3, and 1 ml) of three dilutions (1/10, 1/20,
1/50) of raw fungal fermentation medium.

Fig. 4a. Control; no raw fungal
medium added.

Fig. 4b. Add'n of 5 ml of 1/10
dilution of raw fungal medium.

Fig. 4c. Add'n of 5 ml of 1/20
dilution of raw fungal medium.

Fig. 4d. Add'n of 5 ml of 1/50
dilution of raw fungal medium.

Fig. 4e. Add'n of 3 ml of 1/10 dilution of raw fungal medium.

Fig. 4h. Add'n of 1 ml of 1/10 dilution of raw fungal medium.

Fig. 4f. Add'n of 3 ml of 1/20 dilution of raw fungal medium.

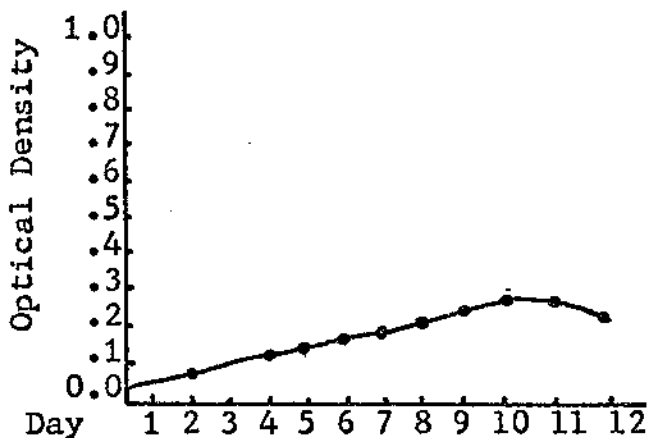Fig. 4i. Add'n of 1 ml of 1/20 dilution of raw fungal medium.

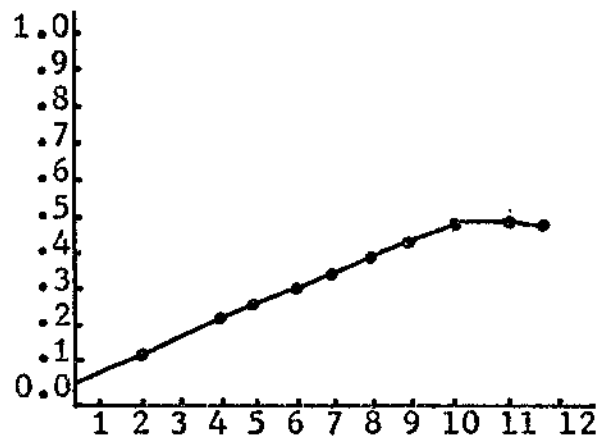Fig. 4g. Add'n of 3 ml of 1/50 dilution of raw fungal medium.

Fig. 4j. Add'n of 1 ml of 1/50 dilution of raw fungal medium.

Figure 5. Growth of <u>Microcystis</u> <u>aeruginosa</u> subjected to three
levels (5, 3, and 1 ml) of three dilutions (1/10, 1/20, and
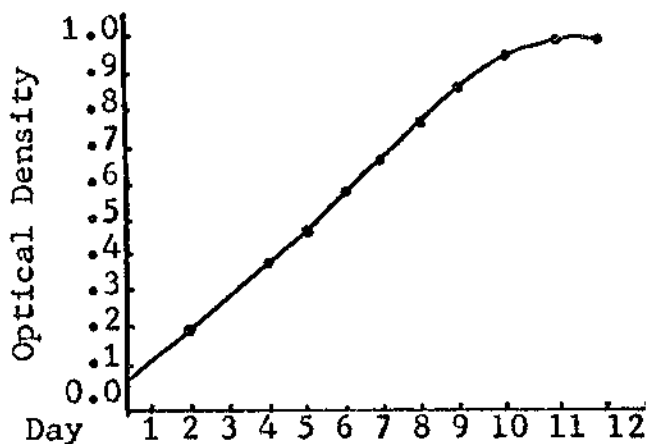1/50) of raw fungal fermentation medium.
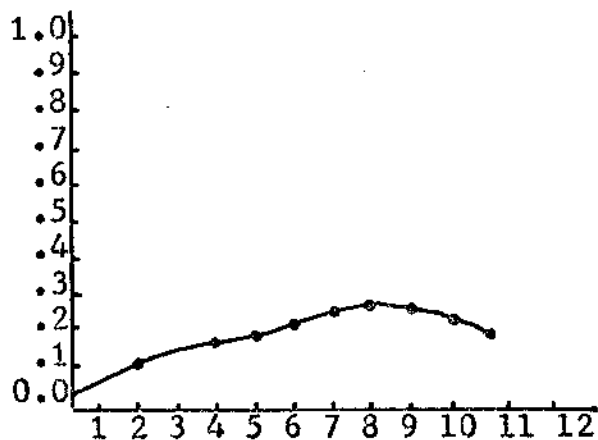
Fig. 5a. Control; no raw fungal
medium added.

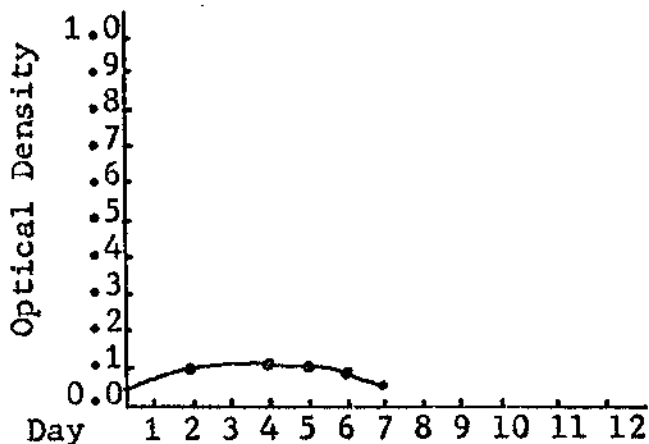Fig. 5b. Add'n of 5 ml of 1/10
dilution of raw fungal medium.

