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Impact of Toxic Clones of Blue-Green Algae on Water Quality as Related to Aquatic Animals

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IMPACT OF TOXIC CLONES OF BLUE-GREEN ALGAE ON
WATER QUALITY AS RELATED TO AQUATIC ANIMALS

By

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COMPLETION REPORT

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INTRODUCTION

The literature indicates that toxic blooms of blue-green algae occur widely. Gorham (1966) cited references describing blue-green algal poisonings from Russia, Argentina, Australia, Morocco, Bermuda, Brazil and Finland. In North America these blooms have been most frequently reported from North Dakota, South Dakota, Minnesota, Iowa, Wisconsin, Illinois, Michigan, Alberta, Saskatchewan, Manitoba and Ontario (Gorham, 1966). Several species of blue-green algae have been associated with these toxic blooms in North America, but the most commonly occurring toxic species are Anabaena flos-aquae, Microcystis aeruginosa and Aphanizomenon flos-aquae. All three of these species occur from time to time in New Hampshire and they occur in both toxic and non-toxic forms.

Problems concerning the toxicity of these species occur when heavy concentrations of them decompose naturally. The products of decomposition plus toxic substances from within the cells cause death or illness to many mammals, birds and fishes at that time. Also, associated with the decomposition of large amounts of organic matter, low oxygen concentrations may occur. The combination of toxic substances and low oxygen levels is rapidly fatal to many aquatic organisms. This is the usual manner of toxic manifestations.

Fatality may occur also in relationship to high numbers of the toxic forms of these algae when such a concentration is treated with copper sulfate. Copper sulfate has been used since 1904 to control unwanted concentrations of algae in lakes and for many years in New Hampshire by the New Hampshire Water Supply and Pollution Control Commission. Two such instances occurred here in New Hampshire and led us into this area of research. In 1964 and in 1966, fish kills were associated with normal copper sulfating of lakes. In both cases Aphanizomenon flos-aquae was identified as the predominant algal species (Sawyer, et. al. 1968). In the latter instance, mortality of more than six tons of fish occurred.

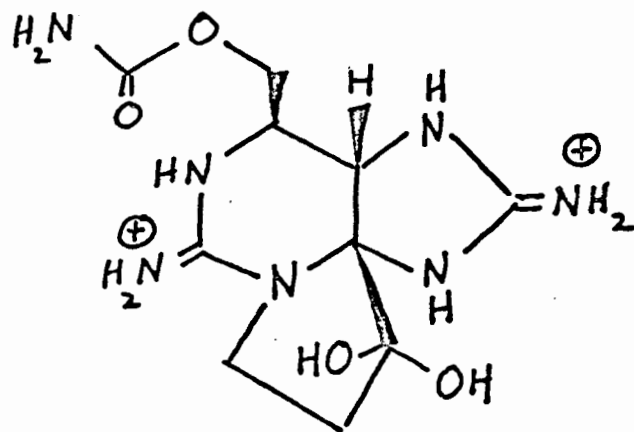
Our research has led us into two pathways. One has been concerned with the biochemistry and identification of the toxin produced by Aphanizomenon flos-aquae. Such a task requires the sophisticated approach detailed below. At times the work seems far removed from the original goals of the project, but upon retrospect it is better for the ecologist and physiologist to know as exactly as possible the characteristics of the toxin with which they are working.

A second direction that our work took concerned a series of studies of the effects of the Aphanizomenon toxin on aquatic animals in our lakes. In this part of the project we studied the effects of Aphan toxin on a zooplankter, a mollusk, a fish, and an amphibian. Our efforts culminated in a good chemical picture of the toxin, and a strong understanding of its physiological effects on a variety of organisms.

Chemistry of Blue-Green Algal Toxins

Aphanizomenon flos-aquae VFDF

In 1968 Sawyer et al demonstrated the presence of a potent toxin in Aphanizomenon flos-aquae cells. The toxin was dialyzable, heat- and acid-stable, alkali-labile, and was soluble in water and ethanol, but not in less polar solvents. The toxin was a very fast death factor (VFDF) that possessed some saxitoxin-like physiological properties. The same year Jackim and Gentile, using laboratory grown cells of Aphan. flos-aquae, reported the partial purification and properties of the toxin. This work was a spin-off from studies initiated by Sawyer, Gentile and Sasner in our laboratory at U.N.H. Cells were disrupted in 0.01 N HCl, the insoluble material removed by centrifugation, the pH of the toxic extract adjusted to pH 5.5, the resulting precipitate removed by centrifugation, and the toxic supernate lyophilized to give partially purified material. This was further purified by preparative paper chromatography followed by chromatography of the toxic fraction on a silica gel column. Three toxic fractions were obtained, the most toxic of which, when chromatographed on paper, gave three Weber reagent-positive spots, one of which corresponds in R_f and reaction with Weber reagent, ninhydrin, and Jaffe reagent sprays with saxitoxin (Structure, Fig. 1), the paralytic shellfish poison (PSP) produced by species of the red tide dinoflagellate Gonyaulax.



SAXITOXIN

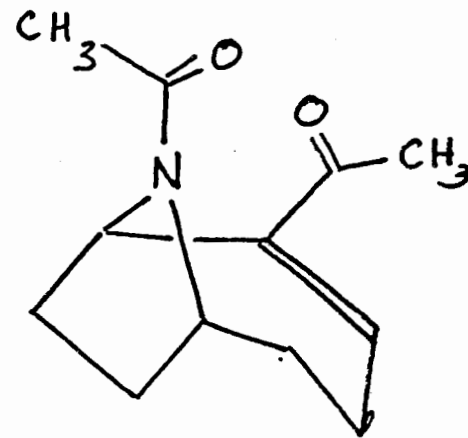
ANABAENA FLOS-AQUAE TOXIN
(N-Acetyl derivative)

Figure 1

The infrared spectrum of material eluted from this zone of the paper chromatogram appeared very similar to that of saxitoxin. It had an activity of 1.5-2.0 ug per MU (=500-667 MU per mg). These authors concluded that Aphan. flos-aquae toxin may be identical to saxitoxin or a slightly modified product.

