HEAVY METAL TOXICITY ON BLUE-GREEN ALGA

by

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ABSTRACT

In this study, the growth response of Anabaena flos-aquae, which is a filamentous, heterocystous blue-green alga, towards heavy metals is described.

The experiments were long term, batch type experiments, and algae were incubated in media containing Hg(II) as mercuric chloride ($HgCl_2$),Cd(II) as cadmium chloride ($CdCl_2$), Se(IV) as sodium selenite (Na_2SeO_3), Ni(II) as nickel chloride ($NiCl_2.6H_2O$),Cr(VI) as potassium chromate(K_2CrO_4) and As(V) as sodium arsenate (Na_2HAsO_4). $7H_2O$) in solution.

Growth is measured spectrophotometrically and optical density is used in the expression of results.

In all cases, metals were found to be inhibitory on the growth, the degree of inhibition depending upon the type of the metal ion and the concentration in the medium.

It was found that in media contaminated with 10,20 ppm selenium and 0.2 ppm nickel, the growth of cells failed complelety.

ÖZET

Bu çalışmada, ağır metallerin mavi-yeşil bir alg türü olan Anabaena flos-aquae'nın büyümesi, büyüme hızı üzerindeki etkileri incelenmiştir.

Uzun dönemli ve kesikli (batch) tipindeki deneylerde Hg(II), civa clorür, Cd(II), cadmiyum clorür, Se(IV), sodyum selenat, Ni(II), nikel klorür, Cr(VI), potasyum kromat ve As(V), sodyum arsenat eriyikleri olarak kullanılmıştır.

Büyümeler spectrophotometri ile ölçülüp, sonuçlar optik yoğunluk olarak belirtilmişlerdir.

Sonuçta, bütün metallerin büyümeyi önleyici olduğu, bu önlemenin derecesinin de metal iyonunun cinsine ve besleyici ortamdaki miktarına bağlı bulunduğu anlaşılmış ve 10 ve 20 ppm selenyum ve 0.2 ppm nikel eklenen ortamlarda büyümenin tamamen durduğu gözlenmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

A_o	Absorbance value at the beginning of selected time interval
A_1	Absorbance value at the end of selected time interval
D	Optical density
t_o	Initial time
t_1	Final time
γ	Dilutions of algal suspension
μ	Specific growth rate

I. INTRODUCTION

1.1. ALGAE

1.1.1 Species and Characteristics

Algae belong to the subkingdom of the Thallophyta, which lack root systems and leaves and which are by all odds the most primitive of all plants. However in one important respect, they resemble the higher plants: they possess the pigment called chlorophlly and can do photosynthesis.

The algae are ciefly water plants, dwelling in oceans, seas, lakes, ponds, rivers, ditches and other water bodies. Some species, however are found on stones, the bark of trees, and so on, generally in moist environments that are not subjected to direct sunlight. The algae are varied in size and shape. Some consist of individual microscopic cells which may be less than 1 micron in diameter, others form flat sheets, or narrow filaments or immense stemlike structures that may be more than 700 feet long.

These primitive plants are divided into nine phyla:

-Blue green algae (or Cyanophyta)

-Euglenophyta

-Pyrrophyta

-Green algae (or Chlorophyta)

-Brown algae (or Phaeophyta)

-Chroysophyta

-Red algae (or Rhodophyta)

-Cryptophyta

-Chloromonophyta

The Cyanophyta are thought to be the most ancient of all chlorophyll-bearing organisms on earth. Many have an ability in adjusting to high temperatures and to low temperatures of polar regions. These slimy algae contain a blue pigment (phycocyanin) in addition to cholorophyll and other pigments. The blue masks out the other hues and gives most species a dark blue color, there are other colors, however, ranging from orange to black. The blue green algae may be solitary or incidentally clumped cells round and oval, globular or platelike colonies of cells enclosed in mucilage. The form taken by the majority of Cyanotphytes is that of a thread of cells (a trichome) in which there is no division of labor. However, there are unbranched filaments in which there is differentiation of some cells to form heterocysts and other cells to form akinetes.

Heterocysts are formed by metamorphosis of a vegetative cell located either intercelary or terminal in a trichome. There is a second

wall layer, layed down internal to the original cell membrane. Heterocyst adjoins other cells in the trichome with a pore that is left at either pole. When the heterocyst matures and the wall is completed, these pores become plugged with mucilage and the pluggs show clearly as shining nodules when the plant is viewed microscopically. The contents of the heterocyst become homogenous and there are chemical changes in nucleic acids as other material is synthesized. The cell wall consists of a inner, thin, cellulose layer, a median pectic layer and an outer mucilage layer. Reproduction in the Cyanophyta is primarily by binary fission. Growth in multicellular plants occurs by both apical and intercolony cell division.

Despite possessing a simple conservative morphology, the Cyanophyta show a highly refined physiology and a complex metabolism which makes them biologically interesting and economically important. An interesting aspect of the Cyanophyta physiology is the ability of several forms to fix atmospheric nitrogen. The amount of nitrogen fixed is about 1.2 per cent of the weight of the sugar used. At least twenty species are nitrogen fixers, mostly in the genera Anabaena, Nostoc and Cylindrospermum. Heterocysts are the places for nitrogen fixation and when this occurs simultaneously with the photosynthetic process the fixation makes use of H as a donor. In culture it has been shown also that some Cyanophytas can fix nitrogen in darkness if supplied with sugar.

Generally blue green algae fall into three main groups, the first being filamentous blue green algae which possess characterically differentiated cells, called heterocysts and to which Anabaena flos-aquae,

the algae used in our experiments, belongs, second, filamentous blue green algae which lack of heterocysts and, third, unicellular blue green algae Anabaena species, are solitary and euplanktonic or occur as many plants embedded in a soft matrix, forming gelatinous masses on substrates, or intermingled with other algae. Cells are spherical barrel-shaped, or sub-cylindric and the trichomes are straight, regularly coiled or entangled. Heterocysts are conspicuous and akinetes are large, solitary, two or several together. They often occur in water blooms and play a role in the many disturbances resulting from unbalanced conditions in lakes and reservoirs[1].

1.1.2 Reaction to Toxicity

Algae can detect very low levels of heavy metals in the environment. Therefore, the utilization of algae as bioassay organisms is now commonly accepted. The response of algae to nutrients as well as to toxic material has been investigated.

In bioassay alga senses qualitatively and quantitatively the composition of its milieu through incoming chemical and physical signals. These signals influence one or more of the metabolic pathways in the organism, and the more important the effected pathways are to the driving of organism through its life cycle or to the maintanance of the total life process, the easier can the effect be detected in the organism or in the culture as a whole. By detecting effects on single metabolic or morphological processes (e.g. active transport of com-

pounds, induction and derepression of enzyme synthesis, cell motility or division) the cell system can act as a multitransducer instead of being only a general toxicity sensor.

Although some potentially toxic metals may be necessary micronutrients at very low concentrations, due to some environmental characteristhics. algae respond to increasing levels of heavy metals such as copper, nickel, mercury,silver or cadmium by reduction of growth rate, photosynthesis, and of nitrogen fixation. The critical concentrations for toxicity in question of a particular metal may vary at different times during the growth of an algal culture being as well dependent upon other chemical and physical conditions.[2]

1.2. HISTORICAL BACKGROUND

1.2.1 Mercury

Mercury is an extremely toxic pollutant,enters the environment from both natural and anthropogenic sources. It is inhibitory to many components of the biosphere and much of this metal eventually enters the hydrosphere. Therefore the susceptibility of the algae, which are the primary producers in many aquatic ecosystems, to heavy metal toxicity has been the subject of several reports.[3][4]

Algae accumulate mercury either by surface adsorption or by active uptake and the cell membrane is reported to be the primary site of mercury toxicity. Cell lysis plays a major role in the inhibition

of growth and acetylene reduction and a significant but less apparent role in the inhibition of photosynthesis.

Stratton et al.[5] found that 96 per cent of the free mercury could be adsorbed in only 5 minutes by Anabaena inaequalis cells at an initial level of 2000 ppb mercury. After adsorption mercuric ion induced cell lysis, the time required for lysis was dependent upon the concentration of the metal added to the system. Binding was irreversible and cells were affected by mercury at different time intervals. For exampleabout one half of the cells in the filament were lysed in 15 min at 100 ppb mercury and all cells were lysed within 1 h.