Fig. 5c. Add'n of 5 ml of 1/20
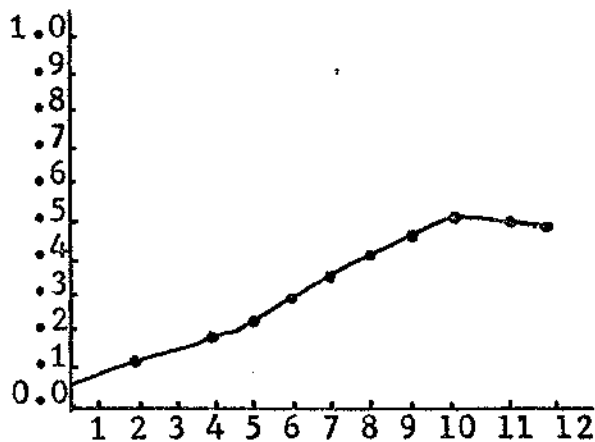dilution of raw fungal medium.

Fig. 5d. Add'n of 5 ml of 1/50
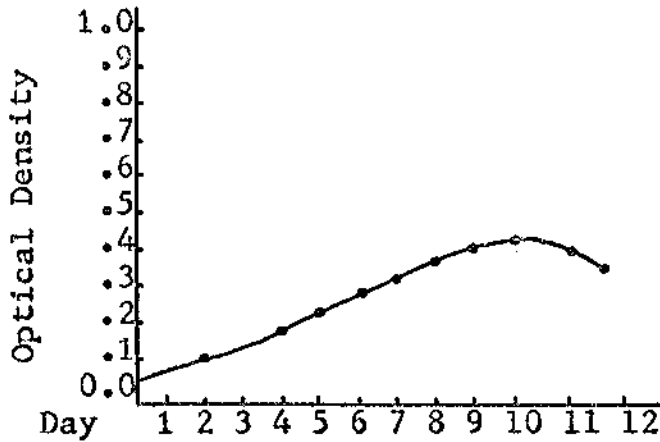dilution of raw fungal medium.

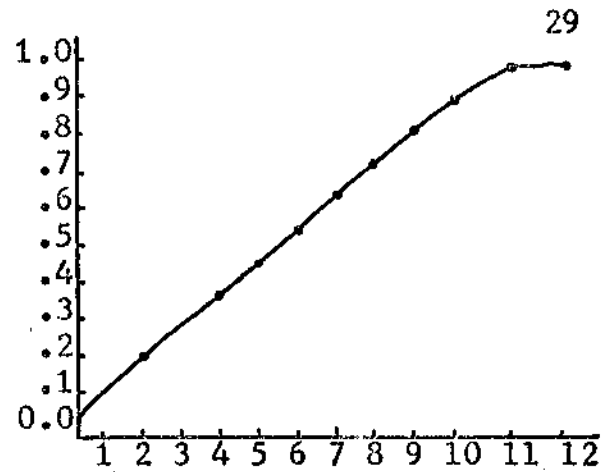Fig. 5e. Add'n of 3 ml of 1/10 dilution of raw fungal medium.

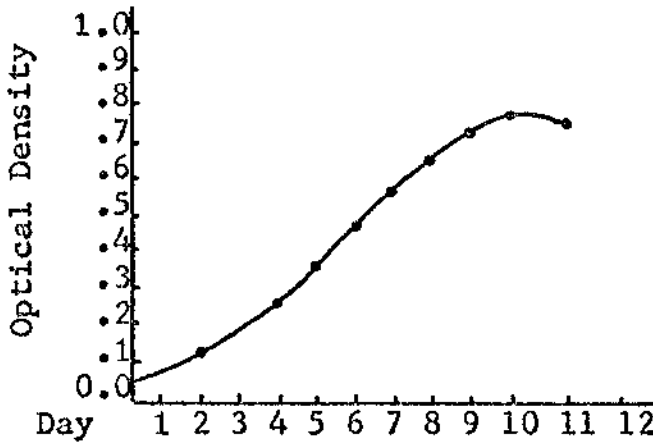Fig. 5h. Add'n of 1 ml of 1/10 dilution of raw fungal medium.

Fig. 5f. Add'n of 3 ml of 1/20 dilution of raw fungal medium.
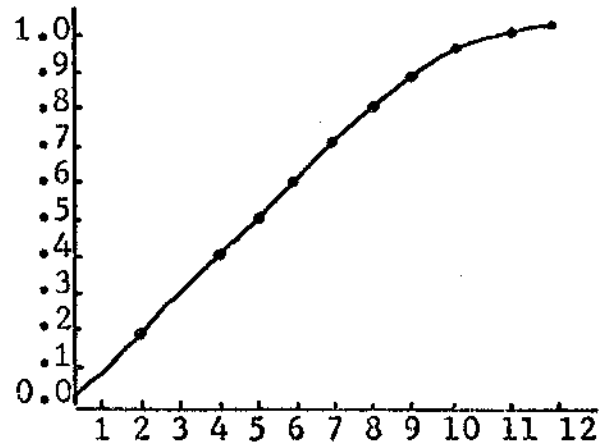