In 1973 Alam et al reported the purification and properties of Aphan. flos-aquae toxin from cells obtained from natural blooms. Cell paste was extracted with water, acidified to pH 3 with HCl, at 80-90°C, the extract taken to dryness, the residue extracted with ethanol, and the toxic ethanol extract taken to dryness. The toxic residue was extracted with chloroform to remove lipoidal material and the toxic chloroform-insoluble fraction subjected to preparative high voltage electrophoresis. The toxic ninhydrin- and Weber reagent - positive zone was eluted from the paper and chromatographed on IRC-50 resin (H⁺ form). The toxicity was eluted with 1M acetic acid and this fraction was further purified by preparative thin-layer chromatography to give a chromatographically homogeneous material that had a potency of 745 MU per mg. Table 1 shows a thin-layer chromatographic comparison of Aph. flos-aquae toxin with saxitoxin in various solvent systems and Table 2 a comparison of the color reactions given with the various spray reagents. From these results it appears that the Aph. flos-aquae toxin isolated by Alam et al (1973) is not identical with saxitoxin. Positive reactions of the toxin with Weber, diacetyl- -naphthol, and Benedict-Behre reagents, however, do indicate that this toxin may be a substituted guanidine derivative.

Table I. Thin-layer chromatographic comparison of
Aphan. flos-aquae toxin and saxitoxin. (Alam et al. 1973)

| Solvent system | R_f | |
|--|------------------------|----------------------|
| | <u>Aphan.</u> toxin | Saxitoxin |
| <u>t</u> -Butanol:acetic acid:water (2:1:1, v/v) | 0.31 | 0.39 |
| Phenol:water (4:1, v/v) | 0.40 | 0.35 |
| <u>t</u> -Amyl alcohol:pyridine:water (7:7:6, v/v) | 0.57 | 0.38 |
| <u>iso</u> -Propanol, conc. NH_3 , water (100:5:10, v/v) | 0.01 | 0.07 |
| n-Butanol, pyridine, water (1:1:1, v/v) | 0.10 | 0.17 (major) 0.31 |
| n-Butanol, acetic acid, water (12:3:5, v/v) | 0.15 | 0.32 (tails) |
| n-Propanol, acetic acid, water (4:1:1, v/v) | 0.26 | 0.36 (major) 0.56 |

*On glass plates coated with cellulose MN 300 powder. Chromatograms sprayed with Weber reagent.

Table 2. Color reactions given by Aphan. flos-aquae toxin
and saxitoxin on paper chromatograms (Alam et al. 1973)

| Spray reagent | <u>Aphan.</u> toxin | Saxitoxin | |
|--|------------------------|----------------|---------------------------|
| | | This report | Literature |
| Ninhydrin | + (purple) | - | - (5), yellow (6,2) |
| Bromcresol-green | blue | blue | blue (6,2) |
| Weber (PCF, penta- cyanoaquoferriate) | + (red) | + (red) | + (5) |
| Jaffe (picric acid) | - | + (red) | + (5) |
| Diacetyl- - naphthol | +(purple-red) | +(purple-red) | |
| Benedict-Behre (3,5- dinitrobenzoic acid) | +(red) | +(purple-red) | + (5) |
| Sakaguchi | yellow-orange | - | - (5) green-yellow (6) |

The toxin purified by Alam et al (1973) showed no absorption maxima at wavelengths longer than 220 nm in the ultraviolet and visible region. The infrared spectrum of a KBr pellet showed a broad absorption band at 3350 cm^{-1} indicative of O-H or N-H stretching due to the presence of an hydroxyl or amino group; a strong band at 1640 cm^{-1} that could be due to C=O, C=N, or C=C stretching; a band at 1730 cm^{-1} indicative of C=O stretching; bands at 1480 and 1400 cm^{-1} ; and a broad band in the $1150\text{-}1000\text{ cm}^{-1}$ region. The infrared spectrum differed from that of saxitoxin in a KBr pellet, that has a prominent band at ca. 1340 cm^{-1} and a very prominent complex absorption in the $1700\text{-}1600\text{ cm}^{-1}$ region (Shantz et al, 1966).

A paper high voltage electrophoretic comparison of Aphan. flos-aquae toxin and saxitoxin in pH 6.41 buffer showed Aphan. flos-aquae toxin to move less rapidly than saxitoxin toward the cathode, indicating it to be less basic than saxitoxin (Alam et al, 1973). Hydrogenation of Aph. flos-aquae toxin did not destroy its activity in contrast to the behavior of saxitoxin that becomes non-toxic after hydrogenation (Shantz, et al, 1966).

Aphanizomenon flos-aquae other toxins

Prescott (1948) reported that decomposed cultures of Aphan. flos-aquae showed the presence of sufficiently high concentrations of hydroxylamine (NH_2OH) and of hydrogen sulfide (H_2S) to cause fish mortalities.

Microcystis aeruginosa FDF (Microcystin)

In 1959 Bishop et al reported the isolation of the FDF of Microcystis aeruginosa from cells mass cultured in the laboratory. The toxin could be extracted from the cells with methanol, 95% ethanol, or 0.1 N sodium carbonate. The dialyzable material in the extract was purified by paper electrophoresis to yield a toxic peptide (peptide 2, later named microcystin (Konst, et al, 1965), that had a LD₅₀ of 0.466 ± 0.013 mg per kg (IP, mouse). All the activity in crude preparations could be accounted for by this peptide. The amino acid composition of the peptide is shown in Table 3. Failure of the peptide to form a N-2,4-dinitrophenyl amino acid and the resistance of the peptide to attack by proteolytic enzymes indicated that the toxin might be a cyclic peptide. The presence of D-serine in the peptide was indicated by the disappearance of serine when hydrolysate of the peptide was treated with D-amino acid oxidase. None of the other amino acids seemed affected, indicating in general the L-configuration for the other amino acids.

In 1970 Rama Murthy and Capindale reported the isolation of a toxic peptide from the same strain of M. aeruginosa used by Bishop et al (1959). The toxin was also extracted from the cells using sodium carbonate but further purified by column chromatography on DEAE-Sephadex instead of paper electrophoresis.