Stratton et al. also correlated the amount of mercury required for complete lysis with cell concentration due to the following equation:

$Y = 0.19\ X - 0.05$ ($r = 0.9899$) where

$Y = \log_{10} Hg^{2+}$ (ppb)

X = cell / ml ($\div 10^5$)

It was apparent that heterocysts were not lysed by mercury because of their extremely thick cell envelope and additional extracellular layers.

In general mercury toxicity can be related to a slow uptake of mercury ions into the cell. Mercury is accumulated intracellularly by some algae and once within th cell, mercury could interact with numerous enzyme systems. Mercury's affinity for S-H and S-S groups of protein is firmly established. Inhibition of biosynthesis of lipids, especially galacto-lipids, one of the major chloroplast lipids and chlorophyll in photosynthetically grown frehwater algae by $HgCl_2$ and

methly-$HgCl_2$ has been demonstrated by Matson et al.[6] The same group also obtained significant inhibition of galactosyl transferase activity in isolated chloroplasts of Euglena gracilis. The conclusion was that mercury was binding with the proteins in question and thereby preventing normal function. Owing to the common occurance of S-H groups in cells, it is probable that inhibition of cell division is caused by a similar inactivation of proteins involved in cell division. In this way mercury can inhibit the transport of carbon compounds, the synthesis of pigments, the photosynthetic transfer of electrons and the activity of many enzymes.

According to Huisman et al.[7] the rapid initial uptake of mercury is partly a purely physical adsorption of mercury to cells(the so-called water-washable mercury fraction) and partly a chemical binding at outer cell structures. The latter quantity of mercury is restricted by the number of available binding sites and to a lesser extent, by the free mercury (II) concentration and by competition with other metal ions (e.g. calcium, magnesium and iron). This chemically bound mercury can be washed off partly with a cysteine solution. Huisman et al. concluded that the noncysteine washable mercury (NCW mercury) was not mainly responsible for the deliterious action of mercury. They supported this conclusion by their observation that the total mercury content of cells as such could not be correlated with inhibition of growth and photosynthesis. A total mercury content of cells not lethal at $15^{o}C$, caused complete inhibition of growth and photosynthesis at $30^{o}C$. Cells growing in mercury containing medium at $15^{o}C$ and with a total mercury content lethal at $25^{o}C$ recovered upon

being transferred into fresh, mercury-free medium at 25°C. This recovery indicated that the NCW mercury of cells was non-toxic to the algae. NCW mercury might not reach its target and this might not be able to inhibit essential functions in a direct way due to immobilization in outer cell structures. Hannan et al.[8] who found a marked reduction of photosynthesis although only 2 per cent of the total mercury content of cells was found to be present in chloroplast, described the same phenomenon. The reversibility of inhibition suggests that the cysteine washable mercury is not bound to sulphhydryl groups but to carboxyl or phospate groups. These mercury ions can be readily exchanged for other metal ions, e.q. calcium ion, leading to a decrease of inhibition by mercury.

Many authers related the mercury toxicity to an interference with cell permeability with regard to the mode of action of mercury. Kamp-Nielsen[9] assumed inhibition of photosynthesis by mercury(II) to be caused by a loss of potassium ions from the cells, due to disturbance of the potassium of membranes. The potassium-loss can occur at a very low mercury dependent upon the mercury concentration of the medium.

In an X-ray energy dispersive study Jensen et al.[10] also showed that in mercury treated cells the potassium signal is lost and the calcium signal is enhances.

Huisman et al.[7] postulated the mechanism of mercury toxicity according to the mercury influences on potassium-sodium dependent ATPase, an enzyme localised in cell membranes. Inhibition of this potassium-sodium pump causes a loss of potassium from cells and directly

influences active transport of nutrients, e.g.nitrate and bicarbonate ions.

The toxicity of organic mercurials was demonstrated by Harris et al.[11] when they obtained 50 per cent inhibition of photosynthesis in freshwater photoplankton and of the marine diatom Nitzschia delicatissima populations exposed to organomercurial fungicides at concentrations as low as 1.0 ppb.

Nuzzi[12] tried concentrations of $HgCl_2$, ranging 0.74 µg/l-66.6 µg mercury/l, and concentrations of phenly mercuric acetate (PMA), ranging from 0.06 µg mercury/l-15.0 µg mercury/l, which were the concentrations equivalent to those found in the sea, on Phaeodactylum tricornutum, Chlorella sp. and Chlamydomonas sp. At the end of 16 days incubation he fpund that all the organisms were inhibited by both mercury compounds, PMA being more toxic than $HgCl_2$. Also unchanged growth of P.tricornutum, after addition of phenyl acetate, showed that the toxicity of PMA was due to the mercury portion of the molecule.

It was again found that, cells grown in sublethal concenrations of mercury became morphologically abnormal, for example P. tricornutum became highly vacuolated and a normally biradiate cell assumed an ovoid shape where Chlorella sp. formed giant morphologically abberrant cells.

In a study Ben-Bassat et al.[13] pointed out that raising the initial mercury concentration gradually from 0-1.0 mg/l caused an increasing lag period in the growth of Chlamydomonas and a medium containing 2 mg/l mercury retarded the growth of the culture completely. In the same work it is also suggested that the loss of the mer-

cury into volatile form through some biological processes, thus allowing it to leave the system, at the same time the permeability to the cells was reduced.

Likewise,Davies[14] demonstrated a linear decrease in specific growth rate of Isochrysis galbana with increasing intracellar levels of the metal.

The toxic effects of mercury salts $Hg(NO_3)$ and $HgCl_2$ on the growth rate of two planktonic diatoms Fragilarie crotonensis Kitton and Asterionella formosa Hass were investigated by Tompkins and Blinn[15]. Theyfound that Fragilaria crotonensis showed total inhibition at 0.1 ppm and a 4 day increase in lag phase along with a 2-4 fold reduction in growth rate at 0.05 ppm. Asterionella formosa showed a gradual increase in lag phase and reduction in growth rate with increasing concentrations of mercury up to 0.25 ppm with total inhibition at 0.5 ppm. Here, also it is pointed out that mercury salts in cultures of both species with soil extract additives were less toxic than were in totally defined systems. It is observed from the typical 8-16 called stellate colony to form large cylindrical stacks composed of 25 to 30 colonies.

Hutchinson and Stokes[16] experimented the effects of mercury on the growth of Chlorella and it appears that growth in mercury is reduced at 0.2 ppm concentration.

References up to now have not included the phenomenon of mercury loss from the culture media. These losses from stored aquous solutions have been explained in four ways:

(a) Chemical reduction of mercury (II) to mercury (I) followed by

disproportioning of the mercury (I) to mercury (II) and mercury (0) which evaporates rapidly

(b) Metabolic activity of microorganisms, comprising either reduction of mercury (II) to elemental mercury or formation of organic mercury compounds which leads to loss of mercury, resulting from evaporation of these products,

(c) Adsorption of mercury on the wall of the solution container,

(d) Leakage of mercury through the wall of the solution container

Materials best suited for minimizing adsorption and providing satisfactory conditions for storing mercury at low concentrations were found to be Pyrex glass, polycarbonate, and Teflon.[17] Furthermore, samples enriched with nutrients lost considerably more mercury than did non-exriched samples.

After a series of experiments Baier et al.[17] suggested that mercury lost from sterile solutions can possibly be explained by the combined phenomena of wall adsorption, conversion to organic forms by bacteria introduced during initial mercury additions and subsequent sampling and volatilization.

Huisman and Ten Hoopen[18] pointed out the fact that growing cultures of algae had greater mercury loss than the same medium without algae, might indicate that in experiments the reduction of mercury (II) by algal reducing agents gave rise to losses of mercury. Ben-Bassat et al. made similar observations using the green algae Chlorella pyrenoidosa.

