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Title REQUIREMENTS FOR THE GROWTH AND MAINTENANCE OF APHANIZOMENON FLOS-AQUAE IN CULTURE

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Aphanizomenon flos-aquae from Upper Klamath Lake, Oregon was introduced into culture in a precipitate-free medium developed by modification of McLachlan's ASM medium. This medium ASMT No. 8 contained no organic substances other than ethylendiaminetetraacetate (EDTA) and Trishydroxymethylamino methane (Tris), and was developed by modifying the concentrations of certain elements, by adding sodium bicarbonate and a 1/25 dilution of Arnon's trace element solutions B and C.

Cultures of Aphanizomenon in this medium have been maintained in combined daylight and supplemental fluorescent illumination at 15° C. The alga has shown good growth, and has retained the morphological integrity of the colonial flake for more than 14 months.

The results of experiments to obtain a medium further improved for the culture of Aphanizomenon, by additional variation of

the concentration of elements in ASMT No. 8 are also reported.

Preliminary experiments utilizing ultra-violet irradiation and chlorine treatment for elimination of bacteria indicate that bacteria possibly may be removed from cultures of Aphanizomenon. These experiments have determined that exposures can be used that materially reduce bacterial numbers without apparent change in the morphology of the alga.

Earlier experiments were made with the alga assumed to be in a unialgal condition as no other alga was demonstrated to be present in the stock cultures. Problems with a contaminating alga, Oscillatoria sp., have since appeared and have not been entirely solved. The complete elimination of Oscillatoria has not been achieved as filaments of this alga were found to grow between filaments of Aphanizomenon, both in the lake and in cultures. Attempts at removal by repeated washing were found to be ineffective. The suggested solution by re-isolation from stock cultures exhibiting, both microscopically and macroscopically, no contamination, has not yet been employed.

REQUIREMENTS FOR THE GROWTH AND MAINTENANCE OF APHANIZOMENON FLOS-AQUAE IN CULTURE

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

REQUIREMENTS FOR THE GROWTH AND MAINTENANCE OF *APHANIZOMENON FLOS-AQUAE* IN CULTURE

INTRODUCTION

Upper Klamath Lake in southern Oregon is a shallow eutrophic lake receiving a heavy load of colloidal, humic material from influent sources. During the summer months, an aesthetically disturbing cyanophycean bloom develops that discolors the water, and the decaying algal material near the shoreline produces noxious odors. The bloom is suspected of causing occasional kills of fish by lowering the content of dissolved oxygen at night below that necessary to sustain life. *Aphanizomenon flos-aquae* is the dominant species in this nuisance bloom and, in the literature, there are reports of suspected cases of toxicity of *Aphanizomenon* to animals.

Earlier investigations (33, 34) were made to determine the ecological factors contributing to the algal problem in Klamath Lake. These investigations were aimed at finding the reasons for the blooms, and if possible, controling them to make the water in the lake available for recreation and agricultural use. With the advent of the need for more water in southern California, a new interest in Klamath Lake has arisen.

The plan to divert water from the lake into drainage systems in California brought up the question of whether *Aphanizomenon* and

other algae would be transfered with the water, and cause problems in these systems.

To answer this question, requirements for the alga to survive in these systems must be known. In order to determine these requirements, a culture study was necessary.

This investigation is an attempt to introduce the alga not only into culture, but into a pure (unialgal, bacteria-free) culture as well. After developing such a culture, it will be possible to determine the light, temperature and nutrient or mineral requirements necessary for the growth of Aphanizomenon in natural habitats, and to thoroughly study the physiological requirements of the alga. To do this, a medium had to be found in which Aphanizomenon would maintain the morphology of the colonial flake form exhibited in nature. Light and temperature requirements for the growth of Aphanizomenon in culture also had to be determined in order to hold light intensities and temperature constant when further physiological studies are made.

The hypothesis that Aphanizomenon could be placed in culture, and could be maintained in its colonial flake form was the genesis of this investigation. This thesis summarizes attempts to introduce the alga into culture, and the results of experiments on attempted purification of the culture.

LITERATURE REVIEW

Introduction

In addition to the papers available when McLachlan (24, p. 3), remarked that little work had been done on Aphanizomenon flos-aquae there have since appeared a number of papers dealing with various aspects of the study of this organism. These papers have involved field investigations all over the world, cytology and culture among a wide variety of other topics. This review will mention a number of these reports.

Natural History of Aphanizomenon

Description of the Alga

Aphanizomenon flos-aquae (L.) Ralfs. is a member of the Nostocaceae. The organism has a macroscopic form that is commonly termed a "flake". When floating in the water, these flakes have the appearance of chopped grass. Each flake consists of a number of laterally coherent filaments. Smith (44, p. 585) states that the trichomes have delicate confluent sheaths. The cells of the trichome are cylindrical or barrel-shaped, and longer than wide. The heterocysts are intercalary and cylindrical. Akinetes are cylindrical and five to twelve times as long as broad and remote from the heterocysts.

Life History

By observing naturally occurring and cultured populations, Rose (41) was able to trace the life history of Aphanizomenon from akinete to macroscopic flake and back to akinete. He found that akinetes formed the previous summer germinated simultaneously in a laterally appressed condition. The young "sporelings" or filaments remained laterally appressed after germination, and through cell division gave rise to the macroscopic flake. He further noted that a heterocyst could develop, toward the middle of the filament, soon after the division of the sporeling to form several cells. Rose (41, p. 133) was

> not prepared to state that the heterocysts have a function in Aphanizomenon. From the experience with this blue-green it would seem that it is practically functionless.

He did state that akinetes are usually formed when conditions become unfavorable for the vegetative existence of the alga. Lund (23, p. 265) has reported that Aphanizomenon

> can produce resting spores [akinete] but [does] not do so every year, or only occasionally in benthic habitats.

An akinete, Rose further observed, is formed from a vegetative cell near the middle of the trichome. Thus, all the akinetes in a flake would be joined together by their lateral sheaths. After the disintegration of the vegetative cells of the filaments, usually in early

winter, these joined akinetes sink to the bottom. These akinetes would germinate the following spring to form a new flake, and the cycle would continue.

Phinney and McLachlan (32, p. 47) and Phinney and Peek (34) have reported the common occurrance of akinetes in bloom populations of Aphanizomenon and the occurrance of large numbers of akinetes in the upper bottom sediments during the winter months. The akinetes in sediments from Klamath Lake did not appear in laterally attached clusters as reported by Rose.

Dodd (6) reported the observation that lateral movements of the filaments cause changes in the size of flakes. These movements stopped after a few hours storage under laboratory conditions. Dodd (6, p. 118) also said that

> ... cell division occurs in one plane only, resulting in an increase in the length of the filament. The number of filaments in the flakes is increased by the breaking of existing filaments... usually at some point between heterocysts.

Increase in the number of flakes was found to be due to mechanical fragmentation of existing flakes.

Fogg (10) has presented a review of the characteristics of the gas vacuoles or pseudovacuoles of the Cyanophyta. He discussed their distribution in nature, their physical and chemical characteristics and theories of their nature, function and ecological significance. He noted that the vacuoles disappeared and were apparently

destroyed under increased pressure. The vacuoles of Aphanizomenon did not disappear when plants were placed under reduced pressure for five hours. On the basis of these and other experiments, it was concluded that the vacuoles did contain gas. Klebahn (as reported by Fogg) theorized the gas vacuoles to be surrounded by rigid impermeable membranes that provide resistance to changes in pressure. A recent study by light and electron microscopy of Aphanizomenon by Bowen and Jensen (3) has indicated that the vacuoles consist of packed arrays of cylindrical, electron transparent vesicles that collapse under pressure. They found that single vesicles were bounded by a single membrane 2 mμ wide. The reversible disappearance of the gas vacuoles under pressure is accompanied by a reversible collapse of these vesicles. Following application of pressure, there were no vesicles present but only short membraneous elements resembling typical "unit membranes". The authors concluded that aggregates of these vesicles can be identified with the gas vacuoles usually seen with the light microscope.

Ecology

Studies of the ecology of Aphanizomenon in the field have been the subject of numerous reports. Reports from all over the world (2, 16, 17, 18, 19, 30, 45, 47, 50) indicate the cosmopolitan nature of this alga.

Aphanizomenon is frequently a principal component of blooms of Cyanophyta species (32, p. 7, 34, 41, 43). Conditions necessary for these blooms can be brought about in a variety of ways. There are blooms that occur naturally in shallow eutrophic lakes where blue-green species predominate and produce the bloom during the summer months. These bloom species are indicators of the presence of organic wastes, and a high nitrogen level (37). The presence of humic leachate from a natural marsh land in part accounts for a heavy bloom of blue-green algae, particularly Aphanizomenon, in Upper Klamath Lake, Oregon (34).

The disturbance of lakes by man has been followed, in many cases, by various nuisance blooms. Wherever

> lakes receive drainage from tilled land, sewer effluents, excreta from farm animals and barnyard drainage, or refuse from city streets and factories, or from summer resort centers, [these lakes] become veritable garden spots for algae. (38, p. 1)

This material acts on the algae in the lake just as fertilizer does on plants on the land.

In many instances, Aphanizomenon appears to be the last alga in an annual succession of algal blooms, being preceded by Microcystis and Anabaena (17). According to Hammer (17), Aphanizomenon apparently is restricted to waters with 170 to 7300 ppm total solids. The alga was rarely present until the temperature had risen to 20°C. The temperature range of most blooms was 22.5 to 26.5°C.

There have been reports (24, p. 49), however, that this alga has been found growing under the ice in the winter. Hammer also indicated that the alga appeared to be directly influenced by phosphate.

Aphanizomenon has been the subject of a group of studies at Oregon State University (25, 33, 34). A survey (32) of aquatic habitats in Oregon indicated that this alga is widespread. These habitats included two natural basins (ox bow lakes and sloughs) and three artificial or man made basins (gravel pits, farm ponds and log storage ponds). A thorough study has been made of one habitat of Aphanizomenon in Montana (24). This study included an area description of the habitat (Ninepipe Reservoir), collection and counts of plankton and chemical analysis of water. Included in the Oregon State investigations is a comprehensive study of Aphanizomenon in Upper Klamath Lake, Oregon (33, 34). The field investigation consisted of counts of plankton and chemical analysis of not only water from Klamath Lake, but influent water sources and effluent water as well.

Several suggestions have been made for possible control of this alga in water sources. Control through treatment with copper sulphate (8 and 1/3 lbs/million gallons of water) (36) and 2, 3 dichloronaphthoquinone (5 μg and up/l of water) (9) were suggested. Chemical treatment would be inadvisable in Klamath Lake (34). The effect of the chemicals on the associated fauna of the lake and the expense would make this treatment prohibitive. A biological control

would not be possible due to lack of consumption of this alga by other organisms (33, p. 14).

Phinney and Peek (34, p. 27) have made two suggestions for possible control in Klamath Lake. First, a

> diversion of all influent humic waters to reservoirs from which they...[could be used] for irrigation of agricultural land.

Another suggestion was the addition of a slurry of some colloidal, inorganic material to the lake, or its tributaries, in sufficient amounts to reduce light intensity below the compensation point. The authors stated that the economic feasibility of these treatments would have to be studied further.

Toxicology

The study of the toxic effects of bloom producing species of Cyanophyta, including Aphanizomenon, has involved observations on animals in the field and in the laboratory and effects on man.

Field Investigations

Prescott (35) listed three ways by which Aphanizomenon has killed fish. First, it has become so abundant that it lowered the dissolved oxygen level during the night below that necessary to sustain life. Second, bacterial decay of the alga, disintegrating due to crowding, rapidly depleted the oxygen supply available for gill-bearing

animals. Third, was the possibility that the alga produced a poison.

There are a number of reports in the literature of the death of cattle after drinking water containing the alga. Early reports indicated that the death of cattle was due to their having drunk water containing algae because no more deaths occurred after the lake was fenced off (29). Another report stated that domestic animals were found dead after drinking water containing Aphanizomenon that had been concentrated near the shore by the wind (8).

A study of diseased ducks on a lake in Manitoba, showed that both Clostridium botulinum, and blue-green algae (including Aph. flos-aquae) were present. No definite conclusion could be reached as to whether either one or both were responsible for the death of ducks (2).

Laboratory Investigations

Prescott (36, 38) performed experiments to determine whether Aphanizomenon was producing a poison that could kill fish. He placed fish in water containing more than sufficient oxygen and large quantities of alga. Although the dissolved oxygen level stayed above that necessary to sustain life, the fish died. He found hydroxylamine to be present but no experiments were made on its toxicity.

Most laboratory investigations of toxicity have been made using

Aphanizomenon, (1) included in a mixed sample containing other algae, and (2) in what was believed to be a unialgal condition. No tests of toxicity have been made utilizing pure cultures of Aphanizomenon flos-aquae.