Table 3. Amino acid composition of the
toxic peptides of Microcystic aeruginosa.

| Bishop et al. 1959. | | Rama Murthy and Capindale 1970. |
|---------------------|------------|------------------------------------|
| Amino acid | Mole ratio | Amino acid |
| Aspartic acid | 1 | Aspartic acid |
| Glutamic acid | 2 | Glutamic acid |
| Ornithine | 1 | Ornithine (?) |
| Serine (D) | 1 | Serine |
| Alanine | 2 | Alanine |
| Leucine | 2 | Leucine |
| Valine | 1 | Valine |
| | | Arginine |
| | | Threonine |
| | | Proline |
| | | Glycine |
| | | Isoleucine |
| | | Tyrosine |
| | | Phenylalanine |

The procedure gave almost a quantitative recovery of the toxicity present in the cells, and the product appeared to be a single component by electrophoresis and chromatography. It has a LD₁₀₀ of 0.1 mg per kg (IP, mouse). This toxic peptide migrated as a cation on electrophoresis at pH 9.1, whereas the microcystin of Bishop et al (1959) migrated as a slow moving anion at this pH. An amino acid analysis of the peptide (Table 3) showed the presence of seven amino acids in addition to the amino acids reported for microcystin. Although this peptide appears to differ from microcystin, the authors believe that both are probably derived from the same toxic material present in the cells.

Microcystis aeruginosa diarrhea toxin

In 1974 Aziz reported the presence of a non-dialyzable material in laboratory grown cells of M. aeruginosa that had marked diarrheagenic activity in quinea pigs. The material was released from the cells by repeated freezing and thawing of the cells and by shaking the cells with glass beads. The author suggests that water blooms of blue-green algae may be the cause of diarrhea epidemics where no common etiology is known. No information on the further chemical characterizations of the diarrhea-producing toxin was given.

Anabaena flos-aquae VFDF

In 1966 Stavric and Gorham reported the purification of a VFDF from An. flos-aquae and its partial characterization as a low molecular weight substance absorbing strongly at 229 nm and containing an α,β -unsaturated ketone grouping and a tertiary amine group.

In 1975 Carmichael et al described the isolation of the VFDF from laboratory grown cells of An. flos-aquae by absolute alcohol extraction, extraction of the residue from the alcohol extract with chloroform, and extraction of the chloroform soluble fraction with acidic water. The toxin was extracted by the acidic water, indicating that it was a basic substance. The toxin had a MLD of 0.3 mg per kg (IP, mouse).

The structure of the N-acetyl derivative of the An. flos-aquae VFDF was shown to be a 2,9-diacetyl-9-azabicyclo,[4,2,1]non-2, 3-ene (Figure 1) by Huber in 1972 on the basis of X-ray crystallography.

Lyngbya majuscula dermatitis-producing toxin

In 1971 Moikeha et al isolated the causative agent of severe contact dermatitis encountered in swimmers in Hawaii from cells collected from a natural bloom of L. majuscula. The toxin was soluble in polar organic solvents, insoluble in water, and only slightly soluble in petroleum ether. It was extracted from lyophilized cells with chloroform-methanol (2:1) and purified (I) by column chromatography on silicic acid followed by preparative thin-layer chromatography or (II) by saponification of the crude lipid extract, passage of the toxic non-saponifiable fraction through an alumina column, passage through anhydrous calcium oxide column, and preparative thin-layer chromatography. The toxin produced severe dermatitis when injected intradermally into mice. It was unstable to heat and light and its infrared spectrum indicated the presence of hydroxyl groups, and C=C, and probably C=N, bonds.

More recently Hashimoto et al (1971) extracted a dermatitis-producing toxin from cells of Microcoleus lyngbyaceus (syn. Lyngbya majuscula) collected from a bloom in Okinawa. The toxin was a neutral molecule soluble in organic solvents, and it was purified by column chromatography on silicic acid, liquid-liquid partition, gel filtration of Sephadex LH 20, and dry column chromatography on silicic acid. The toxin showed absorption maxima at 203, 214, 270, and 275 (shoulder) nm in methanol, gave a positive test for a phenolic compound, and is assumed to be a phenol. The toxin appears to be identical to the dermatitis-producing toxin described by Moikeha et al, (1971).

It has been postulated that ciguatera, a disease characterized by gastrointestinal and neurological symptoms and caused by the ingestion of a wide variety of tropical marine fish, may be the result of a toxin that originates in a benthic alga, perhaps the blue-green Lyngbya majuscula, and is transmitted through the food chain (Randall, 1958). Scheur et al (1967) extracted the raw flesh of toxic moray eel, from Johnston Island, with acetone. The toxin was purified through a series of solvent partitioning steps followed by column chromatography on silicic acid and preparative thin-layer chromatography. The toxin, for which the name ciguatoxin was proposed, was obtained as a light yellow viscous oil that could not be crystallized.

It has a MLD of 0.5 mg per kg (IP, mouse). An empirical formula of $C_{35}H_{65}NO_8$ was suggested although later high resolution mass spectral data indicated that the molecule contains at least three atoms of nitrogen (Scheur, 1969). Hydrolysis of the toxin gave glycerol and showed the presence of a series of long chain saturated and unsaturated fatty acids and a nitrogen base that reacts positive to Dragendorff and Jaffe reagents (Scheur, 1967, 1969). The toxin does not contain phosphorus, so although it is a glyceride, it is not a phosphatide.

Yasumoto and Scheur (1969) found that the livers of Johnston Island moray eel were toxic regardless of whether or not the flesh was toxic and that the toxin from the liver was identical to or closely related to the nitrogenous moiety of ciguatoxin (20). Adding to the complexity of the ciguatera picture, Hashimoto and Yasumoto (1965) extracted a water-soluble toxin, for which the authors proposed the name ciguaterin, from the flesh of the grouper. This toxin also gave the typical symptoms of ciguatera poisoning.

Impact of Toxic Blue-Green Algae on Animal Systems

Toxic materials of biological origin attract attention from researchers because of their specificity, potency, and potential utility as physiological or pharmacological "tools". Since many microorganisms elaborate one or sometimes several toxins, a comparative approach utilizing appropriate extraction and testing procedures should point out meaningful similarities and differences in the effect of the bioactive materials.