It is also added that there is a decrease of the "free" mercury (II) in cultures because of binding by algal cell walls. Thinking that

in natural waters the concentrationsof metals are stabilized by the buffering capacity of dissolved, suspended and precipitated material with metal binding properties, Huisman and Ten Hoopen selected a mercury chelating agent as a mercury buffer in culture medium. Due to the needed characteristics (the stability constants of the mercury (II) complex should guarantee sufficient buffering capacity, chelator has to be neither an inhibitor of nor a nutrient to the algae and the stability constants of the mercury complexes have to be known for different temperatures) N-methyl iminodiacetic acid (MIDA) is used in the experiments with Scenedesmus acutus. They concluded that MIDA does not influence algal growth, after one day the unbuffered medium, in which 4×10^{-3} mMol mercury was used, no longer contained mercury and after two days the algae had lost the mercury, adsorbed on their cell walls and in spite of the presence of MIDA, 50 per cent of the mercury was lost in the experiment.

The same group then investigated the effect of temperature upon the toxicity of mercuric chloride to the green algae Scenedesmus acutus in batch experiments. Again a mercury (II) buffer system was used in order to keep the mercury (II) concentrations constant in the cultures. The combined effects of the mercury (II) concentration (using 0.1-0.9 µg/ml) and temperature (from 15 to 30°C) on the algae were tested by estimating rates of growth and photosynthesis. Toxicity of mercury (II) to S. acutus was shown to be enhanced by increasing temperature. As a result of MIDA-mercury (II) buffer use, their results differ from other authers in especially three respects:

They did not observe a lag phase after addition of mercury to

the algal cultures and did not find any correlation between initial cell density and toxicity of mercury as did Kamp Nielsen[9],Ben-Bassat et al.[13] Ben-Bassat and Mayer[19] They also postulated that when mercury is added to a culture medium which contained chelators as in the case of Kamp-Nielsen free mercury concentration will have been reduced by chelation giving rise to a shorter lag period than in the cultures without chelator added and these cultures will be fairly stable leading to reduced growth rates.

Anabaena inaequalis was found sensitive to mercuric ion in the pbb range by Stratton et al.[5]. Growth was inhibited significantly at concentrations of metal ion as low as 2 ppb. On the basis of equivalent cell numbers mercury was found to be toxic to A. inaequalis at 0.009 µg of mercury per 10^5 cells for growth.

Another toxicity study of Stratton[20] involves mixtures of mercuric, cadmium and nickel ions on a blue green alga Anabaena inaequalis.

When both mercuric and cadmium ions were used at sublethal concentrations (0.003 ppm mercury and 0.04 ppm cadmium) and incorpotated into the system simultaneously, they interacted synergistically with cell yield at day 6 and growth rate and antagonistically with the cell yield at day 12.

When cadmium ion was added prior to mercuric ion marked antagonism was observed and the percentage inhibition in this case was about one-fifteenth of the inhibition recorded when mercury was added first, indicating that cadmium may be protecting the cells against the effect of mercuric ion.

When A. inaequalis was treated with a lethal level of mercuric ion (0.01 ppm mercury), the protective effect of adding cadmium ion prior to mercury was even more pronounced than when both ions were used at sublethal levels.

When cadmium was used at a lethal concentration (0.08 ppm cadmium), the addition of mercury prior to cadmium protected the cells against the toxic effects of cadmium. It is suggested that these can be explained on the basis of competition between mercuric and cadmium ionsfor cellular binding sites. Mercuric ion is very tightly bound to most organic ligands and is not readily risplaced by cadmium, however, it is unable to displace most previously bound cadmium ions.

In the experiments with mercuric and nickel ion combinations it came out that with metal ions being used at sublethal concentrations (0.004 ppm mercury and 0.005 ppm nickel) and when mercuric ion was added first or when both metals were added simultaneously, synergism was observed for both the cell yield and growth rate. When nickel ion was added prior to mercuric ion, marked antagonism resulted, indicating that nickel may be protecting the cells against mercury toxicity. This was more pronounced when mercuric ion was used at a lethal level (0.01 ppm). When nickel was introduced at a lethal concentration (0.2 ppm) the addition of mercuric prior to nickel ion offered no protection against nickel toxicity. These are again explained according to the competition between the metals for binding sites on the surface of A. inaequalis.

When mercuric, cadmium and nickel ions are added simultaneously the metal ions gave expected results at day 6 and interacted antago-

nistically at day 12. Where the metal ions were added in sequence the level of antagonism towards the growth of A. inaequalis was usually more marked. Whereever mercuric ion was added first, the highest levels of toxicity were obtained. The addition of cadmium ion before the other metals gave the lowest levels of inhibition, while the addition of nickel ion first, resulted in intermediate levels of toxicity. It is suggested that these results may be related to binding affinities, mercuric ion being bound with the greatest affinity, followed by nickel and cadmium ions, and the greater the binding affinitiy, the more displacement of metal ions would occur resulting in a greater competition between those ions and others. for binding sites which would lead to a greater degree of antagonism.

Bringmann and Küh[21] worked on the inhibition of cell multiplication of Scenedesmus quaddicauda with $HgCl_2$ and pointed out that 0.07 mg/l $HgCl_2$ can be regarded as toxicity threshold for these algae.

According to the studies of Vocke et al.[22] the first significant inhibition of the freshwater algae Ankistrodesmus falcatus, Scenedesmus obliquus, Selenastrum capricornutum and Microcoleus vaginatus occured at 0.05, 0.1, 0.01 and 0.4 mg/l mercury (II) respectively. Algistatic-algicidal responses for Ankistrodesmus, Scenedesmus and Selenastrum were first noted at 0.4 mg/l. Microcoleus approached an algistatic response at 1.0 mg/l. The EC50 values are 0.078, 0.085, 0.033 and 0.253 mg/l mercury(II) for Ankistrodesmus, Scenedesmus, Selenastrum and Microcoleus respectively. The less sensitivity of blue green algae Microcoleus to mercury(II) is explained due to the differences in the test media or to the fact that the Microcoleus was not axenic.

Again in a metal mixture study Wong et al.[23] used arsenic, cadmium, chromium, copper, iron, lead, mercury, selenium and zink altogether. The researchers used a full strength (1 X) of the metal mixture contained 50 µg/l sodium arsenate, 0.2 µg/l cadmium nitrate, 50 µg/l potassium dichromate, 5 µg/l copper sulfate, 300 µg/l ferrous sulfate, 25 µg/l nickel sulfate, 10 µg/l sodium selenite and 30 µg/l zinc chloridewhere ten times, half strength and one-tenth of the mixture of these used. It was found that the amount of cells of Ankistrodesmus falcatus at the steady state were $1X10^6$, $9.3X10^5$, $8.8X10^5$ and $5.7X10^5$ cells per ml for control, 0.1 X, 0.5 X and 1 X metal concentrations respectively and it was understood that the presence of 1 X metal mixture reduce both the rate and the amount of reproduction of A. falcatus, whereas one-half and even one-tenth of the metal mixture have some inhibitory effect.

1.2.2 Cadmium

Cadmium is another potentially hazardous pollutant in the biosphere, due to its toxicity to many life forms and because it is a frequent contaminant in effluents and extremely persistent in most environments. It is rapidly absorbed and accumulated by plants, animals and is toxic to man.

Cadmium effects mainly cell devision of algae, probably through non-specific enzyme inhibition[24] found that untreated Anabaena inaequalis cultures contained 10-50 vegetative cells and the heterocyst per filament, while cadmium treated cultures had 60-100 vegetative

cells and up to 12 heterocysts per filament often in grapelike cultures. Apical cells in treated filaments were devoid of cellular contents. They suggested that increased heterocyst frequency may be due to an inhibition of the heterocyst frequency may be due to an inhibition of the heterocyst spacing mechanism, which is thought to involve glutamine-derived inhibitors or localized nutrient depletion may also be related to altered growth. According to their findings apical cells are probably more sensitive to their larger exposed surface areas. They also proposed that the pigment bleaching of A. inaequalis cultures (cultures yellowed with age) can be induced by CO_2 deprivation and nitrogen stress, since cells are able to utilize their pigments as a source of nitrogen.

Bartlett et al.[25] indicated that cadmium starts inhibiting growth of unicellular green algae Selanastrum capricornutum at 50 µg/l with complete inhibition at 80 µg/l and is algicidal at 650 µg/l. The noticable effect in the growth rate was a shift of the lag growth phase with increasing cadmium concentrations. The same group used copper and cadmium in combination and these cultures produced near normal growth rates upon resumption of growth.

Klass et al.[26] observed that cadmium (II) concentrations as well as 0.006 mg/l $CdCl_2$ had a significant inhibitory effect on Scenedesmus quadracauda and that 0.061 mg/l severely inhibited growth.