Samples of lake water and algae were used in tests on mice (8). A complete symptomology was included. Other reports indicated that mixed groups of algae were toxic to mice (28, 31) and rats (28). McLeod and Bondar (28) made comparisons between lake water containing algae and algal residue, and lake water with these removed. The results showed that death was due to the algae.

In one instance (33, p. 12), intraperitoneal injections of a lethal extract of algae killed mice. Furthermore, the inclusion of dried algal material as the protein source in the diet of small laboratory animals caused their death.

Only two studies reported tests made on toxicity of Aphanizomenon in a unialgal condition. In one, laboratory mice died following intraperitoneal injections of the alga (5). In the other study, investigations of toxins produced by Cyanophyta species indicated that Aphanizomenon possesses only the so-called "Slow Death Factor" (15).

Effect On Man

An excellent review by Schwimmer and Schwimmer (43)

discusses the effects of the Cyanophyta, and other algae, on man and his domesticated animals. The authors bring together widely scattered reports of items of interest in medicine which have appeared in non-medical journals. Included is a listing of instances of suspected algal-induced gastroenteritis.

Reports of human gastroenteritis have not been traced conclusively to algal sources (20). There is little doubt, however, that the blue-greens cause disagreeable tastes and odors (36). Probably this offensiveness accounts for the lack of reports of human gastroenteritis (51).

One interesting publication was that of a case of sensitization, resulting in contact type allergic dermatitis, to not only a species of Anabaena, but to phycocyanin extracts from the cells of this alga (4).

Laboratory Investigations On Aphanizomenon

Pigment Analyses

Various pigments have been reported (12) for Aph. flos-aquae including chlorophyll a, α-carotene (in small amounts), flavacin, aphanin, aphanizophyll, aphanicin and phycocyanin. The analyses for these pigments were made on material assumed to be unialgal. Four of the pigments (aphanicin, aphanin, flavacin and aphanizophyll) have been characterized (47), and a structural formula given for

aphanin (48). Rabinowitch (40, p. 478) mentioned that proteins associated with phycobilins are probably globulins with isoelectric points at pH 4 to 5, and that phycocyanin from Aphanizomenon had a isoelectric point of 4.75. He also stated that phycocyanin from Aphanizomenon flos-aquae had a molecular weight of 208,000.

Svedberg and Katsurai (45) found that the absorption spectra of phycocyanin from Porphyra tenera and Aph. flos-aquae were not identical though they had identical molecular weights.

Nitrogen Fixation

Aph. flos-aquae does not have the ability to assimilate elementary nitrogen (13, 52). Experiments made by Williams and Burris (52) on unialgal, bacteria-free cultures of the alga [obtained from Gerloff (14)] using N^{15} enriched N_2, indicated no ability to fix nitrogen.

Culture Techniques and Materials

The terms unialgal cultures and pure cultures are used here as defined by Bold (1). Unialgal cultures contain a single species of alga in the presence of other micro-organisms, while pure cultures contain only a single species of alga with no other organisms present.

A number of contributions have been made in the areas of isolation, purification and use of various media in the culture of Aphanizomenon.

1. Isolation

Attempts to use either the bacteriological technique of streaking on agar or a micromanipulator have proven unsuccessful for the isolation of Aphanizomenon flos-aquae according to Gerloff et. al. (14). The technique giving success was that of subculturing from a heavy suspension of mixed algae collected from the habitat. Repeated cultures were selected for best appearance, washed and examined until unialgal cultures were obtained.

A similar method employed by McLachlan et. al. (27), was to wash the material four to eight times in sterile water, and transfer it to flasks of medium thereby obtaining a unialgal culture.

Rose (41) obtained unialgal cultures of Aphanizomenon by germinating akinetes. Filaments from germinated akinetes were removed from the lake water in a vial, and transfered with a pipette to another vial before contaminating algae became abundant. He further stated that the akinetes would not germinate below temperatures of 8^{o} C and that akinetes collected in the summer failed to germinate, while those collected in December germinated readily.

2. Purification

Three treatments have been employed in attempts to obtain pure cultures of blue-green algae. One procedure suggested by

Pringsheim (39, p. 95) involved the use of a 25 p. p. m. solution of chlorine for two minutes. This solution had been used by Fogg (11) in the purification of Anabaena cylindrica. No report has been made by any investigator of the use of chlorine in attempts to purify cultures of Aphanizomenon.

Another treatment employed was antibiotics. McLachlan (25, p. 116-117) reported having no success with these materials in attempting to obtain pure cultures of Aphanizomenon. Tchan and Gould (46) have stated that the blue-green algae in some manner protect the bacteria. They noted that the bacteria could not be removed by using any single antibiotic of the many tested.

The most successful treatment used in purification experiments has been ultra-violet light. This was used in one instance to obtain a bacteria-free culture of Aphanizomenon flos-aquae (14). The light used was of the wave length of 2750 Å. The treatment period was from 5 to 30 minutes with samples being removed every 5 minutes. These samples were then diluted to increase the chances of obtaining filaments without bacterial contamination.

3. Media

Rose (41) reported success in growing Aphanizomenon in unialgal cultures. He found that redistilled water supported satisfactory growth. Other media, known at that time did not support growth

of Aphanizomenon.

Successful cultures of Aphanizomenon have been obtained using a modification of one of the media developed by Chu (14). McLachlan (24, p. 76) made further efforts with an improved (by Rodhe) Chu medium containing ferric citrate-citric acid complex as the source of iron. The alga was not, however, maintained in the characteristic flake form in either of these media (24, p. 53).

Still other media, notably ASM (modification of Provasoli's artificial sea water medium), ASMT and 11X, were tried (27). A soil extract was added to each of these media.

> After a prolonged period of growth in soil extract medium, all strains [of Aph. flos-aquae] became partially or completely non-colonial (27, p. 157).

The loss of iron from culture media containing chelated iron as sequestrene (EDTA) has been determined (21). After three months in the dark at pH 9, ten percent of the iron remained. Greater amounts remained at lower pH levels. At a pH of 6, there was no loss after three months in the dark. With a pH of 7, there was a loss of 18 % and at pH 8, a 30 % loss after three months in the dark. Although this report was concerned with vascular plants it has considerable significance for algal culture work.

Iron has been reported as having a definite effect on the size of Aph. flos-aquae flakes. Guseva (16) noted that large flakes of the alga were formed when it was grown in unialgal culture in sterile

medium with iron in concentrations of 0.2 to 0.5 mg/l. The large flakes broke down into smaller ones, however, at higher concentrations.

Experiments to determine optimum pH for culturing Aph. flos-aquae have been performed. McLachlan (25, p. 95) attempted to vary the pH of a modification of Chu's number ten medium to observe pH effect on the alga, but reported that none of the efforts were particularly successful. Trukhin (49) indicated that a pH of 8.0 gave best culture results for this alga.

MATERIALS AND METHODS

Materials

Experimental Algal Organism

The organism used in this study, Aphanizomenon flos-aquae, is a dominant form in a blue-green water bloom in Agency Lake and Upper Klamath Lake, Klamath County, Oregon. It appears annually associated with Anabaena circinalis, A. spiralis and Anacystis aeruginosa (Microcystis). In late summer, the alga commonly occurs in abundance only in Klamath Lake. The collections of Aphanizomenon for this investigation were made from the two lakes at various times during the summers of 1963-65.

Water, Salts and Glassware

Distilled water was used in all media. Early in the study, the distilled water was deionized, later, it was redistilled in an all glass still.

All salts used for sources of elements in the media, were of reagent grade.

Only borosilicate glassware was used in this study. Bottles used to contain stock mineral solutions, flasks used for stock cultures and flasks used in most growth experiments were washed in

water containing Dreft detergent, or RBS 5 Concentrated Cleaning Solution (Fisher). The glassware was then rinsed seven times in tap water and seven times in distilled water. Flasks used in experiments on the effect of phosphate were acid washed (3 N HCl), rinsed seven times in distilled water and three times in glass distilled water.

Chlorine

Chlorine used in attempts to obtain bacteria-free cultures, was prepared from a commercial preparation of sodium hypochlorite. Following the instructions on the bottle label, one tablespoon (15 mls) of the commercial solution in one gallon (5.64 l) of water gave a solution containing 200 mg. of chlorine per liter.

Methods

Collection

The alga was collected and concentrated by straining lake water through a no. 20 mesh, bolting silk net. The concentrated material was then placed in flasks or jars of (1) lake water; (2) micropore filtered lake water; or (3) sterile ASM or modified Rodhe's culture medium (Tables 2 and 3), and transported to the laboratory.

On one occasion, a transfer needle was used to remove single flakes of the alga from the lake. These flakes were then placed in

flasks containing sterile medium, and taken back to the laboratory.

In the laboratory, all colonies were washed in either micropore filtered lake water or sterile culture medium, and approximately 25 flakes were placed in each culture flask.

Isolation

Attempts were made to obtain unialgal cultures by removing individual colonies from the containers, with a sterile needle, and washing the flakes in two changes of sterile medium. The inoculating needle was bent in the form of a broad hook, rather than a loop, to minimize the transfer of unsterile medium, or lake water. Other attempts to isolate the alga consisted of a series of seven washes. Individual flakes were moved through the sterile medium rinses with a needle.

Sterilization of Media

All media used in the culture work were autoclaved for fifteen minutes at 20 pounds per square inch pressure. Media that were autoclaved were allowed to stand 48 hours to allow dissolved gases to come to equilibrium before algal material was transfered into them. Other items used in tests requiring a sterile technique were also autoclaved.

Culture Conditions

Initially, the stock cultures were maintained in 30 mls of medium in 125 ml Erlenmeyer flasks, with no more than 25-30 flakes per flask. Later, to conserve culture space, the stock cultures were maintained in 280 mls of medium in 1000 ml Erlenmeyer flasks, approximately 125 flakes being placed in each new flask at each transfer period.

The cultures were grown under several conditions of illumination. Some were grown in daylight in a north facing window without and with continuous supplemental light from Ken Rad warm white and deluxe warm white fluorescent tubes. Other cultures were grown in a converted upright food freezer which was equipped with two three tube, circular fluorescent fixtures. One fixture was mounted in the bottom, and one in the top of the cabinet. Here, red cellophane was used to reduce the intensity of the blue wave lengths. (Table 1 gives the approximate light intensities at each location).

Notice must be made that the light intensities in the window varied from day to day throughout the year, and that the values given in Table 1 can only be the order of magnitude of the intensities.

Table 1. Approximate light intensities under which Aphanizomenon was cultured

Location	Intensity (foot-candles)
Cabinet	ca. 100
Shelves (window)[1]	260-340
Shelves[2]	
Noon-heavy overcast	180-290
Noon-bright sunny	220-300
Night-supplementary only	90-120

[1]Shelves with no supplementary light from fluorescent tubes, readings taken August 6, 1964.

[2]Shelves with supplementary light from fluorescent tubes, readings taken in May, 1965.

Temperature was maintained between 13 and 15° C in the culture cabinet. In the window with supplemental light, this same temperature was obtained by immersing cultures in a water bath at 13°C. Water from a refrigerated water cooler was circulated through copper tubing lying in the bath to maintain the desired temperature. The flasks were immersed in the bath so that the water level on the outside of the flask was at the level of the medium inside the flask. In the window without supplemental light, there was no cooling, and the temperature was approximately 22°C, that of the room.

The best results, with regard to the culture of the alga, were obtained at 15° C in a north window with supplemental light from fluorescent tubes. All stock cultures are being maintained under these conditions. Currently, Aphanizomenon has resisted all attempts to grow it under light from fluorescent tubes alone. It is hoped this difficulty will be alleviated shortly.

Transfer Techniques

Stock cultures were transfered by pouring the contents of the flask into a petri dish and transfering the flakes of Aphanizomenon individually, with a sterile inoculating needle, to a second petri dish containing sterile medium. The flakes were rinsed by tilting the dish and agitating the medium. The individual flakes were removed with a sterile needle and placed in a new flask. The top of the flask was flamed before insertion, and after removal of the needle. The washing was omitted at times to prevent exposure to contamination from the air. When transfering the contents of large stock flasks (1000 ml Erlenmeyers), the material was first placed, a flake at a time, in a 125 ml Erlenmeyer with 30 mls of medium, and the contents of this flask were poured into the larger flask containing 250 mls of medium.

Determination of Growth

Algal Growth

One method of observing the effect of various experiments on the alga was to record the relative morphological changes in color, size, form, number of flakes and other general features. This was a purely arbitrary, subjective method entirely dependent upon the observer. The increase in the number of flakes was taken as an indication of growth.

A second method utilized was measuring growth by determining the increase in dry weight. This entailed harvesting the alga on glass fiber filters followed by drying in a desiccator. Prior to the harvest, the glass fiber filters were dried for 24 hours in the desiccator, and weighed. Following the harvest, the filters and alga were dried for 48 hours and weighed. The difference in weight was noted as dry weight of alga.