It may be important also to study the harmful effects on aquatic macroorganisms such as shellfish and crustaceans that may experience potent toxic concentrations during bloom conditions. Some shellfish species are apparently not affected by several blue-green and dinoflagellate toxins. This may suggest that mechanisms for maintaining electrogenic membrane systems differ in bivalves and vertebrates (Sasner, 1973; Twarog and Yamaguchi, 1975). Preliminary investigations have revealed the involvement of neuro-muscular systems with the general site of action of a wide variety of aquatic microorganisms toxins. It is appropriate that their effects be studied at the membrane level on excitable tissue where a more specific mode of action can be determined. Such an approach has public health implications as well as providing potentially useful compounds with which to challenge neuro-muscular preparations in the course of studying their normal physiology. General reviews (Sasner, 1973 and Shantz, 1970) reveal the paucity of information on the chemical and physiological characteristics of compounds elaborated by aquatic microorganisms.

In eutrophic fresh water environments that undergo seasonal microorganism blooms, the blue-green algae are the most common toxic offenders. The state in the eutrophication process of an aquatic ecosystem can often be associated with the frequency and density of such blooms. In addition, secondary conditions not associated with specific poisons, may result from the rapid, massive growth of algae.

The conditions such as oxygen deficiency, nutrient imbalance, hydrogen sulfide production and increased bacterial growth may be harmful to organisms higher in the food chain. It is important, then, to distinguish between the harmful effects of biotoxins associated with algal species and the so-called, secondary but equally harmful effects of altering the physical-chemical environment via massive algal blooms. Obviously the problems of sorting-out such effects are compounded when toxic algal species grow to massive bloom proportions. Increasing interest in the parameters associated with algal blooms stems from at least 2 areas of interest. First, the eutrophication is usually associated with the rate of biological productivity and the degree of oxygen and thermal stratification (Greeson 1969). In short, bloom conditions can affect the economic and aesthetic value of a lake or pond. This may have direct bearing on the aquaculture industry, (i.e., fish farming), sport fishing and recreational utility of ponds and lakes. Second, the biotoxins associated with some "bloom-ing" species may provide a potential source of compounds of biomedical interest. It is clear that several aquatic toxins from microorganism affect the ionic mechanisms of electrogenesis associated with biological membranes and/or the neuro-transmitter system controlling information flow between contiguous cells.

To the comparative physiologist, these active substances may be useful in discovering the similarities and differences in electrogenic and transmitter systems in a wide variety of organisms. To the medical physiologist, these aquatic toxins may serve the same kind of role as poisons from terrestrial plant sources. For a list of some of the more commonly exploited plant poisons and their pharmacological properties, see Sasner, 1973.

The massive mortalities of livestock and water fowl as well as the unpleasant odor and water color during toxic blooms are the primary concerns of the layman. Gentile (1971) has reviewed the interactions of blue-green algal materials with other organisms at the same and higher trophic levels i.e., other algae, zooplankton, and fish. We feel these associations or effects are very basic to the understanding of the latter stages in the eutrophication process. While we have answered several questions concerning the role of the toxic blue-green algae, we have discovered many more that need answers.

A schematic diagram of our testing program for blue-green algal toxins is outlined below.

BLUE-GREEN TOXIC EFFECT
ON

- | | | | |
|---|---|---|---|
| A) Growth and Fecundity - <u>Daphnia magna</u> | B) Heart rate Filtration - <u>Daphnia magna</u> | C) O ₂ consumption -small fish -bivalve gill | D) Neuro-muscular systems -amphibians |
|---|---|---|---|

The toxic blue-green alga Aphanizomenon flos-aquae was harvested from several New Hampshire lakes during bloom conditions (4.0×10^8 cells/liter) that caused water coloration and musty odor. We used DeLaval separators at lakeside to spin the cells from hundreds of liters of water/day. Cell residues were frozen until processed in the laboratory. Crude extracts were prepared by lyophilizing the algal suspensions. Dried material was then reconstituted with lake water and tested on whole organisms. Purified toxin was used in experiments on isolated tissues. In this case, the thick Aphanizomenon suspensions obtained from centrifugation were acidified (pH 3.0), heated to 80-90°C, chilled and then centrifuged at 10,000 X g for 15 minutes to remove solids. This procedure was repeated three times to insure maximum yield. The combined supernatants were neutralized, dried and then extracted three times with 95% ethanol and dried again. The resulting residue was dissolved in water, acidified to pH 3 and HCl and extracted with chloroform and the aqueous layer dried again. The residue was purified by high voltage electrophoresis, ion-exchange column chromatography and thin layer chromatography. The toxicity was increased from 2.5 to 745 MU/mg during the purification procedure. The material obtained is much less potent than saxitoxin, which has 5500 MU/mg (2). (See Alam, et al, 1973). Purified samples of Anabaena toxin were obtained from W.W. Carmichael, Botany Department, University of Alberta, Canada.

Straited, smooth and cardiac muscle contractions or tension development were recorded using Grass FT-03C transducers or RCA 5734 electro-mechanical transducers with signals displayed on a Model 5 Grass Polygraph or Beckman RP Dynograph. Extracellular action potentials were recorded with Ag-AgCl electrodes. Intracellular measurements employed 3 M KCl filled glass microcapillaries with a tip resistance between 10-20 M Ω and a low tip potential. Action potentials from spontaneously active tissues (hearts) were recorded with a "hanging microelectrode" (Woodbury and Brady, 1956). All resting and activity potentials were displayed on a Tektronix 502A oscilloscope preceded by a Keithley Model 605 electrometer. Appropriate Ringer solutions acted as a vehicle for the various toxins used in these studies. Control solutions were prepared by extracting uninoculated culture medium and suspending the residue in appropriate saline solutions.

The rapid decline in numbers of planktonic cladocerans and copepods have been associated with dense blue-green algal blooms by several doctoral students at U.N.H. Gentile and Maloney (1969) were unable to grow Daphnia catawba and Bosmina longirostris in the presence of Aphanizomenon cells. Stangeberg (1967) reported that cell extracts of Microcystis aeruginosa killed Daphnia longispina and Dillenberg and Dehnel (1960) noted immobilization of Daphnids in a water supply containing Anaebaena and Aphanizomenon.