Hutchinson and Stokes[16] found that all cadmium concentrations above 0.05 ppm had an inhibitory effect on the growth of Chlorella vulgaris. In another study of theirs, cadmium and selenium in combination showed antagonisms for the same alage. 0.05 ppm cadmium was

found to reduce growth to 55 per cent and 0.05 ppm selenium to reduce it to 75 per cent. In combination, the growth was 79 per cent of the control. At 0.05 ppm cadmium and 0.1 ppm selenium growth was stimulated compared with the control.

It has been reported that considerable inhibition of growth of Chlorella ellipsoidea was observed when the cells were grown in freshwater medium constaining 2.5 ppm cadmium (27).

Hart and Scaife[28] reported that a cadmium (II) concentration of 0.25 mg/l as $Cd(CH_3CO_2)_2.H_2O$ inhibited the growth rate of Chlorella pyrenoidosa cultures in the logarithmic growth phase.

Rosko and Rachlin[29] reported EC50 value of 0.06 mg/l cadmium (II) as $CdCl_2$ for Chlorella vulgaris.

Chlorella salina has been found to grow well at salinities varying from zero to 16 PPT. Therefore Wong et al.[27] studied the growth responses of the algae using different salinities at cadmium contaminated samples. They observed typical sigmoid curves of growth with lag periods varying between 2 to 4 days depending on the amount of cadmium present in the culture media. In general, the higher the cadmium content in the culture medium, the longer the lag time was required. In cultures in which the cadmium contents were greater than 0.05 ppm a slower growth rate was observed. At zero salinity they did not find significant retardation of growth in 5 ppm cadmium contaminated medium, and concluded that cadmium tolerance of Chlorella salina CU-I is surprisingly high so that even in high salt and cadmium contaminated media this alga can still survive and grow well.

The same group also studied the cadmium accumulation by the same algae. They found that although the algal growth rate was not affected very much, accumulation of cadmium in the algal cells strongly depended on the amount of cadmium present in the medium without sodium chloride. The cadmium content in cells which had been cultured in 0.001 ppm cadmium contaminated complete medium was 3.9 ppm. It was also found that the algal cells accumulate 27 per cent of the total cadmium present in the culture medium. At higher cadmium concentrations such as at 0.1 ppm level, the cadmium content in the cells was as high as 142 ppm. As a result, they observed a linear relationship between the accumulated amount of cadmium in the algal cells and the initial cadmium concentration in the culture medium. Also in their salinity studies they found that sodium chloride can greatly suppress the cadmium uptake by the algal cells. In 0.1 ppm cadmium concentration, the cadmium content in the algal cells were found to be 124 ppm in medium without added sodium chloride 25 ppm in medium with 5 PPT sodium chloride, 18 ppm in medium with 10 PPT sodium chloride and only 7.6 ppm in medium with 15 PPT sodium chloride. Similar declines in cadmium uptakes were observed for media containing 0.001 ppm, 0.005 ppm, 0.01 ppm and 0.05 ppm cadmium with different salinities.

In another study, growth of the algae exposed to various levels of cadmium was not affected by 0.1 mg/l cadmium. Levels of 0.2 and 0.5 mg/l were algistatic and 1.0 mg/l was lethal to the cultures. Algal populations survived in subihnibitory and algistatic cadmium exposed cultures were found to be mainly Scenedesmus sp. and Chlorella sp.[30] In the same study it is found that cadmium accumulation by

the cultures is substantial with approximately 80 per cent removal until culture growth is severely inhibited.

Cossa[31] found cadmium uptake to be greater in exponentially growing cultures of marine diatoms than in other growth stages. At the end of this experiment approximately 67 per cent of the cadmium in solution was accumulated by the mixed algae culture even with severly inhibited physiological conditions in the cultures. With cadmium, copper and chromium present in the melium, nearly 50 per cent of the 100 µg/l cadmium was accumulated.

The growth of Anabaena inaqualis was inhibited significantly by cadmium concentrations greater than 0.02 ppm and completely at 0.06 ppm on 12th day[24]. No significant effect could be found upon the lag phase of growth on the culture doubling time, and retardation phase was reached sooner. It was also understood that cell sensitivity increased directly with exposure time. This and the observed cellular abnormalities led to a suggestion by Stratton and Corke[24] that metal toxicity resulted from effects of cadmium taken up by cells rather than cadmium at the cell surface.

The response of Anabaena inaqualis towards combinations of mercuric, cadmium and nickel ions was investigated by Stratton and Corke[11].

Effects of mercuric and cadmium ions, in combination, on the growth of A. inaequalis was mentioned above (see Mercury section).

When cadmium and nickel ions were used at sublethal levels (0.05 ppm nickel, 0.05 ppm cadmium), they interacted antagonistically towards both the cell yield and growth rate. There was no sig-

nificance as to the order of metal addition.

When cadmium ion was used at a lethal level (0.1 ppm), the addition of nickel ion prior to cadmium protected against the toxic effects of cadmium ion.

When nickel ion was used at a lethal concentration (0.2 ppm) there was no significant protection offered by the addition of cadmium ion first. It implied that nickel may be more tightly bound than cadmium since protection against cadmium toxicity is achieved by adding nickel prior to cadmium addition. In this preparation there were twice as many cadmium ions present. When nickel was used at a lethal level, however, there were 5 times more nickel ions present and they could have displaced previously bound cadmium. Therefore the researchers concluded that cadmium addition did not protect against nickel toxicity.

When the experiment was performed with 3 metal combination, the addition of nickel ion first resulted in intermediate levels of toxicity.

In a study, Devi Prasad and Devi Prasad[32] reported that cadmium concentrations of 5 ppm and above were lethal to Scenedesmus obliquus and Ankistrodesmus falcatus while 10 ppm was lethal to Chlorococcum spp. At lower concentrations (0.1-1.0 ppm) they pointed out that there was a slight stimulation in the growth of the three algae and explained this stimulation to be the result of some enzymes or stimulation of general cellular metabolism.

In one of their experiments with nickel and cadmium used together, each at 0.5 ppm, more increased growth was observed than when they

were used individually. This trend was reflected in the higher concentration (5 ppm) also as 5 ppm cadmium was not lethal when used along with nickel, to A. falcatus.

A combination of cadmium and lead, each at 0.5 ppm, also gave considerable increase in growth of A. falcatus when compared to the control as well as to when they were used individually.

From these results, they concluded that when used in combinations cadmium and nickel seemed to have interacted antagonistically as the stimulation of the growth at lower concentrations was more and the toxicity was reduced at higher concentration when compared to their individual effects. The antagonism between cadmium and lead was more pronounced as there was 50 per cent increase in growth when both were used at 0.5 ppm. It is known that the activities of enzymes like ATPase, succinic dehydrogenase, which are enhanced by cadmium, are inhibited by lead and the activities of enzymes like alkaline phosphatase, cytochrome oxidase, glucose 6-phosphodehydrogenase, etc. which are inhibited by cadmium are enhanced by lead.

Vocke et al.[22] worked on the freshwater algae with different cadmium concentrations. According to their findings cadmium (II) was extremely inhibitory, causing significant inhibition of Ankistrodesmus falcatus, Scenedesmus obliquus and Microcoleus at 0.01 mg/l and Selenastrum capricornutum at 0.05 mg/l. All the algae responded algistatically-algicidally at or below 0.3 mg/l. A significant stimulatory response was noted for A. falcatus with 0.001 mg/l. They also found that EC50 values for test algae are 0.033-0.253 mg/l for cadmium.

The toxicity threshold of cadmium as $Cd(NO_3)_2 . 4H_2O$ is found as 0.08 mg/l for green algae Scenedesmus quadricauda by Bringmann and Kühn[21].

With repeated subcultures at inhibitory levels of cadmium Whitton and Shehata[33] tried to increase the resistance of Anacystis nidulans to this metal. Subcultures were made from a strongly inhibitory level of metal to one just lethal to the present strain. Any flask showing growth was used as inoculum for further subculture. The process was repeated 25 times, leading in each case to increased resistance, when resistant populations were subcultured 20 times in basal medium and then returned to the original level of metal, there was a considerable increase in the lag on first subculture. The original resistance was nevertheless regained by the second subculture, so presumably resistance is due to the selection of spontaneous mutants. All the tolerant strains grew well in basal medium and showed growth curves quite similar to that of the wild type.