Bacterial Growth or Presence

A dilution series was made to find out what dilution would give the optimum plate count (7, p. 492-493) of between 30 and 300 colonies per plate. All dilutions between one and one/one millionth were tried and it was determined that no dilution was required. One ml

was withdrawn from a medicine bottle containing remains of three flakes of Aphanizomenon that had been dispersed by vigorous shaking. This one ml sampling technique was used to check the effect of experimental treatments to eliminate bacteria from cultures.

To prepare plates for counting, the alga flakes were placed in screw cap medicine bottles (8 oz.) containing 100 mls of sterile medium. The bottles were shaken vigorously to break up the flakes and filaments and disperse bacterial cells lying between the algal filaments. Following shaking, the optimum volume of the suspension (1 ml) was withdrawn with a sterile pipette and placed in a petri dish. In early procedures, Difco nutrient agar (23 g/l) was then poured into the plate. Later, this agar was supplemented with 5 g of glucose per liter of agar. All plates were incubated at temperatures of approximately 22^{o} C in the laboratory, or 28^{o} C in an incubator, for 48 hours before counting. Counts were made directly by the unaided eye.

EXPERIMENTAL PROCEDURES AND RESULTS

Basic Media Used

The four media used at various times in the culture work were ASM (26) (McLachlan's modification of Provasoli's artificial sea water medium ASP), Rodhe's (24, p. 76), Gerloff's modification of Chu's number ten (14) and Peek's[1]. (See Tables 2 and 3).

ASM Medium

ASM was never used without modification in attempts to culture Aphanizomenon. This medium was amended sometimes with Tris, or modified by addition of humic extract or garden loam, and was used in primary isolation attempts.

Rodhe's Medium

As used in this study, Rodhe's had a pH of 6.6. This medium and modifications of it, were used in preliminary isolation attempts after collection of the alga.

The algal flakes placed in Rodhe's and modified Rodhe's became colorless, floating, amorphous masses in the bottom of the flasks within twelve days of inoculation. This time was shortened to five days in the case of the biphasic cultures.

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[1]The composition of this medium was obtained from Dr. Chester A. Peek early in 1964.

Table 2. Concentration in mg/l of medium of the major nutrient minerals in the basic media

Element	Compound	Medium			
		ASM	Rodhe's	Gerloff's	Peek's
S	$MgSO_4$	49.26	5.0	-	-
S	Na_2SO_4	-	-	41.3	8.85
Mg	$MgCl_2.6H_2O$	40.65	see above	20.9	17.55
Ca	$CaCl_2.2H_2O$	14.71	-	35.9	18.32
Ca	$Ca(NO_3)_2.4H_2O$	-	60.0	-	-
N	$NaNO_3$	85.034	-	41.3	122.00
N	$Ca(NO_3)_2.4H_2O$	-	see above	-	-
K	K_2HPO_4	17.42	5.0	-	-
K	KCl	-	-	8.6	3.82
P	K_2HPO_4	17.42	5.0	-	-
P	$NaH_2PO_4.H_2O$	-	-	8.2	8.90
Si	$NaSiO_3.9H_2O$	-	20.0[1]	58.2	16.72
C	Na_2CO_3	-	-	20.0	-
Na	$NaSiO_3.9H_2O$	-	20.0[1]	see above	see above

[1] omitted from medium as used here.

Table 3. Concentration in mg/l of medium of the minor nutrient minerals in the basic media

Element	Compound	Medium			
		ASM	Rodhe's	Gerloff's[1]	Peek's
Fe	$FeCl_2$	0.54	-	-	-
	Na_2EDTA	3.00	-	-	-
Fe	Ferric Citrate	-	1.0	3.0	1.11
	Citric Acid	-	1.0	3.0	1.11
B	H_3BO_3	0.62	-	-	-
Mn	$MnCl_2$	1.40	-	-	-
Mn	$MnSO_4 . H_2O$	-	0.03	-	-
Zn	$ZnCl_2$	0.1	0.1	-	-
Co	$CoCl_2$	0.005	-	-	-
Cu	$CuCl_2$	0.000034	-	-	-

[1]Most minor elements are in Hoagland's A-Z solutions which are added as one ml of a 1 to 25 dilution/liter of medium.

Gerloff's Medium

Gerloff's medium is a physiologically unbalanced modification of Chu's number ten medium with a pH of 8.1 after autoclaving. Flakes of Aphanizomenon from cultures in ASM medium and humic extract were used in two tests of the ability of Gerloff's to support growth. The material was checked every two days in both experiments.

For the first test, the transfers were placed in a north window (at ca. $22^{o}C$) and in the culture chamber. Some flakes showed clumping, or formation of amorphous masses, while other colonies became long and stringy. The remaining flakes, which appeared normal, were transfered at the end of the second week. During the third week these colonies maintained normal color and morphology. The flakes exhibited a general decline during the fifth week, becoming amorphous and chlorotic. These cultures were subsequently discarded.

In the second test, three flasks of material were placed in the culture chamber. The colonies in one flask had become amorphous, and in another the flakes were becoming chlorotic by the end of the first week. Ten days after inoculation, the material in all three flasks was yellowing and that in one flask was lysing. By the

fourteenth day, Aphanizomenon in all flasks was chlorotic, and in two flasks the colonies were amorphous clumps. The second test was terminated at this point.

Peek's Medium

Peek's medium was prepared with a humic extract base (one part humic extract to five parts distilled water). The pH of this medium after autoclaving was 8.0. Algal material from ASM medium and humic extract was transfered into flasks of this medium for a test of its effect. These flasks were put on the shelf in the window at ca. 22°C.

After one week in Peek's medium, the flakes of Aphanizomenon lost their original form and became tight rather pale green balls resting on the bottoms of the flasks. At the end of two weeks, the flakes were yellow and still clumped on the bottom. No further attempt was made to use this medium.

Amended Media

Addition of Tris

Trishydroxymethylamino methane (Tris), in the amount of 0.48 mg/l, was added as a buffer to ASM (as a consequence referred to as ASMT). This medium had a pH of 8.9 after autoclaving.

At the beginning of this study, flakes of Aphanizomenon cultured in ASMT showed deterioration after only five days. This deterioration consisted of a loss of the colonial flake form and some lysis. In an attempt to halt deterioration, the concentration of nutrient salts in ASMT was decreased to 50 and 25 % by the addition of distilled water. At these concentrations, the colonies retained flake form and a pale green color for 27 days. There was no evidence of growth, however, as the flakes did not increase in number.

Addition of Yeast Hydrolyzate and Yeast Extract

Yeast hydrolyzate and yeast extract (1% solutions) were added, individually and together, to ASMT, as organic supplements to determine their effect on the growth, color or form of the alga. The amended medium was placed in screw cap culture tubes and inoculated with Aphanizomenon flakes. The tubes were put in the culture chamber and on the continuously illuminated shelf in the window at 15°C.

The algal flakes placed in ASMT containing yeast hydrolyzate, yeast extract or both, disappeared within three days of inoculation.

Modified Media

Two of the basic media (ASM and Rodhe's) were modified by addition of humic extract or garden loam (to make a biphasic culture).

Preparation of the Humic Extract

The water from which the supplemental humic extract was obtained was taken from the Williamson River at the bridge on the road to Kirk, Oregon. The water was collected in five gallon carboys. At Corvallis, the water was put in open plastic containers and placed in the sharp freeze room of a cold storage facility. On freezing, the humic colloids were forced to the center of the ice block. The clear ice was chipped away, and the water containing concentrated colloids and suspensoids (approximately a 3 to 5 fold concentration) was stored in a plastic carboy at 3°C.[2]

Modified ASM Medium

The amount of humic extract added to ASM varied from 25 to 200 mls in a liter of medium. Early in the study, 100 and 200 mls of humic extract in a liter of ASM were used for the stock cultures. The pH of the medium containing these amounts of extract was 7.4 and 7.6 respectively. Material collected from the lake and placed in ASM containing 100 and 200 mls of humic extract was grown for a number of months. Later, to conserve humic extract, the alga was grown in ASM and 100 mls of humic extract as growth in this medium was

[2]Details of this procedure were obtained from Dr. H. K. Phinney on August 16, 1965.

approximately equal to that in ASM and 200 mls of extract.

Tris, in the amount of 0.48 mg/l of medium, was added to ASM medium and humic extract to act as a buffer. The resultant medium had a pH of 9.1. This medium, however, caused the Aphanizomenon flakes to lose their morphological form and become amorphous masses in the bottoms of the flasks.

Twenty mg of sodium bicarbonate per liter of ASM and humic extract were added to provide an additional source of carbon dioxide and to also act as a buffer. The pH of the medium was then approximately 7.8 to 8.0. This amount of sodium bicarbonate appeared to improve the condition and growth of the alga and it was retained throughout the study as a component of this medium and subsequently of ASMT, and its modifications.

Newly transfered cultures began to lyse after several months culture in ASM modified by the addition of humic extract and sodium bicarbonate. Since the pH of the medium was unchanged, altered pH was not responsible for the lysis. As the humic extract was being supplied in the amount of 100 mls in a liter of ASM, new culture medium was prepared containing 200 mls in a liter. All material transfered to medium containing this increased amount of extract showed lysis.

Flasks of ASM medium were then prepared containing humic extract in concentrations of 75, 50, 40 and 25 mls in a liter.

The alga showed lysis at 75 and 50 mls in a liter and poor conditions at the lower concentrations. It thus was reasoned that long storage had resulted in some toxic change in the extract and its use was discontinued and material was transfered to ASMT (50 and 25%).

Biphasic cultures were prepared by adding garden loam in a 1 to 3 ratio to ASM medium. The pH of the resulting biphasic culture was approximately 5. 0. The loam served as a source of organic materials and additional salts. Within five days after inoculation, no Aphanizomenon was present in flasks of biphasic cultures.

Modified Rodhe's Medium

Rodhe's was modified by the addition of 100 mls of humic extract in one liter of medium, resulting in a pH of 8. 2. The ionic concentration of Rodhe's medium and humic extract was also diluted to 50 and 25%, by the addition of distilled water. Biphasic cultures were also prepared by adding garden loam in a 1 to 3 ratio to the medium. The pH was approximately 4. 5.

As noted earlier, the algal flakes placed in these modifications became colorless, floating, amorphous masses in the bottoms of the flasks.

Modifications of ASMT to Obtain Growth Media

The stock culture medium was developed by modifying McLachlan's modification (ASM) of Provasoli's artificial sea water medium (ASP). The original modification by McLachlan consisted of reducing the ionic concentrations to a level that would support fresh-water species, by omission of sodium chloride, and additions of Tris as a buffer (ASMT).

Following the observation of lysis in ASM medium plus humic extract, all algal material was cultured in ASMT with sodium bicarbonate added. The concentrations of certain individual nutrient mineral compounds or groups of compounds were varied independently (Table 4) to determine the relative proportions giving improved growth compared to that obtained in ASMT. The compounds chosen to be varied were those containing elements believed to have a more direct effect on the alga (i. e. phosphorous, nitrogen, magnesium and minor elements). These trials were performed using 10 mls of culture medium in 10 x 150 mm screw cap test tubes. Arnon's trace element solutions B and C were added to the ASMT medium for the first time in this test. These solutions were employed in a 1 to 25 dilution ratio as recommended by McLachlan (25, p. 40). One ml of both stock solutions was added to 23 mls of distilled water. Three

mls of the resultant dilution were added to one liter of medium to give the 1 X concentration. This amount was then varied to observe the effect on the growth and maintenance of Aphanizomenon (Table 4).

Table 4. Component nutrient compounds in ASMT varied individually to study the effect on Aphanizomenon

Compound or groups of compounds varied	Variation tried
K_2HPO_4	1/4, 1/2, 1, 2 X
$MgCl_2$	1/4, 1/2, 1, 2 X
Na_2NO_3	1/4, 1/2, 1, 2 X
Tris	1/4, 1/2, 1, 2 X
Minor elements	1/4, 1/2, 1, 2 X
Arnon's trace elements (solutions B and C)	1/4, 1/2, 1, 2 X

From observations of cultures over a period of three weeks, concentrations were selected which gave best results on the basis of color, and general morphological appearance of the flakes (Table 5).

Table 5. Individually varied concentrations of compounds in ASMT giving best growth and maintenance of Aphanizomenon

Compounds or groups of compounds	Concentration			
	1/4 X	1/2 X	X	2X
K_2HPO_4	+	=	=	+
$MgCl_2$	=	+	+	+
$NaNO_3$	*	*	*	*
Tris	=	+	+	=
Minor elements	+	=	+	+
Arnon's trace elements	=	+	+	+

Code:

(+) Flakes of alga maintained normal form and/or increased in number.