Specimens of the Cladoceran, Daphnia magna, from a growing laboratory population were used for growth and fecundity studies. Prior to testing the effect of Aphanizomenon toxin, the reproductive rates and pre-adult instar development was determined and compared to the rates of Anderson (1932, 1937). The rapid growth between successive instar stages may make Cladocerans more susceptible to toxic substances than other common microcrustaceans, i.e., copepods. During the period of rapid growth the carapace may be more permeable than that at other stages in the life history. It is important then, to know the exact stage during test exposures using toxins.

A modification of the techniques used by Anderson, et al (1937) and Gentile and Maloney (1969) was used in our study. Individual adult Daphnia magna were isolated in 125 ml/erlenmeyer flasks and maintained on a mixed algal culture at 12-15°C. The oxygen concentration was maintained above 5 p.p.m. (5 mg/L) by gentle aeration. Pre-adult animals released from a single adult were used in the growth study. Total length (L), height (H), and carapace length (C) were measured each day.

Pre-adult instars were observed for 11 days before an egg-bearing adult stage was attained. The periods between successive pre-adult molts varied from one to several days with increased variability as the adult stage was reached. Our growth data corresponds closely to that reported by Anderson, et al (1937). This allowed the selection of experimental Daphnids of known age and instar stage for test purposes and eliminated some of the variability that might occur in animals from different age classes.

Animals at different growth stages were challenged with Aphanizomenon flos-aquae toxin by dissolving varying amounts of dried algal material into the test solutions (2.0, 1.0 and 0.5 mg/ml). It is unlikely that blue-green algal material would exceed 2 mg/ml (dry wt.) in nature during bloom. It should be noted that half this amount (1 mg/ml) was potent enough to kill 20 grams mice (injected i.p.) in approximately two minutes. Under our test conditions the growth of reproduction capacity of Daphnia magna was not affected. Gentile and Maloney (1969) reported that their Cladocerans were susceptible to Aphan toxin in the same dosage range used in our test, although several other microcrustaceans were unaffected. These authors described the classical symptoms usually associated with toxicity in higher taxa, i.e., loss of equilibrium, loss of coordination, erratic and slowed activity of the filtering appendages. They observed no effect, however, on heart rate and peristalsis. Our results of heart activity (beats/min.) in Daphnids challenged with Aphan toxin support their observations. We are, however, unable to verify the harmful effects of the toxin to Cladocerans as they have indicated. We found no effects on animals tested at several pre-adult and adult stages. In fact, the Daphnids carried on the reproductive process in "Toxic" solutions at the same rate and time interval as did the control animals. We did find, however, what appeared to be a greater sensitivity to lower oxygen tensions in the test animals than in the controls.

Our observations on Daphnia magna are not consistent with what happens in nature during a toxic blue-green algal bloom. However, there may be secondary effects, i.e., reduced oxygen concentrations, increased metabolite production, fouling of the feeding apparatus, etc., that may reduce zooplankton populations. These secondary or indirect effects of algal blooms may have little to do with toxic algal species since these phenomena have been associated with non-toxic blooms as well.

In the mid 1960's massive fish kills occurred in at least two New Hampshire lakes after treatment of blue-green algal blooms with CuSO_4 , Saywer, et al (1968). Laboratory studies on fish toxicity were then carried out using lyophilized algal material concentrated from lake water and fish species normally found in the lakes where the blue-green algae blooms occurred. White suckers (Catostomus commersoni) and sunfish (Lepomis gibbosus) were shown to be sensitive to Aphan toxin at cell densities above 4×10^5 cells/ml. In addition, Gentile and Maloney, (1969) demonstrated the sensitivity of the eastern golden shiner (Notemigonus cruspleucas), the killifish (Fundulus heteroclitus) and the sheepshead minnow (Cyprinodon variegatus) to Aphan toxin when exposed to laboratory cultures or i.p. injection. Comparative data indicate that fish are approximately 10 times more sensitive to the toxin than laboratory mice (MLD or $\text{LD}_{100} = 10 \text{ mg/kg}$) commonly used in bioassay tests Gentile (1971).

Standard oxygen consumption measurements were made on young of the year (0.3-0.7g) sunfish (Lepomis gibbosus) challenged with dried Aphan samples prepared from material collected at Kezar Lake, North Sutton, New Hampshire. A Gilson microrespirometer was employed as the measuring device with frequent purging of the reaction flask with 100% oxygen. Figure 2C shows representative results obtained from ten animals. Control animals consumed an average 180 uliters O_2 /gram wet wt./hour, while fish subjected to 0.25-0.50 mg/ml (dry wt.) of algal material immediately increased oxygen utilization to an average value of 238 ul O_2 /grams wet wt./hour. This represented a 32% increase within two hours. Increasing the dose level ten times killed all test fish within 30 minutes. The ventilation rates increased markedly in all test animals and animals succumbed with broadly extended opercula.

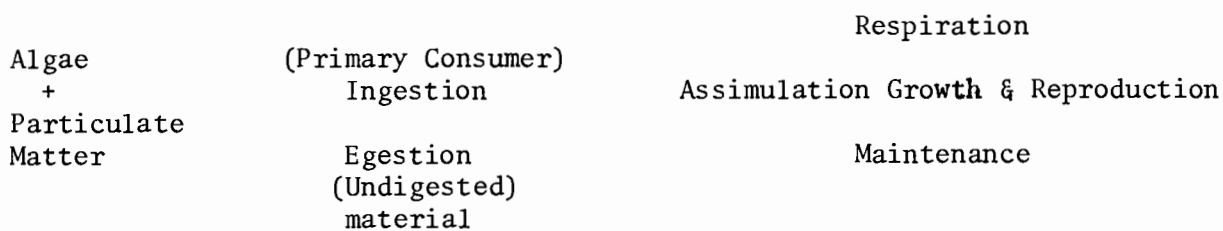
Many of the fish mortalities associated with blooms have been related to anoxia or to harmful substances produced as the algal cells decompose, Mackenthum et al (1945). Hydroxylamine and hydrogen sulfide are two products of bloom decomposition implicated in fish kills, Prescott (1948). While algal toxins, low-oxygen tensions and by-products of bloom decomposition exert deleterious effects on aquatic animals individually, when toxic blue-green algal blooms occur, the possible combination of these factors must be considered. Laboratory studies must be correlated to what goes on under natural conditions and the latter must include the influence of man. The potency of toxins from at least three blue-green algal species (Aphanizomenon flos-aquae, Anabaena flos-aquae, and Microcystis aeruginosa) are altered by pH changes.