They also tested the influence of environmental variables for cadmium toxicity on the wild type and found that K (5-500 mg/l), Cl (120-200 mg/l), NO_3 (10-400 litre^{-1}N) and SO_4 (2.5-320 mglitre^{-1}S) had a negligible effect.

On the other hand when used in combinations, zinc markedly reduced cadmium toxicity to wild type A. nidulans and also to two zinc-tolerant mutants and similarly the presence of cadmium appears to reduce zinc toxicity to the cadmium tolerant mutant.

A mixture of ten metals, arsenic, cadmium, chromium, iron, lead, mercury, nickel, selenium and zinc was found to reduce the reproduction

of Ankistrodesmus falcatus as discussed in mercury section of the report[23].

In anstudy by Les and Walker[34] it has been found that cadmium at concentrations greater than 1.0 mg/l exhibit toxic effects on blue-green alga Chroococcus paris, the lowest concentration which show detectable toxicity being greater than 0.4 mg/l cadmium

1.2.3 Selenium

Generally little information is available concerning the toxicity of selenate to freshwater algae.

Kumar[35] reported an algistatic-algicidal response of the blue green algae Anacystis nidulans at 20 mg/l selenium (VI) as Na_2SeO_4.

Mode et al.[36] found that selenite and selenate inhibited growth of the Scenedesmus dimorphus and Anabaena cylindrica at 40 mg/l concentrations.

Kumar and Prakash[37] reported LD50 values for Anabaena variabilis and Anacystis nidulans at 13 mg/l and 31 mg/l selenium (IV) as Na_2SeO_3, respectively, and at 18 mg/l and 42 mg/l selenium (VI) as Na_2SeO_4, respectively. However it is concluded that the response values obtained from the literature for selenium (IV) are not directly comparable to selenium (VI) response values.

In studies of Hutchinson and Stokes[16] cadmium and selenium in combination showed antagonisms. 0.05 ppm cadmium reduced the growth of Chlorella vulgaris to 55 per cent and 0.05 ppm selenium reduced it to 75 per cent. In combination the growth was 79 per cent of the control.

At 0.05 ppm cadmium and 0.1 ppm selenium, growth was stimulated compared with the control. Haematococcus gave similar patterns of amelioration or metal antagonism with combinations of cadmium and selenium.

Vocke[22] indicated that sensitivity of the algal species to selenium (VI) varied. The first significant inhibition of Ankistrodesmus falcatus. Scenedesmus obliquus, Selenastrum capricornutum and Microcoleus vaginatus occured at 0.01.0.1.0.3 and 10 mg/l selenium(VI), respectively. Microcoleus stimulated by treatment levels of 1.0 mg/l and below. Algistaticalgicidal responses by Ankistrodesmus, Scenedesmus and Selenastrum appeared at 10,4 andl. 7 mg/l, respectively. Microcoleus approached an algistatic response at 50 mg/l.

When Ankistrodesmus falcatus is exposed to a metal mixture of arsenic, cadmium, chromium, iron, lead, mercury, nickel, selenium and zinc, it is found that the growth of the algae is reduced by the presence of metal mixture at 50,50,5,300,25,0.2,25,10 and 30 mg/l concentrations, respectively. In fact, one half and even one-tenth of the metal mixture show an inhibitory effect on these algae[23].

1.2.4 Nickel

It has been shown that nickel enters freshwater systems in various ways[32] and is important since algae constitute the base of many aquatic food chains.

It is known that nickel is accumulated by algae resulting in growth inhibition. Studies performed by Skaar et al.[38] on the growth

of Phaeodactylum tricornutum indicate that it is unaffected by 0.5 mg/l Ni and decreases only slightly in the presence of 1.0 mg/l.

Hutchinson and Stokes[16] reported decreased growth for cultures of Scenedesmus acuminatus. Chlorella vulgaris, Haematococcus capensis and Chlamydomonas eugametous exposed to a range of nickel concentrations (0.05-0.7 mg/l). They found Ni less toxic than Cu,Cd, Ag and Pb for Scenedesmus and showed that growth reduced at 0.5 ppm but at 0.25 ppm was almost as good as in the control. The same group also obtained that Scenedesmus was the most sensitive among other test algae and Chlamydomonas was the most resistant among the same species for nickel.

They also examined the effects of copper-nickel combinations on Chlorella and for all combinations they found synergistic effects. For example, 0.05 ppm copper reduced growth to 95 per cent, while 0.05 ppm nickel was stimulatory. However, in combination, the growth was reduced to 38 per cent of control. For 0.05 ppm copper and 0.1 ppm nickel growth was completely inhibited when the two metals were used together. Similar results were obtained for Haematococcus in which striking synergism can be seen when the effects of 0.05 ppm copper and nickel seperately are compared with the combined presence of both metals in solution.

Likewise studies on combinations of nickel and other metals are discussed in Hg and Cd sections.

Patrick et al.[39] noted changes in the species composition of experimental streams exposed to 0.002-1.0 mg/l Ni, reporting decreased diversity and abundance of diatom species and increased abundance of

green algae and blue-green algae.

Fezy et al.[40] examined the effect of Ni on the growth of the freshwater diatom Navicula pelliculosa observing the differences in the growth rate and number of cells present in laboratory cultures exposed to 0,100,300 and 600 µg/l Ni for 14 days. Nickel concentrations as low as 100 µg/l reduced the population growth rate by 50 per cent and led to decreased 14-day cell numbers. Comparing these experiments to the findings of Hutchinson and Stokes they concluded that short term exposure to Ni (less than 7 days) could have serious effects on natural communities.

Bringmann and Kühn[21] found toxicity thresholds for Scenedesmus quadricauda as 1.3 mg/l Ni when they used $NiCl_2.6H_2O$.

Jensen et al.[10] studied heavy metal uptake by Plectonema boryanum using X-ray energy dispersive analysis. They found that in Ni-exposed cells this element can be detected in the polyphosphate body cell sectors but not in the cell sectors without polyphosphate bodies.

Devi Prasad and Devi Prasad[32] concluded that Ni was not lethal between 0.1 and 10.0 ppm to Ankistrodesmus falcatus, Scenedesmus obliquus and Chlorococcum spp. Also when combined with Cd, Ni showed interaction on Ankistrodesmus, however no interaction was noted with Pb-Ni.

In order to interpret the behaviour of blue-green algae at mining sites Whitton and Shehata[33] tested the tolerance of Anacystis nidulans cultures towards nickel. They showed that by repeated subculturing at inhibitory levels the resistance of to nickel can be increased and found that the strongly inhibitory level of Ni was 0.16 mg/l for wild type whereas it was 1.30 for the tolerant strain.

In another study by Wong et al.[23] a mixture of ten metals(As, Cd,Cr,Fe,Pb,Hg,Ni,Se, and Zn) at the Great Lakes Water Quality Objectivel levels was tested and it was found that tese reduced the reproduction of 4 cultured freshwater algae, Scenedesmus, Chlorella, Anabaena, and Navicula as well as natural phytoplankton from Lake Ontario water.

Wang and Wood[41] found cyanobacteria to be more sensitive to nickel toxicity then the green algae or the Euglena sp. They also examined the effect of environmental variables on nickel uptake for the same algae and a striking pH effect for bioaccumulation was observed.

1.2.5 Chromium

Since large amounts of chromium are released into natural waters through industrial processes it was needed to examine the adverse effects of this excess chromium on ecosystem.

Richards[42] found total mortality of Spirogyra insignis at 2.5 ppm while Oscillatoria limosa did not succumb until the 250 ppm level was reached.

Hervey[43] found that the growth of Chlorella sp. was not inhibited by 3.2 ppm Cr(VI) but that two species of Euglenoids were inhibited by 0.32 ppm.

Mangi et al.[44] used 0.01-10 ppm chromium which was supplied as chromate for Palmella mucosa, Oedogoniumsp., Hydrodictyon reticulatum and Palmellococcus protothecoides. They observed that cell numbers of unicells declined after two weeks and the filamentous forms

showed a loss of weight.