Fig. 5i. Add'n of 1 ml of 1/20 dilution of raw fungal medium.
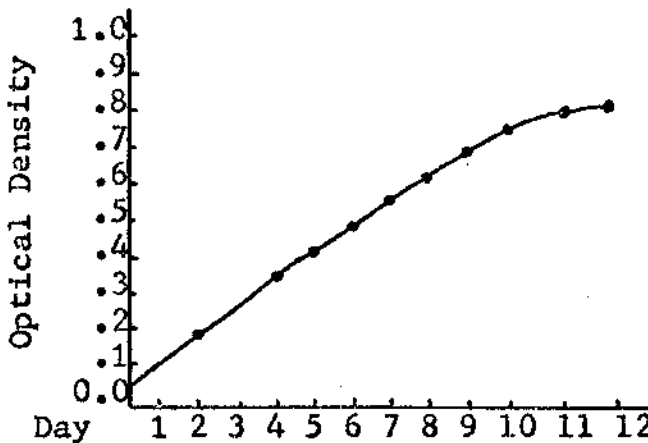

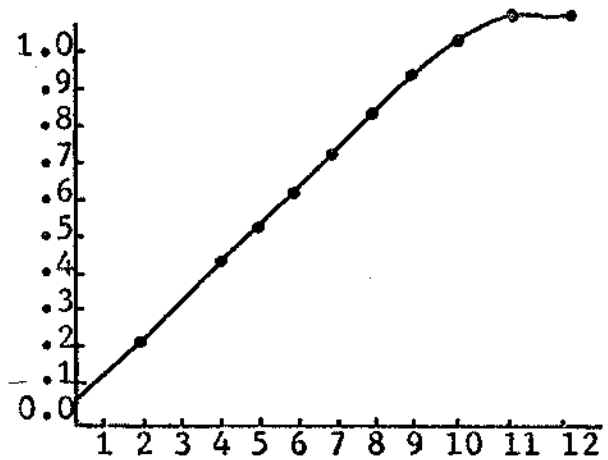Fig. 5g. Add'n of 3 ml of 1/50 dilution of raw fungal medium.

Fig. 5j. Add'n of 1 ml of 1/50 dilution of raw fungal medium.

dilution of each fungal fermentation filtrate. (Where screening was done with test tubes, 0.2 ml of a 1/20 dilution was added to the proportionately smaller algal cultures.)

Preliminary screening indicated eleven instances of early chlorosis or lack of growth in algae, with reference to the control cultures. (See Table III.)

TABLE III

RESULTS OF PRELIMINARY SCREENING OF FUNGAL
FERMENTATION FILTRATES (Aph, Aphanizom-
enon flos-aquae; Ana, Anabaena flos-
aquae; Mic, Microcystis aeruginosa.)

Alga	Filtrate No.	Type Screening	Type Effect	Number of Replicates Affected
1444	22	tube	scant growth	both
1444	19	tube	scant growth	both
Aph	3	flask	scant growth	both
Aph	13	flask	scant growth	one
Aph	49	flask	scant growth	both
Aph	65	flask	scant growth	one
Ana	25	tube	chlorosis	one
Ana	61	flask	scant growth	one
Mic	35	tube	chlorosis	one
Mic	58	flask	chlorosis	one

Figure 6. Curves representing growth of algae in con-
firmatory screening of the 11 fungal filtrates which
appeared to inhibit the growth of an alga in prelim-
inary screening (see Table , p.). Figs. 6a through
6d represent growth of controls of the four algae.
Figs. 6f through 6p represent cultures treated with
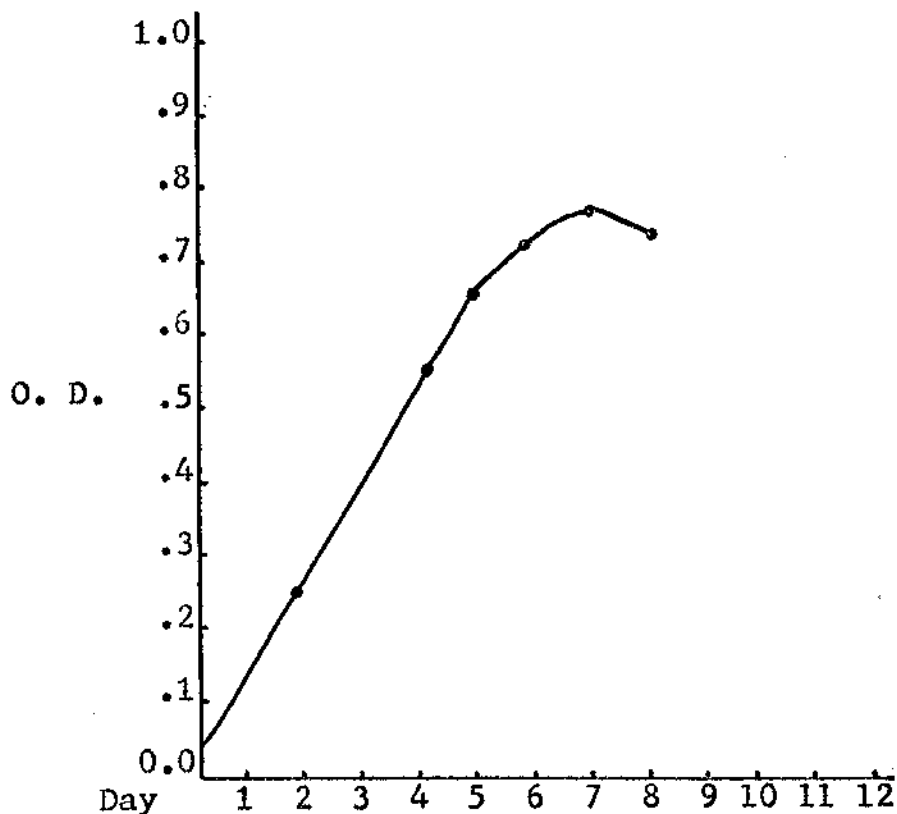fungal filtrates.