They are generally acid stable, and alkaline and temperature labile (Sawyer, et al (1968), Gentile (1971)). Eutrophic lakes are generally warm water ecosystems where dissolved oxygen values can vary over a wide range. During daylight hours photosynthesis by blooming algae maintain relatively high oxygen tensions and consequently high pH values (>8.0) in the photic zone of a lake ecosystem. In the dark, however, the total oxygen demand increases dramatically, the oxygen tension falls and the pH also drops toward more acidic values. This enhances the stability and potency of blue-green algal toxins. The net result is an increased demand for oxygen and less oxygen available--a set of conditions that may severely stress aquatic vertebrates.

In the laboratory studies discussed above, high oxygen tensions were artificially maintained to isolate and study the effect of the bio-toxins. These effects are enhanced by the use of lyophilized cell materials and neutral or slightly acidic test media. In nature the endo-toxic material is released into the epilimnion over a rather long period of time (several days to weeks) and thus the toxins may be diluted and/or destroyed. On the other hand, when algicides like CuSO_4 are used to break up blooms the endotoxins would be released and bacterial decomposition would occur over a shorter time interval.

In addition, sublethal concentrations of copper may cause mucus secretion over the respiratory surfaces of aquatic animals thereby retarding respiratory gas exchange. The significance of blue-green algal toxins in association with the secondary factors mentioned above is clear, particularly since CuSO_4 has been effectively used by the New Hampshire Water Pollution Control Commission to control non-toxic algal blooms. Field observations and laboratory experiments on fish clearly indicated the impact toxic blue-greens could have on organisms higher in the food chain. The variable biological, physical and chemical parameters are easily inferred from the information at hand, however, they remain to be examined quantitatively.

Filter feeding organisms, i.e., bivalves, copepods are the primary consumers that "capture" phytoplankton and particulate matter suspended in the water column. Filter-feeding microcrustaceans play a most important role in the food chain of a freshwater ecosystem, since they serve as a food source for fish. The flow diagram below indicates the pathway of material and energy flow through filter-feeding organisms.



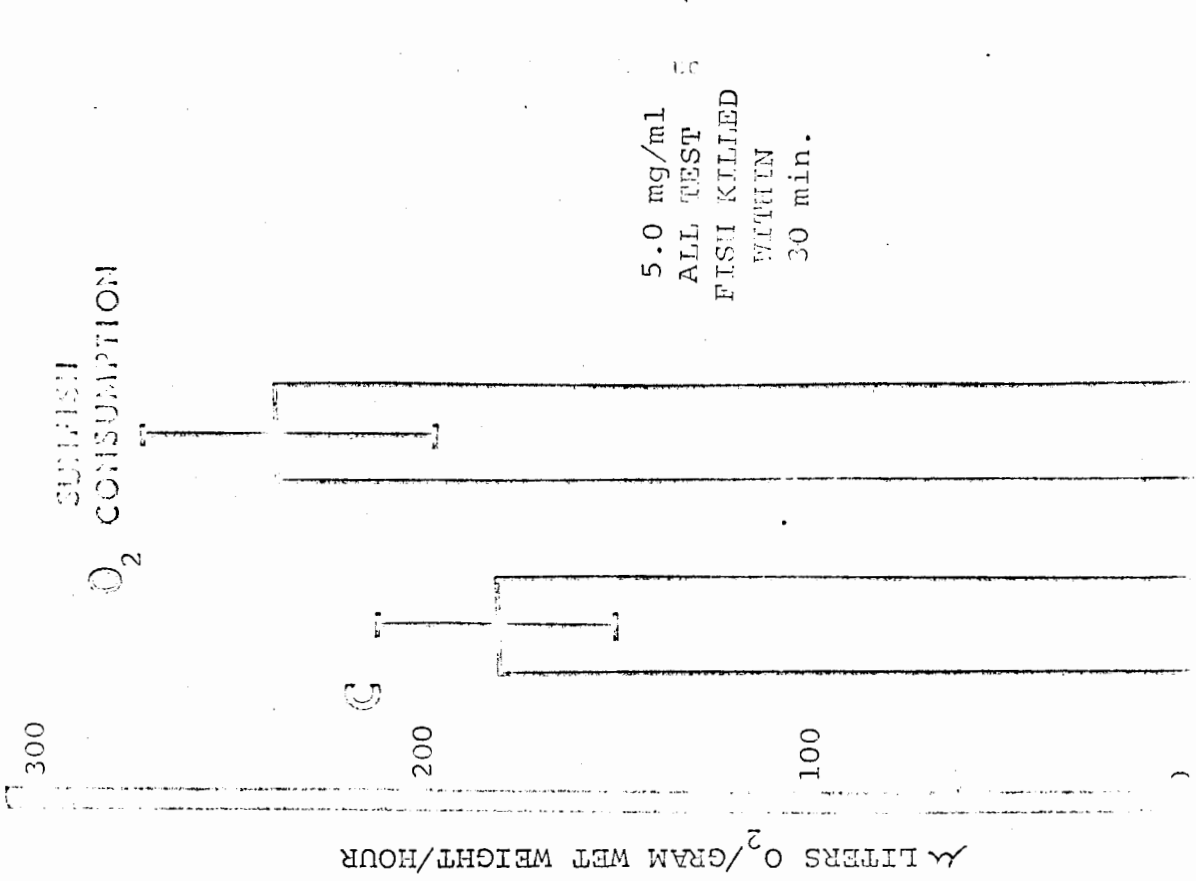
In the marine environment, bivalves serve as biological "storage depots" for toxin accumulation (paralytic shellfish poison = PSP) and pass on the toxicity to animals higher in the food chain. Since PSP has been chemically related to Aphan toxin (Jackim and Gentile (1968) and Alam (1973)), we wanted to study the effects of this toxin on fresh water bivalves.

The abundance of food (algal concentration) controls several physiological parameters at each trophic level in the food chain. There is a direct relationship between rate of filter-feeding by bivalves and a) their size or age, b) the ambient temperature, c) the food concentration and d) bivalve metabolic activity (Capuzzo (1973)). We attempted to control a, b, and c (above) and measure the effect of Aphan toxin on the oxygen consumption of freshwater bivalve gill tissue, that is, the ciliated food-gathering apparatus.