About the mechanism of chromium toxicity, Richards[42] suggests that adsorption occurs on the cell walls of the alga. In their work also Mangi et al.[44] found that a large proportion of the chromium is localised on the cell walls. They also suggested that a sufficient dose of chromium may be adsorbed in or on cells to lower the concentration of chromium significantly which may allow some surviving cells to grow and re-establish the population. Also they though that 'sacrificial lamp' effect can operate in massed algae and the outer most cells, their walls and capsules, may remove enough chromium to protect the innermost cells from limited doses of the metal.

Filip et al.[30] observed that chromium exposure at all levels tested (1-40 mg/l) resulted in a nearly unialgal culture of Oscillatoria sp. In addition they showed that the chromium absorption was 20 per cent, but the mass of chromium removed was much greater as high levels of chromium were added.

Bringmann and Kühn[21] found the toxicity threshold for Scenedesmus quadricauda as 1.2 mg/l $Cr_2O_7^=$

In a study of metal mixture, chromium was used in combination of copper, zinc, nickel, lead, cadmium, mercury, arsenic, tin and selenium.Growth of phytoplankton was not inhibited at metal concentrations expected to occur in a moderately polluted estuary. However,at 5- or 10-fold higher metal concentrations, phytoplankton growth was inhibited both in natural populations and in laboratory cultures[45].

Again, Wong et al.[23] studied the combination of 10 metals in which chromium takes place as discussed in the mercury section.

1.2.6 Arsenic

In general for the effects of arsenic to freshwater algae, little information is available.

Conway[46] reported no detrimental effects to Asterionell formosa when this freshwater diatom was exposed to 0.16 mg/l As(V).

Vocke et al.[22] found the mediam effective concentration (EC 50) values for the arsenic ranged as 0.48-30.761 mg/l As(V). They also concluded that blue green algae Microcoleus vaginatus was more tolerant of As(V) among the Ankistrodesmus falcatus, Scenedesmus obliquus and Selenastrum capricornutum. They also concluded that this could be due in part to differences in the assay media to the fact that the Microcoleus was not axenic or possibly because Microcoleus was prokaryotic organism while the other organisms tested were eukaryotes.

In one of the metal mixture study arsenic is used in combination with copper, zinc, nickel,lead, cadmium, mercury, tin and selenium and a reduction in growth is observed in phytoplankton in high concentrations[45].

In another study it is used at 50 g/l as sodium arsenate on Scenedesmus, chlorella Anabaena and Navicula which have been discussed in the mercury section.

II. STATEMENT OF THE RESEARCH PROBLEM

Insidious build-up of low level concentrations of metals in freshwaters receiving mine waters, industrial effluents, sewage and waste water from power generating stationscan have a harmful effect upon the growth and development of the aquatic organisms, leading to a decrease in productivity of these areas.

When algae were found to absorb heavy metal pollutants readily, grazing predation and finally human consumption were found to be ways for accumulation of heavy metals. Furthermore, algae can detect very low levels of heavy metals in the environment.

These and being the first in aquatic food chain, form the basis for the increasing use and acceptance of algal assays in toxicity studies.

The critical concentrations for toxicity in question of a particular metal may vary at different times during the growth of an algal culture being as well dependent upon other chemical and physical conditions, such as temperature, pH, inoculum concentrations, aeration rates and lightening periods.

In addition, it is already known that, algal species vary endogenically in their responses to heavy metals. For each species harmless, algistatic and algicidal doses change.

These factors mainly necessitate tedicus work in the determination of influence of contamination by each heavy metal on almost each algal species.

Furthermore, the lack of such a research an Anabaena flos-aquae in literature and experimental medium used in this research being different from those cited in literature have quided the author in realization of this research.

The purpose of this study was to investigate the toxicity of mercury, cadmium, selenium, nickel, chromium, and arsenic individually towards the growth of Anabaena flos-aquae in long-term, batch culture experiments where metals were introduced as inorganic salts at three different concentrations.

III. MATERIALS AND METHODS

3.1. ALGAL SPECIES

Anabaena flos-aquae culture has been used throughout the experiment.

3.2 CULTURE MEDIUM

The culture medium that has been used for stock algal cultures and for metal tests is a modification of the original Provisional Algal Assay Procedure medium, being first described by Tözüm and Gallon (Table 3.2.1)

After preparation of the hutrient solution the pH of the medium was adjusted to a value between 7.5-7.6 by adding sodiumbicarbonate ($NaHCO_3$) and allowing sufficient time for a stable pH reading.

In pH measurements Fisher Accumet pH meter Model 620 was used.

3.3. GLASSWARE

All glasware was of Pyrex glass. To ensure the cleanliness of glassware it was washed in detergent solution, rinsed with tap water and soaked in 10 per cent hydrochloric acid solution.Then rinsed throughly

Table 3.2.1. Final concentration of nutrients in culture medium and origin of chemical substance used

Type	Compound	Amount (mg/l)	Origin
Macronutrients	NaCl	877.5	Merck
	$MgSO_4.7H_2O$	36.945	Merck
	$MgCl_2.6H_2O$	29.479	Merck
	$CaCl_2.2H_2O$	27.945	Merck
	K_2HPO_4	17.416	Merck
	$Na_2HPO_4.12H_2O$	35.795	Merck
Micronutrients	$FeCl_3.6H_2O$	0.91	Merck
	H_3BO_3	0.2387	BDH
	$MnCl_2.4H_2O$	1.1999	Fisher
	$ZnCl_2$	0.44	Merck
	$CoCl_2.6H_2O$	1.36×10^{-4}	Merck
	MoO_3	0.0144	BDH
	Na_2EDTA	7.38	Merck

with tap water. Distilled water was used for the final rinse and clean glassware was prerinsed with the type solution before use.

3.4. STERILITY

All flasks and media were sterilized by autoclaving at 121°C and 15 psi (1.05 kg/cm^2) for ten minutes per litre of medium.

3.5. TEST METALS

Growt studies were conducted with mercury (supplied as $HgCl_2$ from Merck), cadmium (supplied as $CdCl_2$ from Mallinckredt), selenium (supplied as Na_2SeO_3 from Fluka), nickel (supplied as $NiCl_2.6H_2O$ from Merck), chromium (supplied as K_2CrO_4 from Merck), and arsenic (supplied as $Na_2HAsO_4.7H_2O$ from Merck) at three different concentrations (Table 3.5.1). The stock solutions of these elements were prepared with distilled water.

Table 3.5.1 Concentrations of heavy metals tested on algae

Concentration of metal and form, ppm					
Mercury $HgCl_2$	Cadmium $CdCl_2$	Selenium Na_2SeO_3	Nickel $NiCl_2.6H_2O$	Chromium K_2CrO_4	Arsenic Na_2HAsO_4
0.001	0.02	0.1	0.02	0.5	0.1
0.004	0.05	10	0.05	10	5
0.008	0.1	20	0.2	20	10

3.6. BIOASSAY PROCEDURE

3.6.1. Inoculation

Inoculations were made from a three week old culture which was in logarithmic growth phase, according to the absorbance readings at 600 nm.

3.6.2. Test System

For long term toxicity testing 500 ml flasks with 375 ml medium were used. Flasks were fitted with 3-hole rubber stoppers. Through the holes three glass tubes were inserted two of which provided the necessary aeration and the other, the sampling. The flasks were bubbled continously with air at a flow rate of 0.3 l/min/l of cells.

3.6.3. Incubation

Cultures were incubated at 24 ± 1 ^{o}C and under continous illumination of 210 ± 5 ft-c for three weeks.

3.6.4. Measurement

Three different concentrations of each metal (Table 3.5.1.) were added to culture flasks on the inoculation date prior to inoculation as aquaeous solutions. Keeping one flask as control respon-

se of algae was measured through growth, taking the inoculation date as the zeroth day and sampling from the fifth day on up to the 16^{th} day.

Optical density was used as an estimate for algal cell concentration and measured at 500 nm by a Varian Super Scan 3 Ultra-Violet-Visible Spectrophotometer.

A common and necessary check upon instrumentation which is to measure optical density on various dilutions of the algal suspension is shown in APPENDIX I.

IV. RESULTS AND DISCUSSION

4.1. GENERAL EXPRESSION OF RESULTS

In growth determination, optical density has been used for estimating algal cell concentrations and response of the algae has been shown on a graph with optical density versus time.