Fig. 6a. Growth of control culture of 1444.
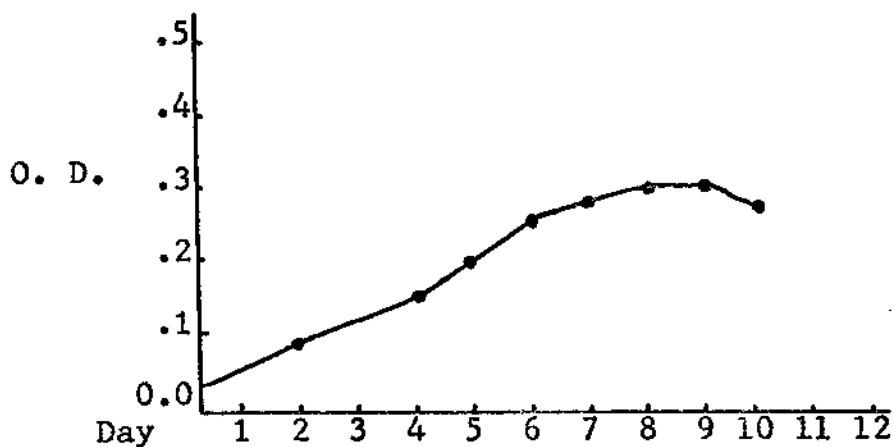
Fig. 6b. Growth of control, Aphanizomenon flos-aquae.

Figure 6, continued.
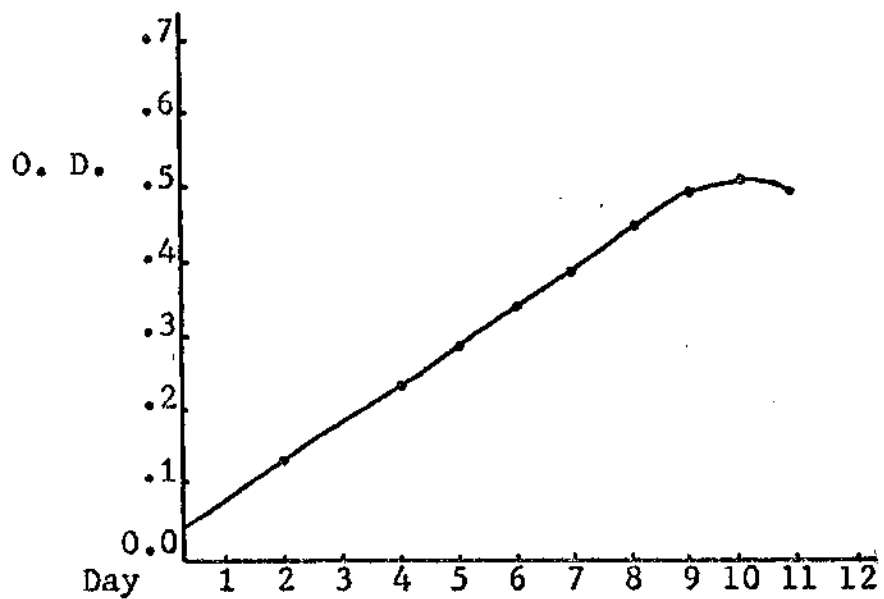
Fig. 6c. Growth of control, _Anabaena flos-aquae_.
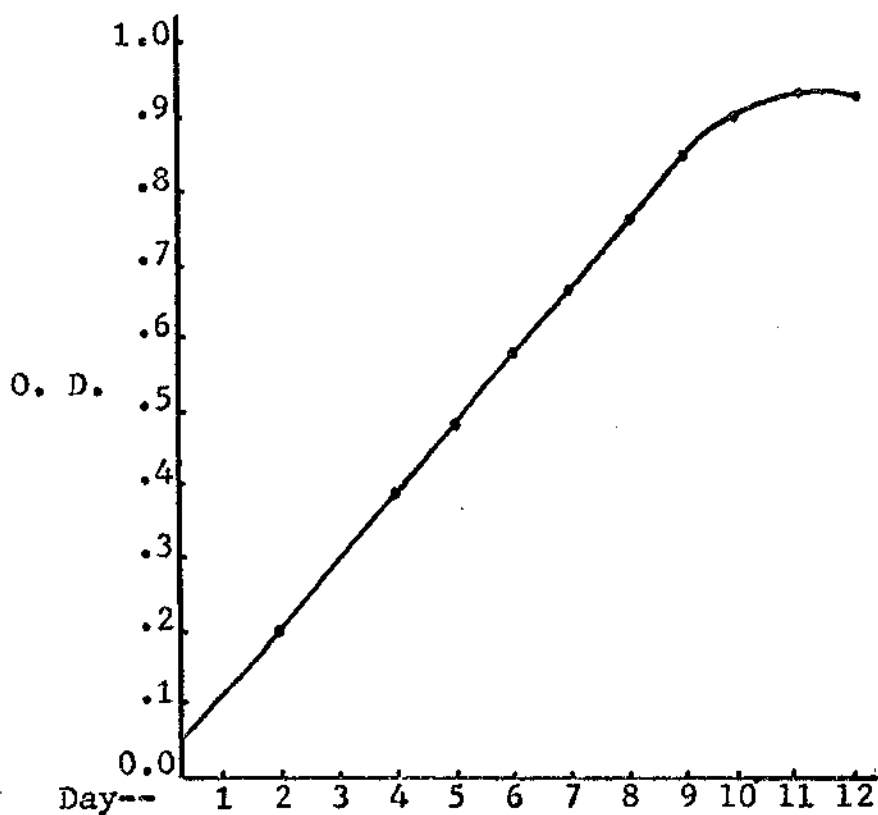
Fig. 6d. Growth of control, _Microcystis aeruginosa_.