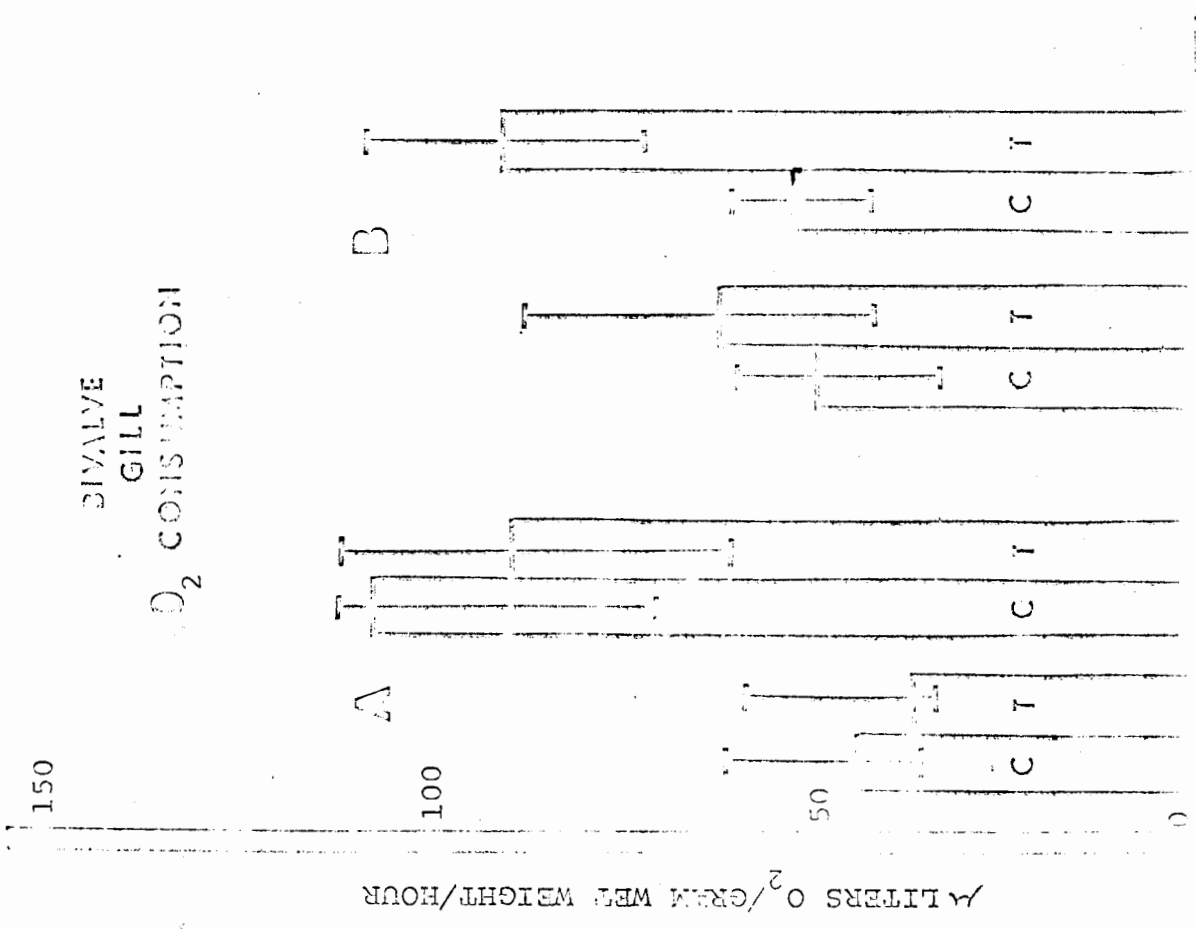
Oxygen consumption rates of excised gill tissues were used as an indicator of the ciliary activity of the filtering mechanisms. Specimens were dissected after exposure to different pre-measurement test conditions. A Gilson Differential Microrespirometer was used to measure oxygen uptake. Bivalves of a size range (45-60mm total shell length) were used to reduce variations due to age or stage of maturity. The gill tissue was placed in 15 ml reaction vessels with 5 mls of filtered lake water or algal culture medium. KOH (15 to 20% soln) was used as the carbon dioxide absorbant.

Figure 2A shows the oxygen consumption values of gills from animals allowed to filter the cells from 1 liter and ten liters of dense Aphan cultures respectively. Controls were allowed to filter the same volume of non-toxic mixed algal cultures. In both tests there was no significant difference in oxygen consumption values, indicating either that 1) the cells were digested and do not affect bivalve tissue or 2) the cells were passed through the digestive tract and egested without decomposition. Long mucus-trapped threads of cells were observed as waste material from the bivalves that fed extensively on Aphan cultures under lab conditions. Microscopic examination revealed that the egested material consisted mostly of whole, unbroken Aphan cells indicating that the endotoxin passed through the animals within intact cells. In another series of experiments, (Figure 2B), gills from previously starved animals were challenged with 0.2 mg/ml and 1.0 mg/ml of lyophilized Aphan material respectively.

The oxygen consumption values increased markedly indicating an effect on the metabolic and/or ciliary activity in the gill tissues. Microscopic examination of gill tissues showed a faster though more irregular pattern of ciliary activity in the gill tissues subjected to Aphan toxin. It is still uncertain, however, as to whether these effects in nature are a direct result of the toxic material or a secondary effect of increased oxygen consumption and lowered oxygen availability. We have observed significant bivalve mortality during and after Aphanizomenon blooms in Kezar Lake.



control 0.25 mg/ml



1 liter 10 liters 0.2 mg/ml 0 mg/ml
culture culture
cleaned cleaned

When excitable systems are challenged with extracts or purified components from toxic microorganisms, the acute sensitivity of neuro-muscular systems and the potency of the active materials is evident. Table 4 lists the range of effects of three biotoxins and generally summarizes their mode of action and site of action in several physiological preparations. At least two different types of responses were recorded for blue-green algal toxins.