Daily specific growth rates have also been used in the expression of the results. For this purpose the following formula is used:

$$\text{Specific growth rate } (\mu) = \frac{\ln A_1 - \ln A_0}{t_1 - t_0}$$

where, A_1 the absorbance value at the end of the selected time interval, A_0 is the absorbance value at the beginning of selected time interval and $t_1 - t_0$ is the number of elapsed days between selected determination of absorbances[47].

In the present case $t_1 - t_0$ is chosen to be unity and starting from the fifth day, tables showing daily specific growth rates have been tabulated.

4.2. EXPERIMENTAL RESULTS AND DISCUSSION

4.2.1. Experiments With Mercury

Keeping in mind that in several studies, growth of the alga was severaly restricted or totally inhibitedat relatively low concentrations of mercury, 1,4, and 8 ppb mercuric ion concentrations have been chosen during the experiments.

Growth response of the algae with time is shown in Fig. 4.2.1.1.

Up to the seventh day there was not much difference between control, 1 ppb and 4 ppb mercury added sets. However, from this day on differences in growth started and although cultures in 1 ppb mercury polluted sample followed control, beginning on the eighth day those in 4 ppb mercury medium remained behind them.

In 8 ppb mercury contaminated medium a lag was observed up to the fifth day, from this day on growth was observed which is much lower than that of the control.

It is not apparent whether the cells growing after five days were a few resistant ones or whether the entire inoculum was initially inhibited.

On the 14^{th} day growth was very close in 4 and 8 ppb mercury polluted media and for the next two days they continued in slightly different values.

Therefore, it is concluded that in short term 8 ppb mercury concontration may have a significant inhibitory effect on growth. However, in longterm although below narmol, growth commences again.

These are the rough observations that are made from the graph. However, when the growth rates have been examined more details can be explaiened.

Daily specific growth rates for mercury toxicity are tabulated in Table 4.2.1.1.

Considering the cell concentrations, it is observed that, the most inhibited culture which is with 8 ppb mercury, reached 5-6 th day concentration of the control culture on the $8\text{-}9^{th}$ days. When it is returned to growth rates, it is seen that the growth rate of the 8 ppb mercury polluted culture is 0.3345 $days^{-1}$ which is slightly a higher value than the growth rate of the control between $5\text{-}6^{th}$ days, being 0.3191 $days^{-1}$.

The are much smaller time intervals before 1 and 4 ppb mercury polluted culture populations reach that of control. For example 4 ppb mercury contaminated culture reaches the $6\text{-}7^{th}$ day population of control on $7\text{-}8^{th}$ days, and 1 ppb reaches even in a shorter period.

Maximum specific growth rates have been observed between $8\text{-}9^{th}$ days for control and medium with 1 ppb mercury as 0.3677 day^{-1} and 0.3400 $days^{-1}$ values respectively. For 4 and 8 ppb mercury contaminated medium it was between $5\text{-}6^{th}$ days being 0.3111 day^{-1} and 0.4243 day^{-1}.

Having smaller values for polluted medium than unpolluted ones with the order of pollution quantity is as expected, except in the 8 ppb mercury medium.

In general, 8 ppb mercury medium showed higher growth rates than control after the fifth day. This seems that mercury initially inhibits

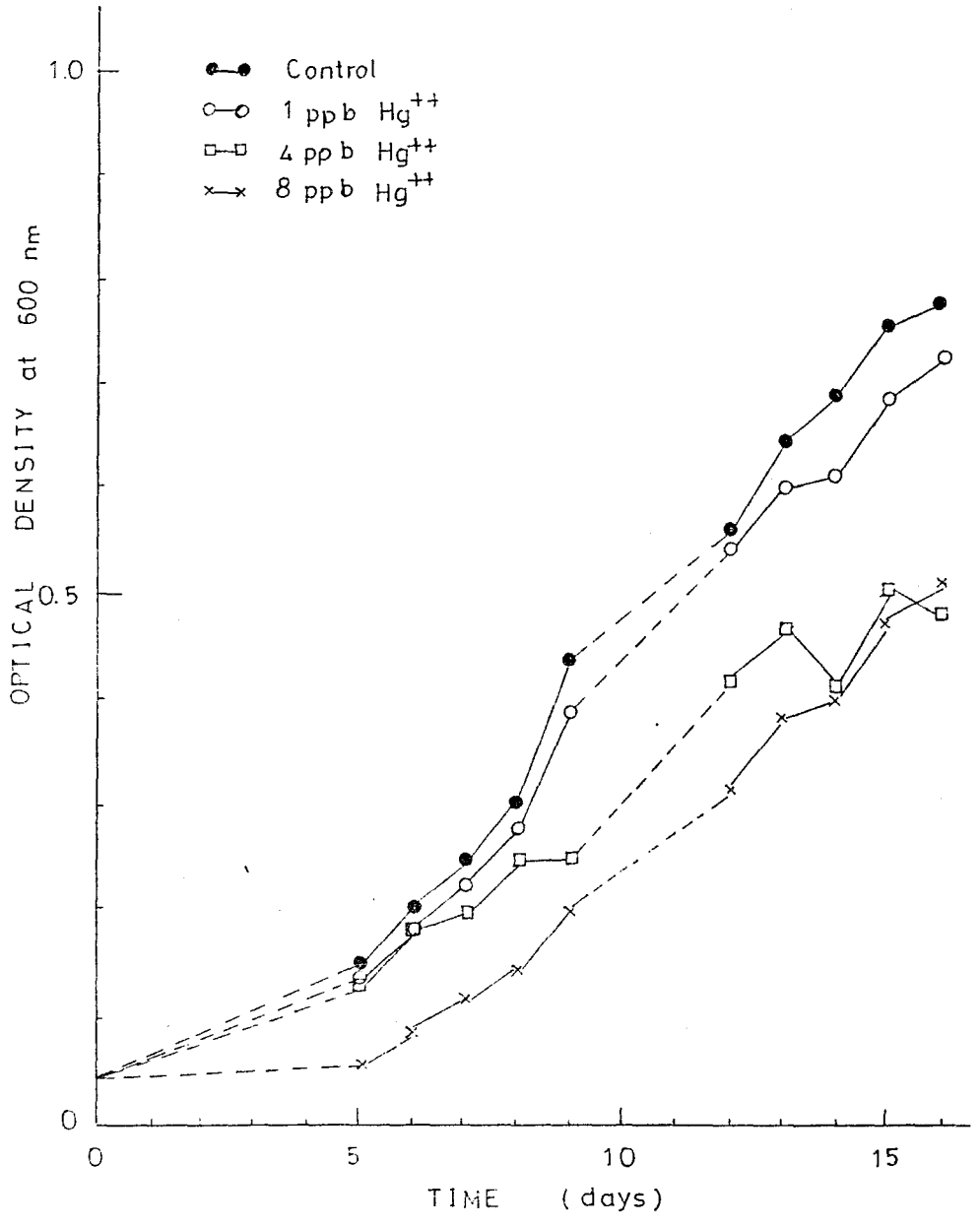


FIGURE 4.2.1.1. Effect of mercury on the growth of Anabaena flos-aquae

Table 4.2.1.1. Growth rates for Anabaena flos-aquae culture at the tested mercury concentrations

Time (Days)	SPECIFIC GROWTH RATES (μ) - Day^{-1}			
	CONTROL	1 ppb Hg^{++}	4 ppb Hg^{++}	8 pp Hg^{++}
5 6	0.3191	0.2823	$0.3111^{(*)}$	$0.4343^{(*)}$
6 7	0.2024	0.1938	0.0722	0.2933
7 8	0.1981	0.2063	0.2261	0.2129
8 9	$0.3677^{(*)}$	$0.3400^{(*)}$	0.0040	0.3345
9 12	0.0824	0.1123	0.1682	0.1552
12 13	0.1353	0.0971	0.1212	0.1746
13 14	0.0686	0.0196	-0.1260	0.0650
14 15	0.0882	0.1104	0.1994	0.1399
15 16	0.0260	0.0605	-0.0462	0.0828

(*) denotes maximum values

the growth of the culture up to the fifth day and from then on somehow growth again starts perhaps with some resistant cells and because the effect of the mercuric ion disappearedafter a time.