Figure 6, continued.
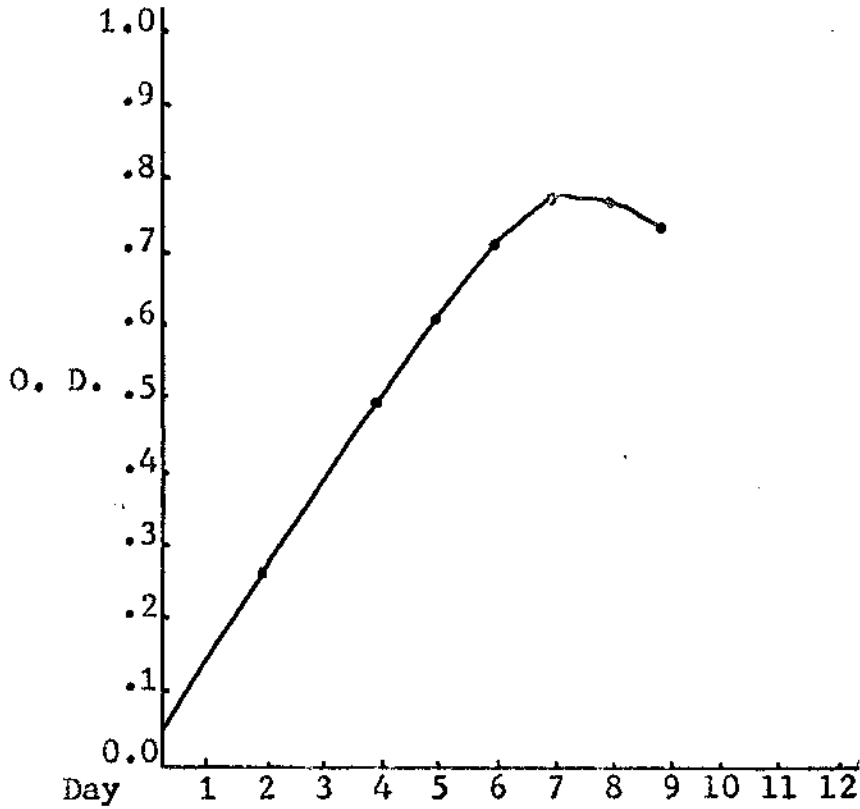
Fig. 6e. Growth of 1444, treated with fungal filtrate no. 22.
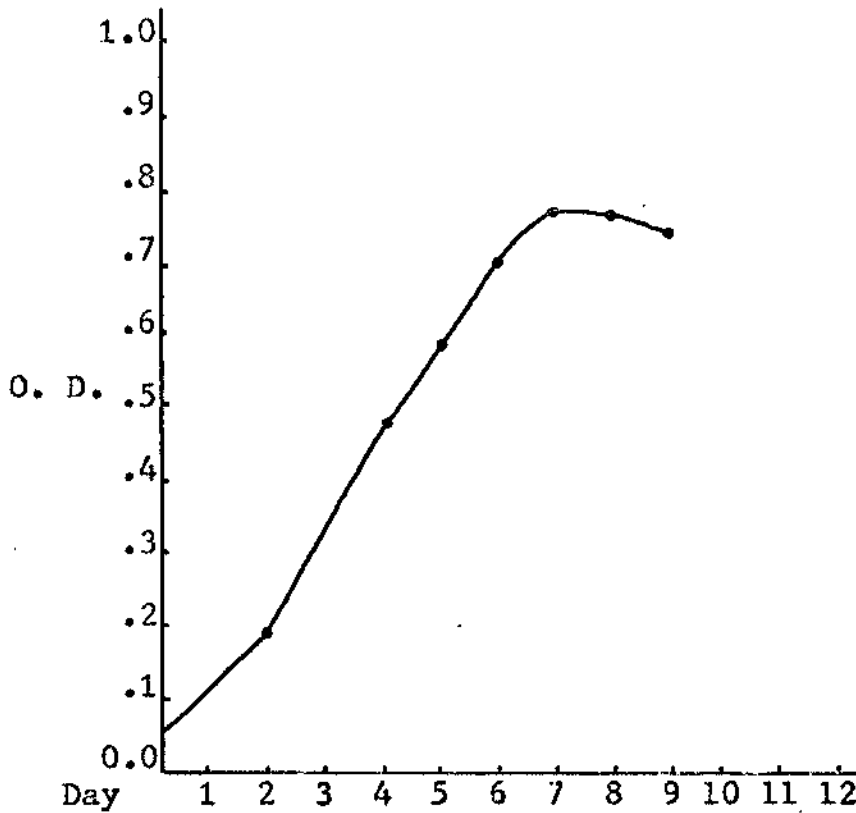
Fig. 6f. Growth of 1444, treated with fungal filtrate no. 19.