(=) Flakes of alga did not maintain normal form or show any sign of increase in number.

(*) No appreciable difference was observed between flakes of alga in various concentrations of nitrate nitrogen.

There are 169 possible combinations of the 13 concentrations which gave good results as indicated in Table 5. Ten of these combinations[3] were arbitrarily selected to test their effects on the

[3]After this experiment was performed, the two combinations ASMT Nos. 4 and 5 were noted to be identical.

growth and condition of the alga (Table 6 gives the combinations, and Table 7 gives the actual concentration of compounds in each combination).

Table 6. Combinations of concentrations in the growth media ASMT Nos. 1-10

	Compounds or groups of compounds[1]				
ASMT No.	Arnon's trace	K_2HPO_4	$MgCl_2$	Tris	Minor Elements
1	1/2 X	1/4 X	2 X	1 X	1 X
2	1/2 X	1/4 X	2 X	1 X	1/4 X
3	1/2 X	2 X	2 X	1 X	2 X
4	1 X	1/4 X	1/2 X	1/2 X	1 X
5	1 X	1/4 X	1/2 X	1/2 X	1 X
6	2 X	1/4 X	1/2 X	1/2 X	1 X
7	1/2 X	1/4 X	1/2 X	1/2 X	1 X
8	1 X	1/4 X	2 X	1/2 X	1 X
9	2 X	1/4 X	2 X	1/2 X	1 X
10	1/2 X	1/4 X	2 X	1/2 X	1 X

[1]The concentration of $NaHCO_3$ was maintained at 20 mg/l while $MgSO_4$, $CaCl_2$ and $NaNO_3$ were as in ASM (Table 2).

Table 7. Concentrations in mg/l of medium of compounds in ASMT and modifications of ASMT, Nos. 1-10

Compounds	ASMT	Modifications									
		1	2	3	4	5	6	7	8	9	10
$MgSO_4$	49.26	49.26	49.26	49.26	49.26	49.26	49.26	49.26	49.26	49.26	49.26
$CaCl_2$	14.71	14.71	14.71	14.71	14.71	14.71	14.71	14.71	14.71	14.71	14.71
$NaNO_3$	85.034	85.034	85.034	85.034	85.034	85.034	85.034	85.034	85.034	85.034	85.034
$NaHCO_3$	-	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
$MgCl_2 \cdot 6H_2O$	40.65	81.20	81.30	81.30	20.32	20.32	20.32	20.32	81.30	81.30	81.30
K_2HPO_4	17.42	4.36	4.36	34.84	4.36	4.36	4.36	4.36	4.36	4.36	4.36
Minor Elements[1]	10.00	10.00	2.50	20.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Trace Elements[2]	-	1.67	1.67	1.67	3.34	3.34	6.68	1.67	3.34	6.68	1.67
Tris	0.48	0.48	0.48	0.48	0.24	0.24	0.24	0.24	0.24	0.24	0.24

[1] Minor element concentrations are given in mls of a stock solution. This solution contains concentrations of each compound such that 10 mls of it added to one liter of medium would give the concentrations shown in Table 3.

[2] Arnon's trace element solutions B and C. a 1 to 25 dilution.

These combinations were numbered 1 to 10 for purposes of notation in subsequent tests, and will be denoted as ASMT Nos. 1-10, since they were modifications of the ASMT medium. The ASMT Nos. 1-10 combinations were called growth media. Approximately uniform flakes of Aphanizomenon were transfered from flasks of ASMT into 30 mls of each of these media in 125 ml Erlenmeyer flasks, and replicates were placed in the culture chamber and on the shelf in the window at 15°C. Observations were made on the cultures for a period of three weeks.

The results of these experiments indicated that four combinations, ASMT Nos. 1, 7, 8 and 9, were most suitable for growing Aphanizomenon.

Further tests were then made on ASMT Nos. 1, 7, 8 and 9 using fresh material obtained from Klamath Lake. The material placed in ASMT Nos. 1 and 7 showed loss of flake form after one week. These two were therefore eliminated as possible stock media. The material in the other two media, ASMT Nos. 8 and 9, showed growth and maintenance of form. They were used for stock cultures and tests in all phases of the culture experiments. Over a long period of culture, flakes in ASMT No. 8 seemed to increase in number more consistently than those in ASMT No. 9, and eventually most of the material was grown in the former. The latter was finally eliminated and all material growing in it was transfered to ASMT No. 8.

Aphanizomenon collected from Klamath Lake has been in culture in ASMT No. 8 for 14 months. The form, color and general condition of the alga have been maintained, and appear somewhat improved over the condition at the time of initial collection from the lake.

Two growth curves were made to determine the rates of growth of Aphanizomenon in ASMT No. 8. Fifteen flakes of alga were placed in each flask of medium and the flasks were randomly placed in the window at 15°C. For the first determination, three flasks were chosen randomly for harvest every two days until the nineteenth day and every four days thereafter. Three flasks were chosen at random for harvest every six days for the second curve. Determination of growth was made from dry weights, and graphs drawn representing the log of dry weight versus time.

As can be seen from the first curve (Figure 1), no leveling off of the log phase of growth occurred. Harvests were made every four days beginning with day 19, in an attempt to obtain a leveling off of growth before all of the material set aside for this experiment was harvested.

In the second experiment (Figure 2), a decrease in rate of growth began at day 25. In future experiments on variation of the concentrations of elements, all harvests of test material were made on day 17 to assure inclusion in the log phase of growth as determined from figure two.

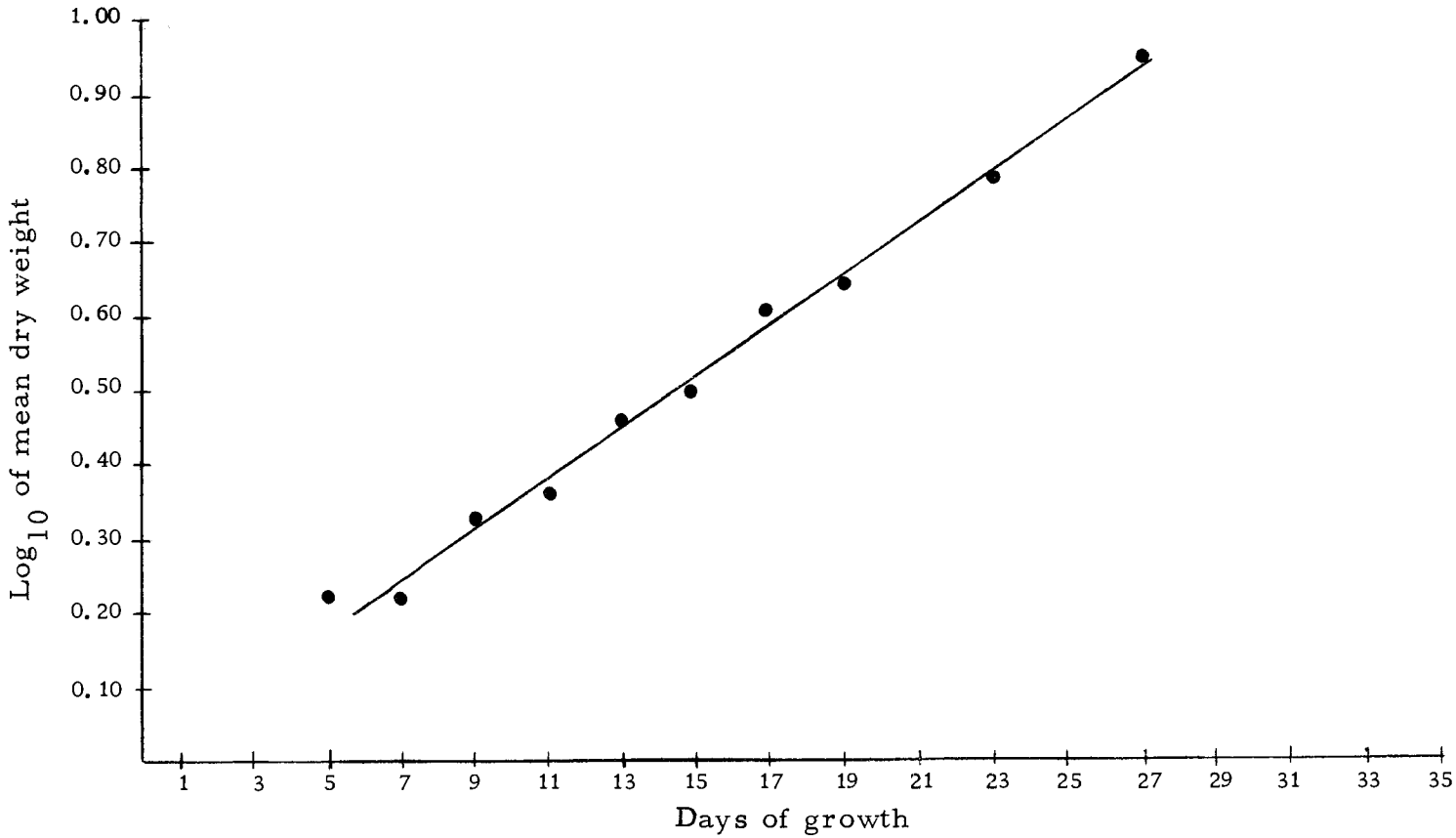

Figure 1. Curve of growth of Aphanizomenon in ASMT No. 8

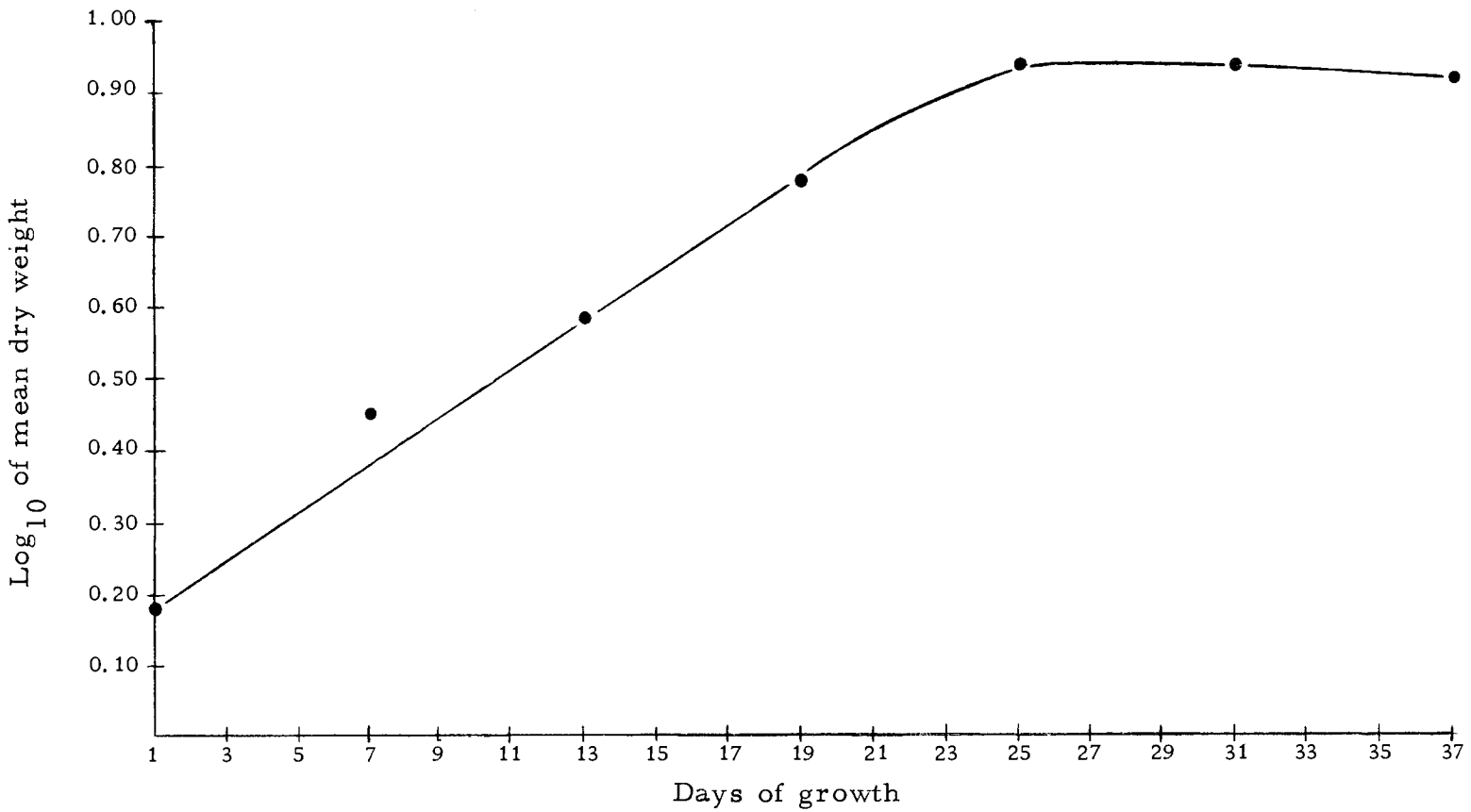

Figure 2. Curve of growth of Aphanizomenon in ASMT No. 8.