The first general type of response involves the destruction of the conduction properties of excitable cells with no transmembrane depolarization. This characteristic of Aphanizomenon flos-aquae toxin and the poisons from the marine dinoflagellates in the genus Gonyaulax. The fresh water blue-green (Aphan) and some marine organisms evidently produce similar acting materials that may block sodium conductance pathways in membrane systems. Aphanizomenon toxin is chemically different from the marine poisons and may not be as specific in its activity since it also blocks calcium dependent membrane systems (Sawyer et al (1968) and Thurberg (1973)).

The second general type of response involves the transmission properties between excitable cells. Anabaena flos-aquae toxin directly affects end plate phenomena in cholinergic systems. The toxin acts like a post synaptic depolarizing agent, that may compete for acetylcholine sites with curare. Recent work indicates that miniature end plate potentials are rapidly abolished in the presence of Anabaena toxin, (Sasner, et al (1975) unpublished, Carmichael et al (1975)). In addition, mechanical records of muscle activity show a rapid decrease in amplitude with indirect (via nerve) stimulation. Since no effect was observed on the action potential reaching the neuro-muscular junction, we must assume, at this time, that the primary site of action of Anabaena toxin occurs at the gap between two contiguous cells.

Table 4

CHARACTERISTICS OF 3 BLUE-GREEN ALGAL TOXINS

| | <u>Aphanizomenon flos-aquae</u> | <u>Anabaena flos-aquae</u> | <u>Microcystis aeruginosa</u> |
|--|--|---|--|
| Location | Temperate lakes of U.S. & Canada | Temp. lakes of U.S. & Canada | Temp. lakes-USA, Can., USSR Eur. |
| Name of toxin(s) | <u>Aphan</u> toxin (VFDF) | Anatoxin-A (ANTX-A), Carmichael, 1975. (VFDF) | Microcystin (FDF) |
| Chemical Characteristics | H ₂ O & EtOH sol; CHCl ₃ insol; acid & heat stable; alkali labile; may be a substituted guanidine deriva- tive; low molecular weight endo- toxin. Not Saxitoxin. | H ₂ O & EtOH sol; CHCl ₃ sol; basic molecule; 2,9-diacetyl-9- azabicyclo 4,2,1 non-2,3- ene. Molecular weight=165; endotoxin. | MeOH, EtOH and Na ₂ CO ₃ sol; dialyzable; cyclic peptide- not destroyed by proteolytic enzymes; 14 different amino acids; endotoxin. |
| General Physiological Effects | Mammals and fish-spastic twitching Coordination loss, resp. irregular- ity. Toxin absorbed thru gill membrane of fish. | Mammals (i.p. or oral)-muscle facicul., coord., loss, respiratory failure. Toxin <u>not</u> absorbed across fish gill membranes. | B ₁ -avitaminosis-like effect. Blood disorders, lowers-hemato- crit, extravasation of int. organs, reduced Hb conc. Gross neuro-muscular effects like <u>Aphan</u> or <u>Anabaena</u> toxin but slower acting |
| Dose Range MLD -whole animal or LD ₁₀₀ | Lyoph. cells=10mg/kg, i.p. mice 1-5mg/ml Purified=745 MU/mg | Lyoph cells-60mg/kg, i.p. mice & rats. Purified (i.p.)-0.3 mg/kg, mice MLD (oral =7.5 mg/kg, rates; 1.8mg/kg, duck; 2.4mg/kg calves; 0.6 mg/kg goldfish. | Lyoph cells=2mg/MU; LD ₁₀₀ =0.1mg/kg |
| -isolated tissues | 10 - 100 ug/ml | 1-50 ug/ml | 0.6-5mg/ml. |
| Transmembrane Resting potentials | No effect | No effect | No immediate effect (<1 hr.) |
| Nerve Action Potentials | Blocked in desheathed nerves | No effect on desheathed nerves. | No immediate effect (<1 hr.) |

Aphanizomenon flos-aquae

Anabaena flos-aquae

Microcystis aeruginosa

| | | | |
|----------------------------|---|---|--|
| Muscle Mechanical Activity | Vert. striated-mech. activity blocked to indirect then direct stim, reversible. Vert & Crustacean heart-rate slowed to block, A.P. reduced, reversible Mollusc heart-no effect at incr. dose levels. (100X) | Vert. striated-mech. activity blocked to indirect then direct stimulation. Facicul., ACh-like effect on motor end plate before blockage, MEPP's reduced and blocked. Vert. smooth muscle-stimulated | Muscle paralysis, coordination loss, reversed by thiamine (100ug/50 gram body wt.) |
| Site of Action | Cell membranes, nerve & muscles. | <u>Motor end plate</u> | Thiamine \downarrow , Thiaminase \downarrow in body tissues |
| Mode of Action | Alteration in membrane ion conductance suggested | Post synaptic blocking agent suggested | Alternation in basic metabolic pathways suggested. |
| References | Sasner, 1973; Alam, et al 1973; Sawyer, et al, 1968; Gentile,1971; Alam, 1973; Thurberg, 1972. | Huber, 1972; Carmichael, et al, 1975, Sasner, et al, 1975. | Komarovsky, 1970; Malyarevskaya, et al, 1970; Gentile, 1971; Rama Murthy and Capindale, 1970 |

The toxin "microcystin" from the alga Microcystis aeruginosa has not been thoroughly tested on neuromuscular systems. Lethal effects of this toxin take considerably longer to act than toxins from the other blue-green algae (i.e., Aphanizomenon and Anabaena). Microcystin has received considerable attention from Russian scientists. This is undoubtedly due to the occurrence of massive blue-green algal blooms and associated fish kills in temperate lakes of the Soviet Union in the late 60's and early 70's. Komarovsky (1970) describes the same symptoms in fish that have been reported in the North American literature; i.e., sluggish movement, loss in coordination, inflammation of skin and gill areas, and death by respiratory failure. In addition, severe changes in blood chemistry coincide with the presence of the toxins. Malyarevskaya et al (1970) reported a decrease in thiamine and coinciding increase in thiaminase activity in the fresh water perch (Perca) challenged with sublethal doses of Microcystis toxin. The role of thiamine is ubiquitous in living systems, and plays an important role in the metabolism of carbohydrates, lipids and proteins. Injection of thiamine chloride (100 ug/50 grams) into body wt. perch afflicted with microcystin reversed the symptoms described above thereby linking the mode of action with B₁- avitaminosis.

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