Figure 6, continued.
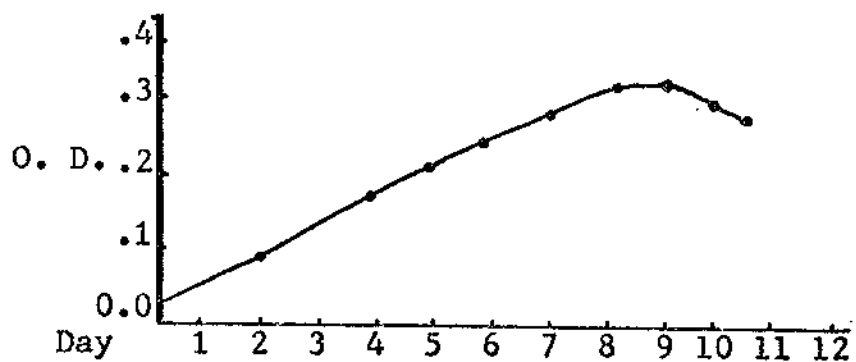
Fig. 6g. Growth of Aphanizomenon flos-aquae
treated with fungal filtrate no. 3.
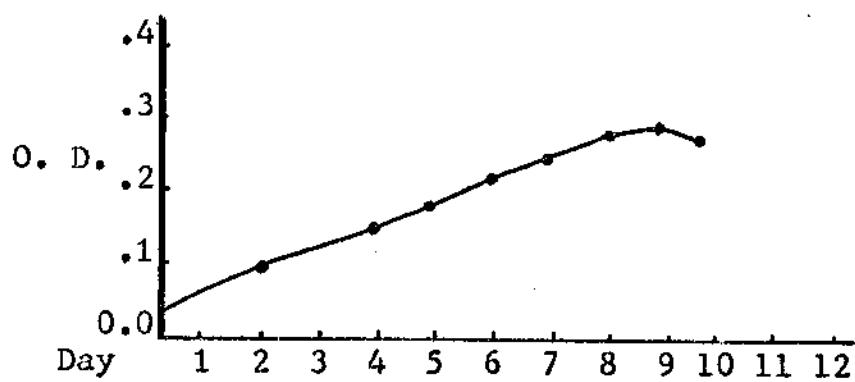
Fig. 6h. Growth of Aphanizomenon flos-aquae
treated with fungal filtrate no. 13.
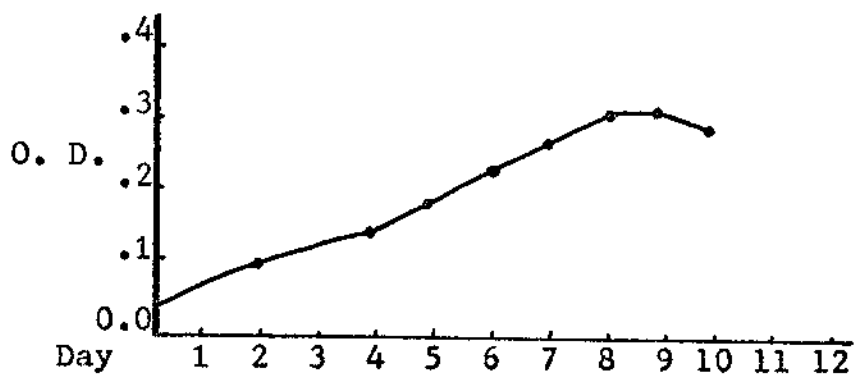
Fig. 6i. Growth of Aphanizomenon flos-aquae
treated with fungal filtrate no. 49.