Modification of Growth Media ASMT Nos. 1-10

Addition of Resins

Ion exchange resins were added to ASM, ASMT, modifications of ASMT (growth media) and Gerloff's medium to reduce the frequency of transfering stock cultures and to observe their effect on the growth and condition of the alga. The resins were of two types, a hydrogen ion type and a hydroxyl ion type.[4] The object was to add the two types of resins to the medium, where the ions (H^+, OH^-) would be replaced on the resin beads by positive and negative ions from the medium and an equilibrium would thus be set up. As the alga utilized the ions remaining in the medium for its metabolism, more ions would be released from the resin beads. This release would allow for a continuous source of nutritional ions for a longer period of time than could be supplied by a medium without added resins.

The two species of resins were placed in suspension in glass distilled water. Each ml of hydrogen ion resin suspension would exchange 3.5 milliequivalents of ions. One ml of hydroxyl resin suspension would exchange 2.0 milliequivalents of ions. The milliequivalents of each ion in each nutrition compound (i.e. $NaNO_3$)

[4]The hydrogen ion type was a weak cation exchange resin termed Amberlite CG (chromatographic) 50 Type 2. The hydroxyl type was a weak anion exchange resin termed Amberlite CG 45 Type 2.

were calculated. The calculation of milliequivalents was accomplished by dividing the mg of the ion, or ionic group in the medium, by the mg/meq. for that particular group. The amounts of resin to be added to the various media are given in Table 8.

Table 8. Amounts of chromatographic resins used in various media

Resin	Medium								
	ASM	ASMT	1	5	7	8	9	10	Gerloff's
$(H^+)^1$	1.40	1.40	1.76	1.34	1.53	2.01	2.51	2.51	0.33
$(H^+)^2$	0.04	0.04	0.05	0.03	0.05	0.06	0.08	0.08	0.01
$(OH^-)^1$	1.82	1.82	2.34	1.75	1.75	2.59	3.09	3.09	0.91
$(OH^-)^2$	0.06	0.06	0.07	0.05	0.05	0.08	0.09	0.09	0.03

[1]mls of resin suspension for one liter of medium.

[2]mls of resin suspension for 30 mls of medium.

Determinations of the ionic concentrations contained in ASM made with a conductivity bridge, before and after addition of the resins, indicated an increase in resistance of the medium as ions were taken up by the resins. The pH of ASM was 6.8 and that of ASMT was 7.0 after addition of the resins. One flask each of ASM, ASMT, Gerloff's, ASMT Nos. 1 and 5 and two flasks each of ASMT Nos. 7, 8 and 9 were prepared by adding the respective amounts of resin in 30 mls of medium (Table 8). These flasks, containing flakes of alga, were placed on the 15°C shelf in the

window. Examinations of the algal material were made over a 25 day period.

The algal flakes lost their form after 23 days in ASM, 15 days in ASMT, 11 days in Gerloff's, 17 days in ASMT No. 1, 17 days in ASMT No. 5, 9 days in ASMT No. 7 and 17 days in ASMT No. 9 media and resins. The flakes became amorphous masses and never recovered their natural appearance. In ASMT No. 8 and resins, however, the flakes maintained their form and a healthy green color, without being transfered, for 25 days. At this time, the flakes lysed and the experiment was terminated. There appeared to be no difference between conditions in the flasks of ASMT No. 8 alone, and with resins; therefore, no further attempt was made to employ the resins.

Varying pH

A study was made of the effect of varying the pH on the growth of the alga. The ninth modification of ASMT (Table 7) was used for this experiment. The basic pH of this modification 48 hours after autoclaving was 7.3. The pH was adjusted by adding 0.005 ml of a 0.01 N NaOH solution to raise the pH 0.1 unit, or by using 0.02 ml of a 0.01 N HCl solution to lower the pH 0.1 unit. Flakes were transfered into flasks with the following pH levels; 7.0, 7.1, ... 8.0 and 8.5. Three replications at each level were made. A pH of 7.7 gave the best maintenance and increase in the number of flakes of the

alga (Table 9). A similar study of the effect of variation of pH on the growth and maintenance of Aphanizomenon has still to be made using ASMT No. 8.

Table 9. Relation of growth and condition of Aphanizomenon to pH of ASMT No. 9

	Days after inoculation				
pH	3	7	11	14	19
7.0	=1, -2	-1, +2	=3	=3	=3
7.1	-2, +1	=1, +2	+3	+3	=3
7.2	-2, +1	-2, ++1	=3	0	0
7.3	-1, +2	=3	=2, -1	0	0
7.4	-3	-3	=2, +1	0(2), -1	=1
7.5	-3	-3	+3	-2, +1	=3
7.6	-1, +2	-3	-1, +2	-2, +1	=2, -1
*7.7	-2, +1	-1, ++2	++3	-1, +2	=1, ++2
7.8	-3	=1, +2	-1, +2	-2, ++1	=2, -1
7.9	-3	-3	-3	-3	0(2), =1
8.0	-1, ++2	-2, +1	=2, +1	0(2), -1	=1
8.5	+3	=1, -2	=2, -1	0(2), -1	0

Code:

(++) The flakes of alga maintained their normal form and increased in number.

(+) The flakes maintained their normal morphological form.

(=) All of the flakes lost their normal morphological form.

(-) Some of the flakes lost their normal morphological form.

(0) Flasks were discarded.

Number shows the number of flasks exhibiting the above characteristics.

*Apparently gave the best growth and maintenance of the alga.

Varying Sources of Iron

The effect of various sources of iron on the alga was determined by using iron-sequestrene (ferric versene complex), ferric citrate-citric acid and disodium ethylenediaminetetraacetate (EDTA) ferric chloride. Two experiments were performed in which each of several 100 ml lots of ASMT No. 8 minus iron were supplied with a different iron source in an amount sufficient to supply the iron normally present in this medium.

In the first experiment, the iron was supplied in the amounts given in Table 10. After the addition of the iron, 10 mls of the medium were placed in each of 3, 10 x 150 mm, screw cap culture tubes. Flakes of Aphanizomenon were placed in each tube, and the tubes were placed in the window on the 15°C shelf. Visual observations of the material were made every day for the first week, and every other day for the second week.

Table 10. Sources of iron varied in ASMT No. 8 to observe the effect on Aphanizomenon

Source	Concentration
Ferric Chloride	Added 0.3 mg of Na_2EDTA and 0.5 mg of $FeCl_3$ to 100 mls of ASMT No. 8 minus iron
Sequestrene	Added 0.133 mg of Sequestrene (for iron requirement) to 100 mls of ASMT No. 8 minus iron
Ferric Citrate	Added 0.05 mg each of Ferric Citrate and Citric Acid to 100 mls of ASMT No. 8 minus iron
None	No additions

Material placed in ferric citrate-citric acid and zero iron did not maintain normal colonial flake form and was eventually discarded. (Table 11).

Table 11. Effect of various sources of iron in ASMT No. 8 on Aphanizomenon: Trial 1

Source	Day 2	7	10	13	15
No iron	+3	-3	=3	=3	0
Sequestrene	+3	++2, +1	++2, +1	+2, 0(1)	+1, 0(1)
Ferric Citrate-Citric Acid	+3	+1, -2	=3	=3	0
Na_2EDTA-$FeCl_3$	+3	+1, -2	+2, -1	=2, +1	+1, 0(2)

Code: (++) The flakes of alga maintained their normal form and increased in number.

(+) The flakes maintained their normal morphological form.

(=) All of the flakes lost their normal morphological form.

(-) Some of the flakes lost their normal morphological form.

(0) Flasks were discarded.

Number shows the number of flasks exhibiting the above characteristics.

In the other two sources, the alga was maintained, and the material in sequestrene appeared to be in slightly better condition.

The second experiment repeated the first one except that iron was added in a more accurate manner (Table 10) and 10 mls of the

medium with each iron source were placed in three 50 ml Erlenmeyer flasks. Observations were once more of a qualitative nature and made for the same time intervals as those in the first trial.

In the second test (Table 12), disodium EDTA-ferric chloride gave a better maintenance of the alga, and it was used as the iron source in ASMT No. 8.

Table 12. Effect of various sources of iron in ASMT No. 8 on Aphanizomenon: Trial 2

	Day			
Source	3	6	9	13
No iron	-3	=3	=3	Nothing visible
Sequestrene	++1, +2	-1, +2	++1, +2	=3
Ferric Citrate-Citric Acid	=1, +2	=1, -1, +1	=2, +1	=1, 0, +1
Na_2EDTA-$FeCl_3$	+3	+3	+3	+3

Code: (++) The flakes of alga maintained their normal form and increased in number.

(+) The flakes maintained their normal morphological form.

(=) All of the flakes lost their normal morphological form.

(-) Some of the flakes lost their normal morphological form.

(0) Flasks were discarded.

Number shows the number of flasks exhibiting the above characteristics.

Varying the Concentration of Nitrate Nitrogen

The inoculum for the experiment on variation of nitrogen concentration was obtained from stock cultures maintained in 30 mls of medium in 125 ml Erlenmeyer flasks. The algal material from the stock flasks was randomly assigned to the various concentrations of nitrogen. The following concentrations of nitrogen were used; 0.0 mg/l $NaNO_3$(OX), 21.0 mg/l (1/4 X), 42.0 mg/l (1/2 X), 84.0 mg/l (1X), 168.0 mg/l (2X), and 336.0 mg/l (4X), with three replications for each concentration. The algal flakes of the inoculum were not grown in medium minus nitrogen prior to inoculation into the test flasks.

The pH of each flask was noted prior to autoclaving, and 48 hours after autoclaving, the pH of a flask of ASMT No. 8 was taken (Table 13).

After a 17 day period of growth at 15°C in the window, the alga was harvested and dry weights recorded (Table 13). The pH of the pooled filtrate from the replicate flasks of each concentration was also determined (Table 13). Qualitative observations made at the time of harvest, showed that the morphological appearance of all flakes in various concentrations of nitrogen was approximately the same, except O X in which the flakes appeared smaller. One flask at the 1 X concentration was lysed.

Table 13. Effect of varying the concentration of nitrate nitrogen in ASMT No. 8 on growth of Aphanizomenon

Concentration	pH before autoclaving and inoculation	pH after 17 days growth	Mean dry weight of alga (mg)
O X	6.8	7.0	2.33
1/4 X	6.8	7.1	4.43
1/2 X	6.8	7.2	5.54
1 X ASMT No. 8	6.9, 7.3[1]	8.0[2]	4.45[2]
2 X	6.8	8.1	4.23
4 X	6.8	7.9	5.03

[1]Reading taken 48 hrs after autoclaving on a flask not actually used in the experiment.

[2]Determinations made on only a two flask sample, as the material in the third flask lysed.

As Table 13 shows, there was quite a range of pH values. It was felt, however, that since the lower concentrations (0 to 1/2 X) appeared to show little difference in pH, and yet a large difference in dry weight (according to the sample means), that the experimental error would not be too great if an analysis was made of these data. The difference in sample means between O X and 1/4 X concentrations was about 2:1 while there was a corresponding difference of

only a tenth of a pH unit.

An analysis of variance was made to compare growth at the various concentrations of nitrogen (Table 14). This analysis was on a completely randomized experiment as the algal flakes in the inoculum were from a number of stock flasks. It was made with unequal sample sizes (22, p. 193-197) because of the lysis of material in a flask of the 1 X concentration.

From this analysis of variance, it would appear that there was no difference in the growth of the alga that could be related to changes in concentration of nitrate. Without further experiments, no conclusions can be drawn from this information due to the difference of pH levels. Certain interesting questions can be asked, however, in regard to these data. (1) Was the result obtained because of lack of preliminary growth of the alga in minus nitrate medium, and thus was nitrogen carried over to the test flasks by the flakes of inoculum, or (2) could Aphanizomenon be fixing nitrogen contrary to a report by Williams and Burris (52)? These are questions that will be subjects of further experiments.

Table 14. Analysis of variance of the data from the growth of Aphanizomenon in ASMT No. 8 containing various concentrations of nitrate nitrogen

	Sample					
	0 X	1/4 X	1/2 X	1 X	2 X	4 X
Observations (mg dry weight)	3.0 2.6 1.4	6.3 3.6 3.4	5.9 4.4 6.3	- 5.7 3.2	2.4 4.7 5.6	5.3 6.5 3.3
Sample Total	7.0	13.3	16.6	8.9	12.7	15.1
Sample Number	3	3	3	2	3	3
Sample Mean	2.33	4.43	5.54	4.45	4.23	5.03

Preliminary Calculations

Type of Total	Total of Squares	No. of Items Squared	Total of Squares per Observation
Grand	$G^2 = (73.6)^2$	1	318.633
Sample	-	-	336.522
Observation	-	-	357.960

ANALYSIS OF VARIANCE

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio
Among-Sample	17.889	5	3.578	1.836 with 5 and 11 d.f.
Within-Sample	21.438	11	1.949	
Total	39.327	16		

Hypothesis: The means of all the concentrations are equal and equal to zero. That is, there is no difference in growth with changes in concentration of nitrate nitrogen.

Alternate Hypothesis: At least one of the means is not equal to the others.

Test using a F-test. Tabular F value with 5 and 11 d.f. at 99% confidence level, is 5.3160, therefore, since the calculated F value (1.836) is less than the tabular value, the hypothesis is accepted (all are equal).

Varying the Concentration of Phosphate Phosphorous

The effect of variation in concentration of phosphate phosphorous was determined in two separate trials. The procedure for both trials was the same except that the pH was adjusted in the second test. The material to be used in these experiments was transfered from a large stock culture to a 1000 ml Erlenmeyer containing ASMT No. 8 minus phosphate phosphorus. The material was maintained in this flask for three days prior to inoculation into the test flasks. The concentrations of phosphate phosphorous were the same for both trials (Table 15).

1. Trial One

In the first trial Aphanizomenon was grown in three replicates of each concentration of phosphate (Table15). Precipitation of phosphate by tris has been reported (26) and a control series of the 1/4 X concentration was prepared by aseptically adding the proper amount of an autoclaved phosphate solution to autoclaved flasks containing ASMT No. 8 minus phosphate. This experiment was performed although no precipitate had been observed in ASMT No. 8 at anytime.

Since no pH was recorded prior to autoclaving, a new set of flasks was made up exactly the same way as the first group, and the pH taken 48 hours after autoclaving (Table 16).

Table 15. Concentrations of phosphate phosphorous supplied in ASMT No. 8

Concentration	Supplied as: K_2HPO_4(mg/l)	$NaH_2PO_4 . H_2O$(mg/l)	KCl(mg/l)[1]
O X	none	none	none
O X	none	none	8.0
1/4 X control[2]	4.0	none	none
1/4 X	none	4.0	8.0
1/4 X[3]	4.0	none	none
1/2 X	none	8.0	8.0
X[4]	none	16.0	8.0
2 X	none	32.0	8.0

[1]Equivalent amounts of KCl were added in cases where the potassium was either reduced, lacking or would be increased due to the variation of the phosphate.

[2]Concentration in ASMT No. 8.

[3]Autoclaved separately, and added aseptically.

[4]Equivalent to the concentration of phosphate phosphorous in the original ASMT medium.

Fifteen flakes of approximately uniform size and condition were transfered to each flask in the first set. The flasks were then placed randomly in the window at 15°C. Periodic observations were made during the 17 day growth period, and the appearance of the material noted. The material was harvested, and the dry weight recorded (Table 16). The pH of the filtrate of each flask was also determined at the time of harvest (Table 16).

Prior to harvesting the material, comparisons for a least significant difference (L. S. D.) test (22, p. 265-270) were made. It was advisable to compare 1/4 X (K_2HPO_4) with the other concentrations as this is the concentration found in ASMT No. 8.

From Table 16 it is apparent there was a great deal of variation of pH values between O X, 1/4 X autoclaved separately and the others, but not, however, between two of the 1/4 X (ASMT No. 8 and NaH_2PO_4) and the 1/2 to 2 X concentrations. It would be applicable, therefore, to analyze these five latter variations by an analysis of variance for equal sample sizes (22, p. 176-180) (Table 17).

Table 16. Effect of varying the concentration of phosphate phosphorous in ASMT No. 8 on the growth of Aphanizomenon: Trial 1

Concentration	pH[1] after autoclaving	pH[1] after 17 days growth	Mean dry weight of alga (mg)
O X	7.0	6.8	1.83
O X (Table 15)	7.1	6.9	1.70
1/4 X (ASMT No. 8)	7.3	9.0	5.37
1/4 X ($NaH_2PO_4 . H_2O$)	7.0	9.2	5.77
1/4 X autoclaved separately	7.3	7.7	2.26
1/2 X	7.2	9.3	6.36
1 X	7.2	9.1	3.77
2 X	7.0	9.0	3.46

[1]Average pH of three replicates.

[2]Mean of dry weight for three replicates.

Table 17. Analysis of variance of the data from the growth of Aphanizomenon in ASMT No. 8 containing various concentrations of phosphate phosphorous.

Samples

	1/4 X (No. 8)	1/4 X	1/2 X	1 X	2X
Observations	5.6	6.3	7.5	4.2	3.0
	5.5	4.9	4.9	3.8	4.0
	5.0	6.1	6.7	3.3	3.4
Sample Total	16.1	17.3	19.1	11.3	10.4
Sample Mean (n=3)	5.37	5.77	6.37	3.77	3.47

Preliminary Calculations

Type of Total	Total of Squares	No. of Items Squared	Obs. / sq. item	Total of Squares per Observation
Grand	5505.64	1	15	367.043
Sample	1159.16	5	3	386.387
Observations	392.20	15	1	392.200

ANALYSIS OF VARIANCE

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio
Among-Sample	19.344	4	4.836	8.319 with 4 and 10 d.f.
Within-Sample	5.813	10	0.581	
Total	25.157	14		

Hypothesis: Means of all concentrations are equal and equal to zero.

Alternate Hypothesis: At least one of the means is not equal to the others

Test using a F-test. Tabular F value with 4 and 10 d.f. at 99% confidence level, is 5.9943, therefore, since the calculated F value (8.319) is greater than the tabular value, the hypothesis is rejected and the alternate hypothesis accepted.

Because the F-test showed a significant difference in treatment means, a further analysis was made using the Least Significant Difference (L. S. D.) between treatment means. The determination of the L. S. D. was made and various hypotheses were tested as shown in Table 18.

Table 18. Results of a test of L. S. D. between treatment means from the first trial of the effect on growth of Aphanizomenon in ASMT No. 8 containing various concentrations of phosphate phosphorous

Comparison	Symbolically	L. S. D.	Difference y_1-y_2 between sample means
1/4 X with 1/4 X(NaH_2PO_4)	$\mu_1 = \mu_2$	0.544	0.40
1/4 X with 1/2 X	$\mu_1 = \mu_3$	0.544	1.00
1/4 X with 1 X	$\mu_1 = \mu_4$	0.544	1.60
1/4 X with 2 X	$\mu_1 = \mu_5$	0.544	1.90

Conclusions:

$\mu_1 = \mu_2$	since	y_1-y_2	$<$	L. S. D.
$\mu_1 < \mu_3$	"	y_1-y_3	$>$	L. S. D.
$\mu_1 > \mu_4$	"	y_1-y_4	$>$	L. S. D.
$\mu_1 > \mu_5$	"	y_1-y_5	$>$	L. S. D.

L. S. D. $= t_{.01}\sqrt{\frac{2\ s^2}{n}}$

where $t_{.01}$ is Student's t at 99% confidence level with 10 d. f.
$s^2 = 0.5813$ from Analysis of Variance (Table 21)
n=3 (sample size)

y_i, y_j are sample means for i^{th} concentration and j^{th} concentration of phosphate phosphorous.

To summarize the conclusions derived from the L. S. D. test; there is no significant difference in growth of Aphanizomenon in media supplied with phosphate as NaH_2PO_4 and as K_2HPO_4 at 1/4 X concentration. Growth in 1/2 X was greater than in 1/4 X (K_2HPO_4 and NaH_2PO_4, since these are equal). Growth in 1 X was less than that in 1/4 X and growth was also less in 2 X. The greatest increase in dry weight(on the basis of sample means) was recorded in 1/2 X concentration. Determination of the reliability of these results is dependent upon further studies of the variation of pH in relation to variation of phosphate concentration. It was, however, interesting to note that possibly better growth could be obtained by supplying the phosphate at a concentration of 8. 0 mg/l (1/2 X). No change has been made in the concentration of phosphate in ASMT No. 8 medium, to the present time.

2. Trial Two

Four replicates of each concentration were prepared in the second trial. A determination was made of the ability of two normalities (0. 1 and 0. 01) of NaOH and HCl to change the pH of ASMT No. 8 medium. The solutions were added to separate flasks of No. 8, in 0. 2 ml amounts, and the change in the pH reading was noted after each addition.

Using this information, and beginning with the day of

inoculation and continuing every fourth day thereafter, the pH of the fourth replicate of each concentration was taken. If any reading varied more than 0.3 of a pH unit, then the pH of that particular set of flasks was adjusted until it was near the average of the readings for the other concentrations.

Observations were made on the alga during the growth period. After eight days growth, the trial was terminated due to heavy contamination with Oscillatoria sp. Prior to this time both macroscopic and microscopic examinations revealed no contaminating alga in any of the tests performed. For all intents and purposes, tests made up to this trial could be assumed to have been made on unialgal material, as no Oscillatoria or other alga, had been observed in the cultures.

Subsequently, Oscillatoria appeared in some, but not all, stock flasks. The stock flask used in this trial gave no sign of containing Oscillatoria prior to this experiment.

The Oscillatoria filaments were noted, by microscopic examination, to lie between the filaments in the flake of Aphanizomenon. This position poses a definite problem. No solution, as yet, has been devised for eliminating this contaminant. Washing will not remove this alga from its position in the flake. An Oscillatoria, appearing to be the same species, has been found occupying this position between filaments in some material taken directly from Klamath Lake. Other material collected from another part of the lake,

showed no signs of contamination. Oscillatoria was noted to be present in large amounts, however, in culture flasks after a period of growth in the laboratory.

The cause of the sudden bloom of the Oscillatoria in the phosphate trial and in stock flasks is not known. It is hoped, however, that further studies will shed some light on this contaminant.

Attempts to Eliminate Bacteria From Cultures

Pasteurization of Akinetes

Attempts to obtain pure cultures of Aphanizomenon were made by germination of akinetes contained in bottom sediment that had been pasteurized.

An Ekman dredge and a gravity core sampler were used to obtain samples of bottom sediment from the bays immediately north and south of Eagle Ridge in Upper Klamath Lake. Entire dredge samples were placed in plastic bags, while the upper layers of the cores were pipetted into screw capped vials. All samples were stored in the dark at 3°C. For pasteurization and germination experiments, samples of mud were suspended in either medium or glass distilled water in a beaker in a water bath, and the suspension heated to 80°C as rapidly as possible. Sub-samples were removed as the temperature of the suspension rose and inoculated into culture flasks.

These flasks were placed in a north facing window at room temperature (ca. 22°C). After a period, they were moved to either the cooled window shelf, or the lighted culture chamber.

No Aphanizomenon appeared in the cultures at anytime following this procedure. Chlamydomonas, a few ciliates and bacteria survived temperatures up to 62°C but nothing survived exposures to temperatures above 62°C.

Lake water containing algae was collected from Klamath Lake and stored in the cold room for a month. Pasteurization was carried out in the same manner as that employed with the sediment. Aphanizomenon again did not appear from germinated akinetes. Chlamydomonas, Oscillatoria (?), Anabaena and Anacystis aeruginosa survived temperatures up to 58°C, with Anacystis being present in greater numbers after treatment at lower temperatures (11-51°C). No organism observable at magnification of 400 diameters survived temperatures above 62°C.

Ultra-Violet Irradiation

Irradiation with ultra-violet was used in attempts to obtain pure cultures. A cardboard box was arranged to enclose the ultra-violet source and to act as a radiation and transfer chamber. A two tube germicidal lamp was so placed as to extend through the top of the box. Prior to the actual exposures of the plant material,

the lamp was turned on and the interior of the box and the various instruments, and accessories to be used, were irradiated for at least an hour. Cooled petri dishes containing 30 mls of medium were inoculated with 6 flakes of Aphanizomenon each, and the open dish exposed to ultra-violet radiation at a distance of 30 cm from the lamp for varying periods of time (Table 19). During the exposure, the flakes were swirled slowly to expose them evenly to the radiation.

Table 19. Times of exposure to ultra-violet radiation

Seconds	Minutes
7	2
15	4
30	8
60	16
90	32

Following treatment, the flakes were transfered aseptically to flasks of sterile medium. The flasks were placed on a shelf in the window at 15°C, and observations made of the effect of irradiation on the alga (Table 20). A standard plate count of a sample from each flask was made 15 days after treatment (Table 21). These counts were made by removing three flakes from each flask containing material maintaining the colonial flake form, or by removing a 1 ml aliquot from flasks containing algal material that had lysed or lost the colonial flake form.