Figure 6, continued.
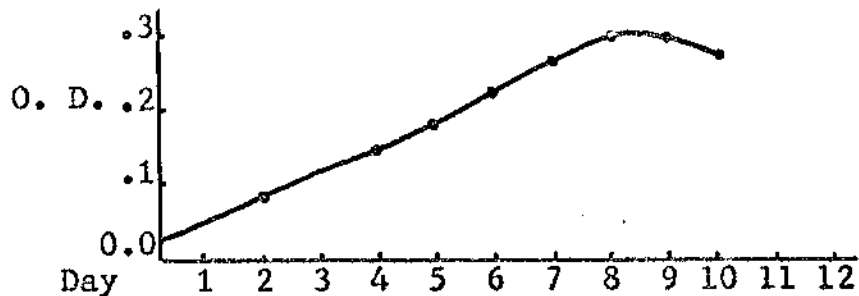
Fig. 6j. Growth of Aphanizomenon flos-aquae treated with fungal filtrate no. 65.
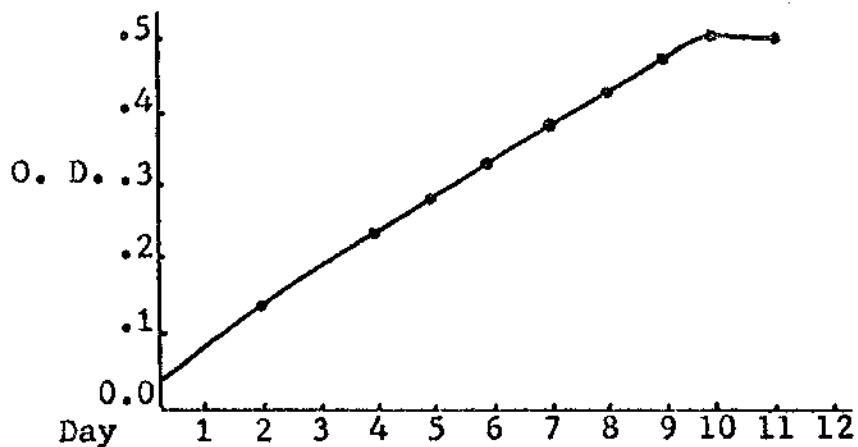
Fig. 6k. Growth of Anabaena flos-aquae treated with fungal filtrate no. 25.
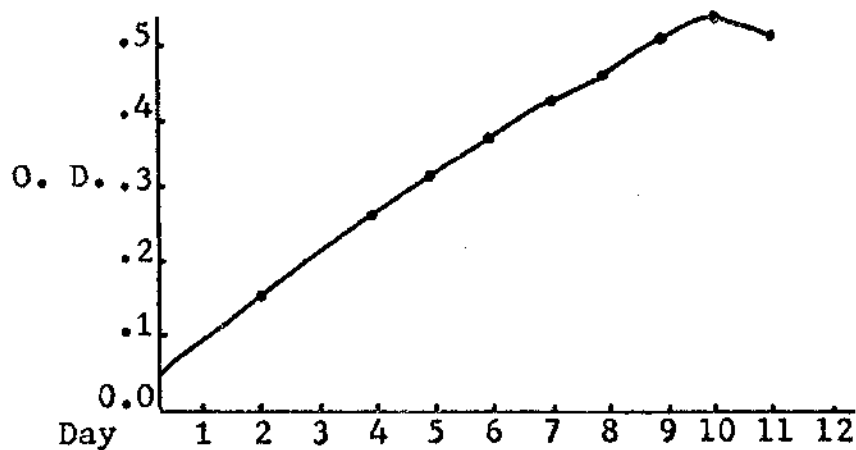
Fig. 6l. Growth of Anabaena flos-aquae treated with fungal filtrate no. 61.
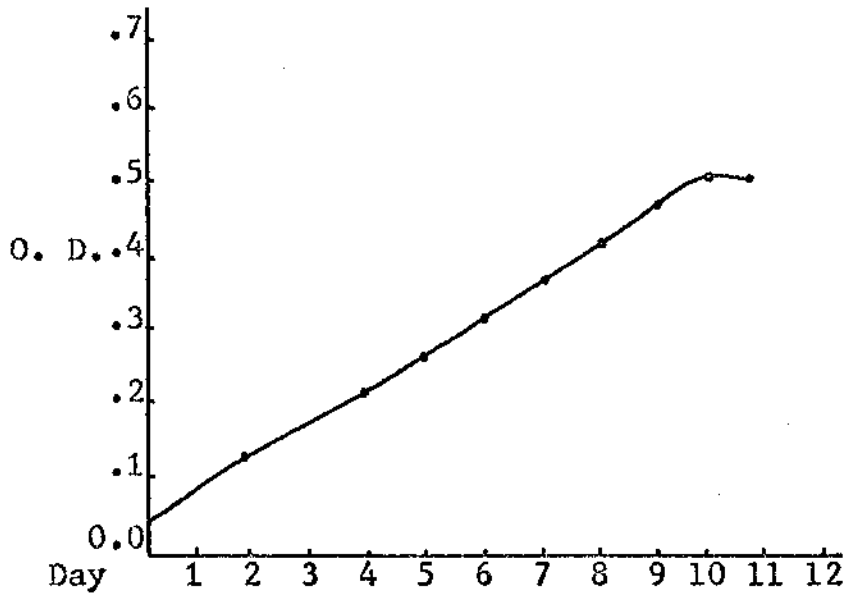
Fig. 6m. Growth of <u>Anabaena</u> <u>flos-aquae</u>
treated with fungal filtrate no. 42.
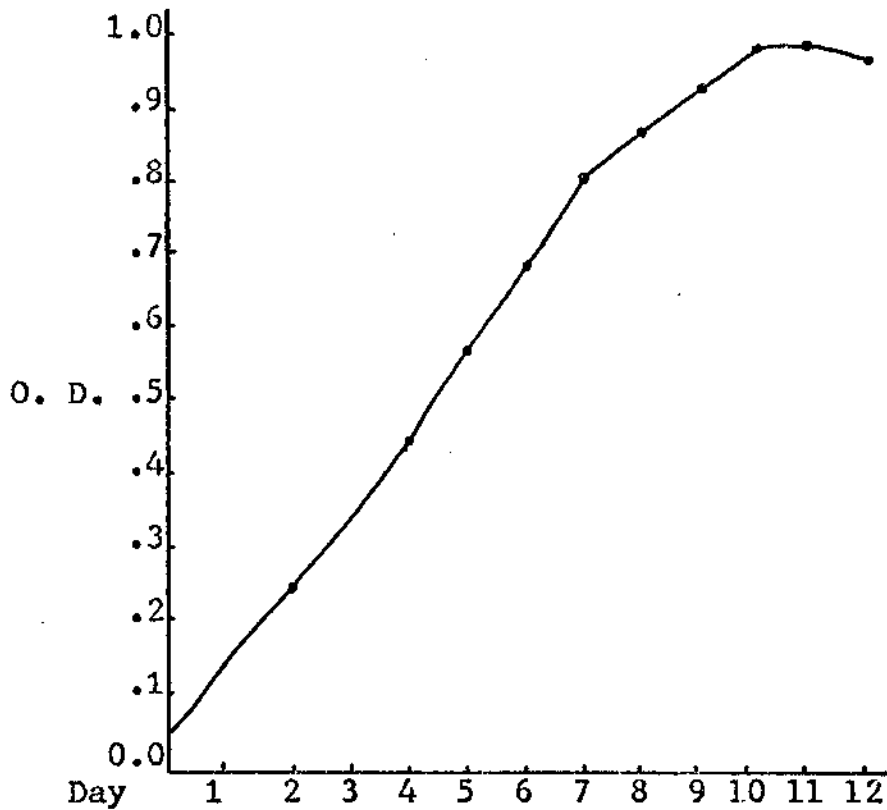
Fig. 6n. Growth of <u>Microcystis</u> <u>aeruginosa</u>
treated with fungal filtrate no. 35.