The 30 and 60 second exposures gave the greatest reduction in the number of bacteria in the flakes (Table 21). Also, it would appear that the alga became less viable with longer exposure, while the bacterial count increased. No exact explanation can be given for this observation as the longer ultra-violet exposures would be expected to have an adverse effect on the bacteria. It is possible, however, that since the plate counts were made some time after treatment that the bacteria increased due to the presence of suitable substrate. This substrate being algal material that disintegrated because of the adverse effect of ultra-violet treatment. This possibility would seem to be logical since greater bacterial numbers were related to longer lengths of exposure to ultra-violet irradiation. Further experiments with plate counts being made at the time of exposure may determine whether this explanation is correct.

A second trial repeated the procedure used in the first trial except that isolates were made at ten second intervals for exposure times from zero to 90 seconds.

Plate counts made seven days after treatment indicated that there was no reduction in number of bacteria until 90 seconds exposure (Table 23). This result is not consistent with that obtained in the previous trial, as in that trial the 30 and 60 second exposures gave the greatest reduction in bacterial number. The above mentioned possibility does not seem to be applicable in the second trial

Table 20. Effects on Aphanizomenon of various lengths of exposure to ultra-violet irradiation: Trial 1

Time	Days after exposure			
	1	6	11	17
7 sec.	++8	++19	++21	++T
15 "	++9	++13	++22	=
30 "	+	++9	++14	++T
60 "	++7	++13	++13	++T
90 "	++12A	+20S	++34S	++T
2 min.	-9	++20S	-20S	0
4 "	++8	=L	-S	0
8 "	++13A	=	=	0
16 "	+S	=	=	0
32 "	=20S	=	=	0

Code:

(++) The flakes of alga maintained their normal form and increased in number.

(+) The flakes maintained their normal morphological form.

(=) All of the flakes lost their normal morphological form.

(-) Some of the flakes lost their normal morphological form.

(0) Flasks were discarded.

Number shows the number of flakes showing the above characteristics.

A indicates adhesiveness, a sticking together of normal flakes

S flakes smaller than at inoculation

L flakes were lysed.

T transfered and maintained in culture

Table 21. Plate counts on the bacteria in Aphanizomenon flakes after ultra-violet irradiation: Trial 1

Time of exposure	Plate count[1]
7 sec.	332
15 "	288
30 "	55
60 "	74
90 "	290
2 min.	233
4 "	> 500
8 "	X[2]
16 "	X[2]
32 "	X[2]

[1]Average number of colonies per three replicate plates.

[2]Colonies too numerous to count.

as the material at the 30 and 60 second exposures in this trial was in better condition than that at the 90 second exposure (Table 22). It is impossible at this time to make a definite conclusion on the effect of ultra-violet irradiation on the bacteria in the flakes.

It is important to note that following 90 seconds exposure, the morphology of the alga was essentially unaltered (Table 22). The significance of the appearance of Oscillatoria in the cultures is not known, however, the condition of the Aphanizomenon seemed to improve for a time after its appearance. Since there was an ultimate loss of algal material at lower exposure times in this trial (Table 22), further experiments will be necessary to observe the effect of ultra-violet light on Aphanizomenon.

Chlorine Treatment

General Procedure

A series of experiments were performed using chlorine treatment in an attempt to obtain bacteria-free cultures of the alga. The procedure for the various trials was basically the same. Whole flakes, or flakes partially dispersed by shaking, were placed in a chlorine solution for a given time. In all trials, the desired concentrations of chlorine were obtained by appropriate dilution of a 200 mg/l stock solution. The treated alga was either washed in sterile

Table 22. Effects on Aphanizomenon of various lengths of exposure to ultra-violet irradiation: Trial 2

Time (sec.)	Days after exposure 1	7	11	31	45
0	+6	++, C, S(22)	++, S, T(22)	++, C, T(>25)	0
10	+6	++, C, S(18)	++, S, T(21)	++, C, T, *(>25)	+(15), C, S, *
20	+6	++(22)	++, T(21)	++, T, *(>25)	0
30	+6	++, C(22)	++, S, T(22)	++, *(>25)	0
40	+6	++, S(27)	++, S, T(27)	++, *(>25)	0
50	+6	++, C, S(25)	-, S, T(32)	++, *(>25)	0
60	+6	-, C, S(>25)	-, C, T(33)	++, *(>25)	0
70	+6	-, C, S(>25)	-, S, T(34)	++, *(>25)	0
80	+6	-, C, S(>25)	-, S, T(32)	++, T, *(25)	=(0)
90	+6	-, C, S(>25)	-, S, T(25)	++, T, *(25)	+(15)

Code:

(++) The flakes of alga maintained their normal form and increase in number.

(+) The flakes maintained their normal morphological form.

(=) All of the flakes lost their normal morphological form.

(-) Some of the flakes lost their normal morphological form.

(0) Flasks were discarded.

Number indicates the number of flakes showing the above characteristics.

S flakes smaller than at inoculation

T transfered and maintained in culture

C a color change from green to yellow

* the appearance of Oscillatoria contaminant

Table 23. Plate counts on the bacteria in Aphanizomenon flakes after ultra-violet irradiation: Trial 2

Time of exposure(sec.)	Plate count[1]
0	54
10	110
20	115
30	203
40	151
50	184
60	151
70	105
80	198
90	1

[1]Average number of colonies per three replicate plates.

medium or one ml of a 0.0056 N sodium thiosulphate solution[5] was added to the chlorine solution for two minutes at the end of the period of treatment, and the alga then transfered to fresh medium with a sterile needle. The mouth of the flask was flamed before and after each insertion of the needle. Certain modifications were made in this general procedure as noted in the description of the trials. A total of eleven trials were made, with the first five designed to determine the range of concentrations of chlorine within which the alga would survive.

Trials 1 and 2

In the first trial, flakes of Aphanizomenon were placed in 5 mls of a 25 mg/l chlorine solution for two minutes. Flakes in the second trial were placed in concentrations of chlorine varying from 4 mg/l to 20 mg/l by 2 mg/l increments, and 25 mg/l for two minutes. Following the treatment, sodium thiosulphate was added and the flakes were washed and transfered.

All flakes treated with concentrations of chlorine from 10-25 mg/l showed lysis. Those flakes treated in trial 2 with concentrations from 4-10 mg/l showed normal flake form and no lysis.

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[5]One ml of the solution of sodium thiosulphate would neutralize 0.2 mg of chlorine.

Trial 3

In trials 3, 4 and 5, the general procedure was modified by including a plate count of the bacteria surviving treatment with chlorine. After a rinse in either sodium thiosulphate, or sterile medium, three of the eighteen treated flakes of Aphanizomenon were placed in medicine bottles containing 100 mls of sterile medium and shaken to disperse the filaments. Aliquots were taken from the bottles to inoculate the plates for counting the surviving bacteria.

Flakes in trial 3 were placed in chlorine concentrations between 1 and 10 mg/l (increasing by 1.0 mg/l amounts) for two minutes. Following a rinse in sterile medium (no sodium thiosulphate) the flakes were transfered to flasks and bottles containing medium. The flakes of Aphanizomenon maintained normal form after treatment with 1 and 2 mg/l chlorine concentrations but not after treatment with concentrations of 3 to 10 mg/l (Table 24). The 2 mg/l concentration appeared to give the most satisfactory results both in effect on the alga and reduction of bacterial population.

The fact that there were no bacterial colonies after treatment at certain concentrations including 2 mg/l (Table 24), may have been due to a fault of technique in making the plate counts. A control was used in subsequent trials to assure that poor technique was not

responsible for reduced numbers of colonies. In these preliminary studies, no supplemental glucose was added to the agar and this lack of glucose could possibly account for the low counts.

Table 24. Results of treatment with chlorine: Trial 3

Concentration of chlorine (mg/l)	Effect on Aphanizomenon	Plate count[1]
1	flakes maintained form	3
2	flakes maintained form	0
3	some flakes clumped	25
4	all flakes clumped	3
5	all flakes clumped	15
6	nothing visible in flask	0
7	lysed, floating amorphous masses	0
8	some flakes clumped, rest lysed	0
9	flakes clumped, white	0
10	flakes clumped, green	0

[1] Average number of colonies per three replicate plates.

Trial 4

In trial 4, the material was immersed for two minutes in each of a series of concentrations of chlorine from 0.00 mg/l to 2.00 mg/l, increasing by 0.2 mg/l increments, and 2.5 mg/l. A check was made of the effect of adding sodium thiosulphate to the 1.0 mg/l concentration. Sodium thiosulphate was added to one group in the 1.0 mg/l concentration and not to the other. No sodium thiosulphate was added to any of the other concentrations.

An adverse effect on the alga of chlorine concentrations above 1.2 mg/l was clearly indicated (Table 25). It is interesting to note that the material treated with 1.0 mg/l concentration and with no sodium thiosulphate lost flake form on the third day, while the material (1.0 mg/l concentration) that had been put through a sodium thiosulphate rinse maintained form up to 43 days.

The eventual loss of the algal material that had been treated with concentrations of chlorine from 0.00 to 1.2 mg/l, could not be definitely attributed to the effect of chlorine because of the length of time between treatment and ultimate loss. In rechecking the number of flakes per flask, it was apparent there was a crowding of material which has been observed to cause lysis and/or clumping in stock cultures.

The low numbers of bacterial colonies observed in this trial (Table 25) can not be accounted for except by the fact that supplemental glucose was not added to the agar. No definite statement can be made that these low numbers resulted from treatment with chlorine.

Table 25. Results of treatment with chlorine: Trial 4

Concentration (mg/l)	Plate count (after 96 hrs incubation)[1]	Effect on Aphanizomenon	
		After 3 days	Ultimate effect
0.0	14	+	=,
0.2	8	+	L (52 days)
0.4	0	+, A	L (41 days)
0.6	3	+	= (25 days)
0.8	7	++	(0) (43 days)
1.0	9	=, *	(0) on third day
1.0[2]	1	+	Flakes S, (0)(43 days)
1.2	140	+	(0) (16 days)
1.4	4	-	(0) (41 days)
1.6	23	=, *	(0) on third day
1.8	1	=, *	(0) on third day
2.0	1	=, *	(0) on third day
2.5	1	=, *	(0) on third day

[1] Average number of colonies per three replicate plates.

[2] Sodium thiosulphate added.

Code:

(++) The flakes of alga maintained their normal form and increased in number.

(+) The flakes maintained their normal morphological form.

(=) All of the flakes lost their normal morphological form.

(-) Some of the flakes lost their normal morphological form.

(0) Flasks were discarded.

S flakes smaller than at inoculation.

A indicates adhesiveness, a sticking together of normal flakes.

L lysed.

* bacteria observed on microscopic examination.

Trial 5

Concentrations of chlorine at 0.5 and 1.0 mg/l and a control at 0.00 mg/l were used in a sequential treatment in the fifth trial. The flakes were treated with chlorine for two minutes and then sodium thiosulphate was added to the control and each treatment, and the flakes were rinsed in sterile medium. This procedure was repeated 5 and 10 hrs later. The algal material was placed in flasks of sterile medium between treatments, and the flasks were set on the window shelf at 15°C. After each treatment, three flakes of Aphanizomenon were transfered to a medicine bottle and a plate count of the surviving bacteria was made.

The results of the bacterial plate counts were inconclusive as they showed an average of one colony per plate for the 0.00 mg/l and 0.5 mg/l concentrations after the sequential treatment times and for the 1.0 mg/l concentration at the initial and 5 hr times. No count was made for the 1.0 mg/l concentration at the 10 hr treatment as the flakes of alga lysed during the period between the 5 hr and the 10 hr treatment.

Some flakes treated with 0.0 and 0.5 mg/l chlorine showed a clumped character after three days. Flakes in these concentrations, not showing a clumped character, were transfered and observed for 53 days in the case of 0.0 mg/l and 65 days for the 0.5 mg/l.

The 0.0 mg/l material was discarded on the fifty-third day. The 0.5 mg/l material showed normal colonial form and indications of growth until the time of writing.

Trial 6

In trial six, four flakes of Aphanizomenon were shaken vigorously until broken down into small clumps of filaments in order to obtain more complete exposure of the bacteria lying between the algal filaments to the chlorine. The clumps formed a cloudy suspension in 100 mls of medium in 8 oz. medicine bottles. After shaking, chlorine was added to the medicine bottles for varying periods (Table 26). Following the exposure to chlorine, sodium thiosulphate was added except as noted. After two minutes, and shaking the medicine bottle 25 times, one ml samples of the suspension were removed with a sterile pipette, and placed in flasks containing sterile medium and in plates for a standard plate count.

The plate counts of bacterial colonies present in the dispersed filaments showed greater numbers of colonies with increasing lengths of exposure to chlorine and increasing concentration of chlorine (Table 26). In this and all subsequent trials the agar used in making plate counts was supplemented with glucose.