Figure 6, continued.
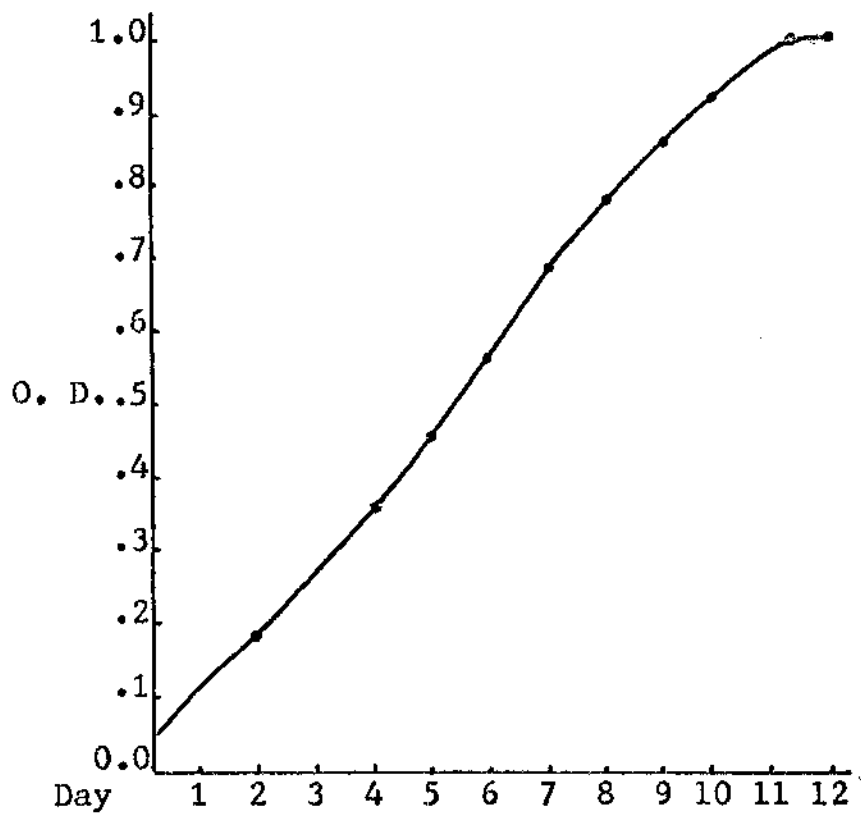
Fig. 6o. Growth of <u>Microcystis</u> <u>aeruginosa</u>
treated with fungal filtrate no. 58.

In order to confirm and quantitate the effects demon-
strated in Table 3, the eleven fungal filtrates were sub-
jected to a confirmatory screening in the growth chamber.
The results are shown in Figure 6.

Of the eleven filtrates which appeared to inhibit
algal growth in preliminary screening, none were substant-
iated in the confirmatory screening under controlled condit-
ions.

CHAPTER IV

DISCUSSION

One factor which prolonged the initial stages of this investigation was the length of time required to learn how to reproduce growth curves of two of the algae, *Aphanizomenon flos-aquae* and *Anabaena flos-aquae*. After much work, it was evident that considerable care had to be taken in handling cultures of these organisms uniformly in growing up inocula, as any disparity in handling or agitation would yield different degrees of fragmentation, i. e., different numbers of growth centers. This in turn would cause growth curves of different flasks to peak at different times.

Under the experimental conditions of this investigation, none of the sixty-five fungi (representing thirty genera) demonstrated the ability to produce a metabolite with a measurable inhibition effect on any of the four algae used.

In future screenings for antialgal metabolites, the chances for success might be enhanced by focusing more on fungi with strictly aquatic habitats. Such fungi would have to have a mechanism for survival against antagonism by algae, and one such mechanism could be the production of an antialgal metabolite.

39

Another helpful step in an investigation of this kind would be the development of a procedure to allow a greater dosage of fungal filtrate to be introduced into the algal culture. This would require a knowledge of the effects of medium L, glucose, and yeast extract on the growing algal cells. Another prerequisite would be a quick, easy, and inexpensive way to monitor the declining levels of these three ingredients in the progressing fungal fermentation.

An antialgal metabolite was not found in this investigation, but this fact does not entirely invalidate the concept of using very small amounts of the fungal filtrates in screening experiments; in a natural, aquatic setting, extreme dilution of any fungal metabolites would be a certainty, and any effectiveness of such metabolites against algal antagonism would presuppose an extreme potency of the inhibiting molecule.

It is also felt that more controlled, precise techniques are necessary to quickly screen a large number of filtrates. The preliminary screening methods used in this investigation obviously allowed variables other than the fungal filtrates under investigation to give a false positive result. This is in keeping with Wyatt's (1968) observation that without rigorous uniformity in handling these strains of algae, a wide diversity of fragility in cultures can be expected. The variable which probably had the greatest damaging effect on

uniformity of growing algal cultures was that of illumination; in addition to the 40-watt lamps intended for illumination, the ceiling lamps contributed to the growth of the algal cultures in a irreproducible way.

CHAPTER IV

SUMMARY

Since many approaches to dealing with algal blooms are inefficient, expensive, or harmful, it was concluded that a biologically-sythesized chemical agent, specifically inhibitory to pre-bloom algal cells, might prove helpful in controlling algal blooms. Fungi were chosen as the biological entities to investigate for such a chemical.

Sixty-five fungi representing thirty genera were isolated from water and mud, and stored on PGA agar slants. Using the spores and mycelial particles from these slants as inoculum, a seven-day shake flask fermentation was carried out using a mineral salts, yeast extract, glucose medium. The mycelium in each flask was filtered off and the filtrates were stored in a freezer until needed.

Four blue-green algae, three of them documented bloom formers, were cultured in a growth chamber in shake flasks of ASM-1 medium, to obtain data on their growth characteristics. This data was used to establish standard inocula for all subsequent experiments.

An experiment was performed to determine the maximum

amount of raw (sterile and uninoculated) fungal fermentation medium the algae could receive without alteration of their established growth curves; for all four algae, 1 ml of a 1/20 dilution per shake flask was selected to represent this amount. Subsequently in all screening experiments, the fungal filtrates were administered in this amount to preclude a false positive test resulting from an effect of residual raw medium rather than a metabolite.

A preliminary screening of fungal filtrates was carried out under casual environmental conditions by being administered to shake flask or test tube cultures of the algae at room temperature. Eleven filtrates appeared to have an effect on an alga in this preliminary screen. Confirmatory screening of these eleven instances, in shake flask cultures under controlled conditions, produced no measurable effects on the growth of the algae.

Observations on handling algae were made, and recommendations were made for possible future screenings of fungi.

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