Table 26. Average number[1] of bacterial colonies per plate after treatment with chlorine: Trial 6

Concentration of chlorine(mg/l)	Times of exposure (seconds)				
	15	30	60	90	120
0.00	62	63	45	33	51
0.00[2]	49	50	50	36	27
0.01	88	56	67	63	64
0.05	32	45	63	34	37
0.10	41	55	92	132	75
0.20	189	202	148	164	326
0.40	326	417	352	389	437
0.60	464	737	800	777	>1000
0.80	>1000	>1000	>1000	>1000	>1000
1.00	>1000	>1000	>1000	>1000	>1000

[1]Average number of colonies per three replicate plates.
[2]No sodium thiosulphate added.

The condition of the alga 14 days after treatment is summarized in Table 27. In general, only one or two flakes per flask returned to normal size. This "normal size" was judged as that in the size range of flakes present in stock cultures at that time. Treated flakes were transfered aseptically to fresh flasks of medium every two weeks.

Table 27. Condition of Aphanizomenon 14 days after treatment with chlorine: Trial 6

Conc. (mg/l)	Time of exposure(seconds)				
	15	30	60	90	120
0.00	I, G(2)	I, G(1)	I, G(1)	I, G(1)	I, G(1)
0.00[1]	I, G(1)	I, G(1)	I, G(1)	I, G(1)	I, G(1)
0.01	I, G(1)	I, G(2)	I, G(1)	I, G(1)	I, G(1)
0.05	I, G(1)	I, G(x)	I, G(1)	I, G(3)	I, G(1)
0.10	R	R	I, G(1)	I, G(1)	N
0.20	I, G(1)	I, G(1)	I, G(1)	I, G(3)	I, G(1)
0.40	I, G(1)	I, G(1)	I, G(2)	I, G(1)	I, G(2)
0.60	I, G(3)	R	I, G(1)	I, G(1)	R
0.80	I, G(1)	R	I, G(1)	I, G(1)	I, G(1)
1.00	I, G(1)	I, G(3)	I, G(1)	I, G(2)	R

[1]No sodium thiosulphate added.

Code:

Number — The number of flakes showing the characters described by the code below.

G — Flakes were green or yellow-green.

I — The flakes increased to a normal size.

N — Nothing visible in the flask.

R — Size of the flakes remained the same as when the treatment was made.

At the end of 42 days, a second plate count was made on flasks containing at least three flakes, the number required to make a plate count using the method described earlier (Table 28).

Table 28. Average number[1] of bacterial colonies per plate 42 days after treatment of Aphanizomenon with chlorine: Trial 6

Concentration of chlorine[2] (mg/l)	Time of exposure(seconds) 15	30	60	90	120
0.01	46	37T	77	23	(0)
0.05	228	(0)	65T	64T	(0)
0.10	(0)	(0)	(0)	(0)	(0)
0.20	88	12	79	(0)	43
0.40	107	549	95	173	(0)
0.60	(0)	(0)	(0)	935T	(0)
0.80	(0)	(0)	89	(0)	85T
1.00	84T	(0)	285T	224T	(0)

[1]Average number of colonies per three replicate plates.

[2]Control flasks had been discarded to provide culture space for other experiments.

Code:

(0) Material for this concentration was discarded earlier, because of deterioration.

T Transfered and maintained in culture for a total time of two months.

The results of plate counts as noted in Table 26, differ greatly from those in Table 28, at least in the higher concentrations of chlorine. No satisfactory explanation can be given for this difference. Speculation leads to at least three possibilities, (1) the techniques of making plate counts differed or, (2) the growth of the alga, in some manner, reduced the number of bacteria present or, (3) there was a residual action by the chlorine. The first possibility is unlikely as all counts were made in the same manner. The second suggestion seems unlikely as logically the bacteria must be utilizing substrate produced by the alga. The alga could, however, produce some inhibitory substance, but no studies were made on this possibility. It is also possible that the chlorine could have some residual effect on the bacteria, further studies may substantiate this possibility.

Trial 7

Trials 7, 8 and 9 were made to test the effect of chlorine on bacteria alone. Four flakes of Aphanizomenon were dispersed by shaking. One ml samples of the suspension were placed in petri plates, and nutrient agar with glucose supplement added. Following incubation for 48 hrs, portions of the colonies present on the plates were placed on slants of nutrient agar and glucose. These slants were then incubated for a further 48 hrs. A small amount of material was then removed with a sterile loop and rinsed in 100 mls of medium in

a medicine bottle. Chlorine was added and the bottle was vigorously shaken to disperse bacterial clots. Sodium thiosulphate was added at the end of the treatment interval. The standard plate count procedure (7, p. 492-493) was then carried out using one ml of treated bacterial suspension for each plate. The plates were incubated at ca. 22°C in trials 7 and 8 and at a constant 28°C for trial 9. Counts were made after 48 hrs incubation.

In trial 7, plate counts of bacteria treated with chlorine concentrations of 0.4 mg/l for 30 to 60 seconds, and 1.0 mg/l for 60 and 120 seconds showed that these concentrations and times of exposure appeared to decrease the number of bacteria (Table 29). Other concentrations and times caused no decrease.

Table 29. Effect[1] of chlorine treatment on bacteria: Trial 7

Time of exposure (seconds)	Concentration of chlorine (mg/l)					
	0.00[2]	0.00	0.2	0.4	0.8	1.0
30	X	X	X	1	X	1000
60	X	X	X	204	X	404
120	X	X	X	X	500	250

[1]Average number of colonies per three replicate plates.

[2]No sodium thiosulphate added.

Code:

X Bacteria covered the entire plate, no single colony countable.

It is necessary to note that only one type of bacterium appeared to be present on the plates in this trial. This bacterium formed a yellow colony. In previous trials, three differently colored colonies (yellow, white and pink) were observed. Other bacteria could possibly be present, but no others appeared on plates prepared.

Trial 8

The plate counts (procedure in trial 7) revealed no decrease in bacteria until treatment for 32 minutes at 0.5 mg/l (Table 30). The counts at the two longer times were below all others except those at 32 minutes. No definite conclusion could be drawn from these data, although it would appear that 32 minutes had the desired effect of reducing numbers of bacteria.

Table 30. Effect[1] of chlorine treatment on bacteria: Trial 8

Time of exposure (minutes)	Concentration of chlorine (mg/l)		
	0.00	0.00[2]	0.50
2	X	X	581
4	X	X	> 500
8	X	X	X
16	X	X	X
32	X	X	3
64	X	X	223
128	X	X	292

[1] Average number of colonies per three replicate plates.

[2] No sodium thiosulphate added.

Code:

X Bacteria covered entire plate.

Trial 9

Times of exposure were used in the region of 32 minutes (for 0.5 and 1.0 mg/l chlorine) which gave the lowest count in the preceding trial (Table 31).

Exposures for 20, 28 and 32 minutes at 1.0 mg/l chlorine gave the greatest reduction in number of bacteria (Table 31). Thirty-two minutes at 0.5 mg/l, that had given lowest counts in the previous trial, showed no decrease in this trial. It would appear that greater reduction is obtained at less exposure time. No reason can be given for this result.

Table 31. Effect[1] of chlorine treatment on bacteria: Trial 9

Time of exposure (minutes)	Concentration of chlorine (mg/l)		
	0.00	0.50	1.00
16	X	> 500	X
20	X	X	2
24	X	X	X
28	X	X	90
32	X	X	21
40	X	X	1000
44	X	X	1000

[1]Average number of colonies per three replicate plates.
Code:
X Bacteria covered the entire plate.

Trial 10

Freshly collected material from Upper Klamath Lake was used in trials 10 and 11. The flakes had been collected individually with an inoculating needle.

In trial 10, the flakes of Aphanizomenon were transfered to 30 mls of cooled medium in 125 ml Erlenmeyer flasks. Four flasks of material were used for controls, and eight were used for treatment with 1.0 mg/l chlorine. The exposure times were 20 and 28 minutes with two control flasks and four treatment flasks for each time. The flasks were maintained at ca. 13° C in the culture cabinet until the exposure time had expired. Two minutes after the addition of sodium thiosulphate, the flakes (including control material) were transfered to flasks of sterile medium and placed in the window at 15°C.

The chlorine at 1.0 mg/l concentration had an adverse effect on the freshly collected Aphanizomenon. Three flasks at 20 minutes and 3 at 28 minutes exposure contained lysed material within 18 days, and two flasks (1 at 20 minutes and 1 at 28 minutes) showed flakes with a yellow color and no growth. The 20 minute controls were maintained up to 63 days, at which time they lysed. The flakes in the 28 minute controls were still growing and maintaining their form at the time of this writing. The flakes have a light green color.

This experiment demonstrates that freshly collected material

is less able to withstand chlorine treatment than material cultured for some time.

The algal material used in this trial was examined microscopically before the test for signs of algal contaminants. None was observed. During the trial, however, Oscillatoria made an appearance about the fifteenth day. The conclusion is that it was present but overlooked in the preliminary examination.

Trial 11

The same concentrations and exposure times were used in trial 11 as were used in trial 10. The flakes were placed in medicine bottles containing 100 mls of sterile medium, followed by a sufficient amount of chlorine solution to give 1 mg/l chlorine (no chlorine for control) and then by vigorous shaking to disperse the filaments. After 20 or 28 minutes exposure time, sodium thiosulphate solution was added for two minutes. Ten mls of the algal suspension were transfered to a fresh flask of medium using a sterile pipette, and the flasks were put in the window at 15°C.

The flakes of Aphanizomenon were able to withstand dispersal and chlorine treatment as the material treated recovered normal flake form and condition. The alga was not maintained indefinitely, however, as the flakes gradually became smaller and finally disappeared. Once again, Oscillatoria appeared although preliminary

examination indicated that none was present.

In general, further tests will be required to determine the actual merit in treating flakes with chlorine. It is apparent from the above trials that concentrations as low as 1.0 mg/l will be required for a period of possibly 20 to 40 minutes. Any increase in concentration above 1.0 mg/l causes lysis and/or deterioration of the alga. The controls in trial 10 again showed that freshly collected material can be introduced into culture.

SUMMARY

This thesis reports the results of investigations of the culture of Aphanizomenon flos-aquae. Aphanizomenon has been introduced into culture in ASMT No. 8 medium, which was a modification of an earlier medium of McLachlan. McLachlan's medium(which he called ASM) was a modification of Provasoli's artificial sea water medium. This ASMT No. 8 medium had no added organic materials other than ethylenediaminetetraacetate (EDTA) and trishydroxymethylamino methane (Tris). No precipitate was ever observed in flasks of ASMT No. 8 medium after autoclaving. Aphanizomenon collected from Upper Klamath Lake, Oregon, and placed directly in this medium has been in culture for 14 months. The alga in culture had the same characteristic flake form as that found in the lake. The color of the flakes was a dark to pale green, and they have shown continuous growth during the 14 months.

The cultured algal material has exhibited definite light and temperature requirements. The alga has shown best growth, color, form and general appearance when grown in a north window with combined illumination from natural and fluorescent light giving total intensities from 120 f. c. at night to 300 f. c. during the day. Currently, the alga has not grown under illumination from artificial sources alone. Best growth and maintenance of the alga

have been obtained at 15°C.

Attempts were made to obtain a culture medium even more satisfactory than ASMT No. 8 by varying the concentration of the nutrient elements. Three elements were tested in the investigation including various sources of iron and different concentrations of nitrogen and phosphorous. Best results were obtained with iron supplied in a chelated form with disodium ethylenediaminetetraacetate. No significant difference in growth was observed when the alga was grown in various concentrations (0.0, 21.0, 42.0, 84.0, 168.0 and 336.0 mg/l) of nitrate nitrogen. Significant differences were noted when the Aphanizomenon was grown in different concentrations of phosphate. The greatest increase in dry weight was obtained in the 8.0 mg/l concentration of phosphate phosphorous.

Ultra-violet irradiation and chlorine treatment hold promise for the elimination of bacteria from cultured algal material. Aphanizomenon can withstand ultra-violet irradiation exposures of 90 seconds without apparent changes in morphology. This exposure time also gave indications of reducing bacterial numbers. The optimum times of 28-32 minutes at a chlorine concentration of 1 mg/l gave greatest reduction of bacteria with a corresponding maintenance of algal morphology.

Earlier experiments were made on material that was apparently unialgal, as no other alga was observed to be present. Large

quantities of Oscillatoria, however, appeared in flasks during a second trial on variations of phosphorous concentrations. Since this time, Oscillatoria has also appeared in stock flasks. The filaments of this alga grown between the filaments of Aphanizomenon in the flakes. Attempts to remove Oscillatoria by repeated washing have been unsuccessful. Oscillatoria believed to be the same species has been found occupying this position between Aphanizomenon filaments in material taken directly from the lake. To obtain unialgal material, it will be necessary to sub-culture stock flasks that exhibit no contamination, and that have never shown contamination during this investigation.

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