For the reason that, in general, mercury toxicity experiments are complicated by thefast reduction and evaporation of mercury. These losses from stored aqueous solutions have been reported by a number of investigators. Early explanations for losses followed along the lines of adsorptive processes that have been observed for many metals with respect to glass and and although the Pyrex glass which is used in the experimentation of this research, is one of the best suited material for minimizing adsorption, in the small concentrations used, it is inevitable to have a loss from the media in this way.

Today it is evident that chemical reduction of mercuric to mercurous ion followed by disproportioning of the mercurous ion to mercuric ion and elemental mercury which evaporates radiply, leakage of mercury through the wall of the solution container and the adsorption of mercury on algal cells, are the other factors that can effect mercury concentrations in a toxicity experiment.(17)(48)

Therefore, although mercury loss from a solution with sodium chloride as in this study, has been found very low(48), one must bear in mind that the afore mentioned concentrations are the beginning concentrations and taking mercury measurements in the solution while continuing the experiments may be useful.

4.2.2. Experiments with Cadmium

Regarding the used concentrations in literature, and trying to find values that are naturally observable, 20,50, and 100 ppb cadmium concentrations have been chosen.

The response with time of Anabaena flos-aquae to this range of cadmium is shown in Fig. 4.2.2.1.

Growth of the algae exposed to various levels of cadmium was not effected significantly up to the eighth day, but from then on differences among them appearel.

Growth continued at 20 and 50 ppb cadmium polluted media, but those grown at 100 ppb cadmium added medium appeared to be inhibited for four days and then on 12^{th} day starting a slower growth.

Thus starting with a 0.041 absorbance value at the inoculation, at the end of the 16^{th} day, control, 20,50, and 100 ppb cadmium contaminated samplesreached to 0.897, 0.788, 0.612 and 0.374 absorbance values respectively and it is apparent that greater concentrations than 100 ppb will have an inhibitory effect on the culture.

In addition to these, during the experiment a color change to yellowish green is observed in 100 ppb cadmium contaminated culture.

In spite of the growth rates, (Table 4.2.2.1.), maximum specific growth rates were observed between 5-6^{th} days for all of the cultures. The order of the magnitude of the rates were 50, 100, 20 ppb cadmium polluted and in control medium.

Having greater growth rates in polluted cultures than control up to the 8 and 9^{th} days indicate that, although cadmium ion inhibits

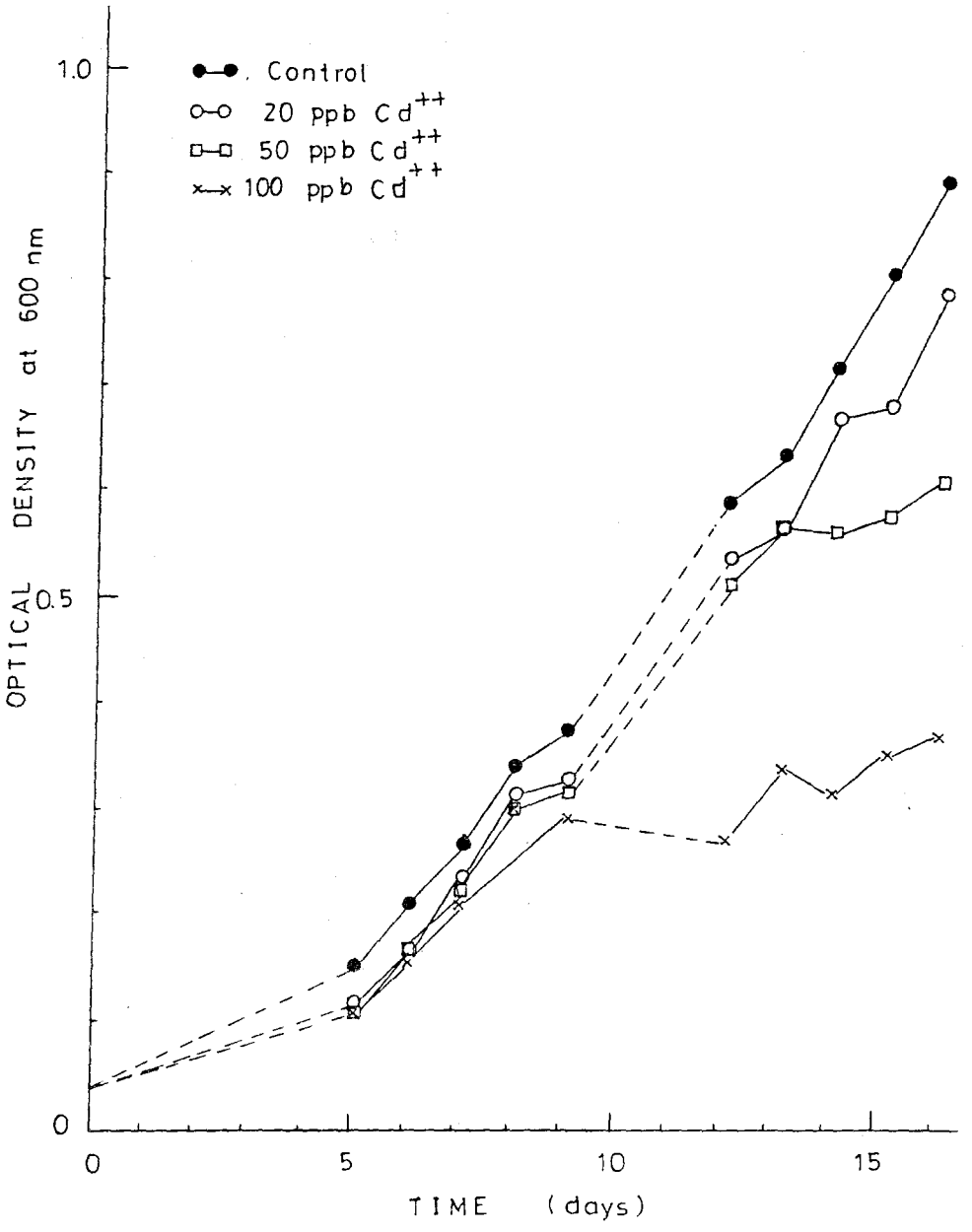


FIGURE 4.2.2.1. Effect of cadmium on the growth of Anabaena flos-aquae

Table 4.2.2.1. Growth rates for Anabaena flos-aquae culture at the tested cadmium concentration

Time (Days)	SPECIFIC GROWTH RATES (μ) - Day^{-1}			
	CONTROL	20 ppb Cd^{++}	50 ppb Cd^{++}	100 ppb Cd^{++}
5 6	0.3034(*)	0.3625(*)	0.3881(*)	0.3704(*)
6 7	0.2361	0.3431	0.2744	0.2651
7 8	0.2501	0.2897	0.3107	0.3280
8 9	0.0827	0.0185	0.0352	-0.0267
9 12	0.1518	0.1637	0.1620	-0.2157
12 13	0.0779	0.0580	0.0941	0.2158
13 14	0.1129	0.1726	0.0035	-0.0606
14 15	0.1201	0.0147	0.0191	0.1040
15 16	0.0983	0.1401	0.0520	0.0521

(*) denotes maximum values

the growth after a time it starts to stimulate the growth rate for a short time and the stagnation in the first five days causes other cultures to be behind the control.

In 50 ppb cadmium polluted medium it is observed that, growth rate started to decrease beginning from the 12^{th} day, this obviously effects the population as well and it can be also seen from the graph. Same phenomenon also appeared in 100 ppb cadmium polluted sample.

Thus, it is concluded that, cadmium ion cause the retardation phase to occur soorer, indicating a delayed toxicity. These findings are also in accordance with Stratton[24].

4.2.3. Experiments With Selenium

The growth response of Anabaena flos-aquae culture to selected selenium range is shown in Fig. 4.2.3.1.

During the experiments selenium was supplied as sodium selenite at 0.1,10 and 20 ppm concentrations. Experiment was started with nearly 0.086 absorbance readings and it took approximately 15 days for the algal growth in control to reach stationary phase.

Cells continued to grow at 0.1 ppm Se added solution, but a slower growth than control is observed. This difference started to increase on the 14^{th} day and on the last day of the experiment there was an important difference among them.On the other hand cells grown at 10 and 20 ppm selenium contaminated medium failed completely within a week and during the experiment no growth was observed. Also in 10 and 20 ppm selenium polluted cultures a white color is observed on

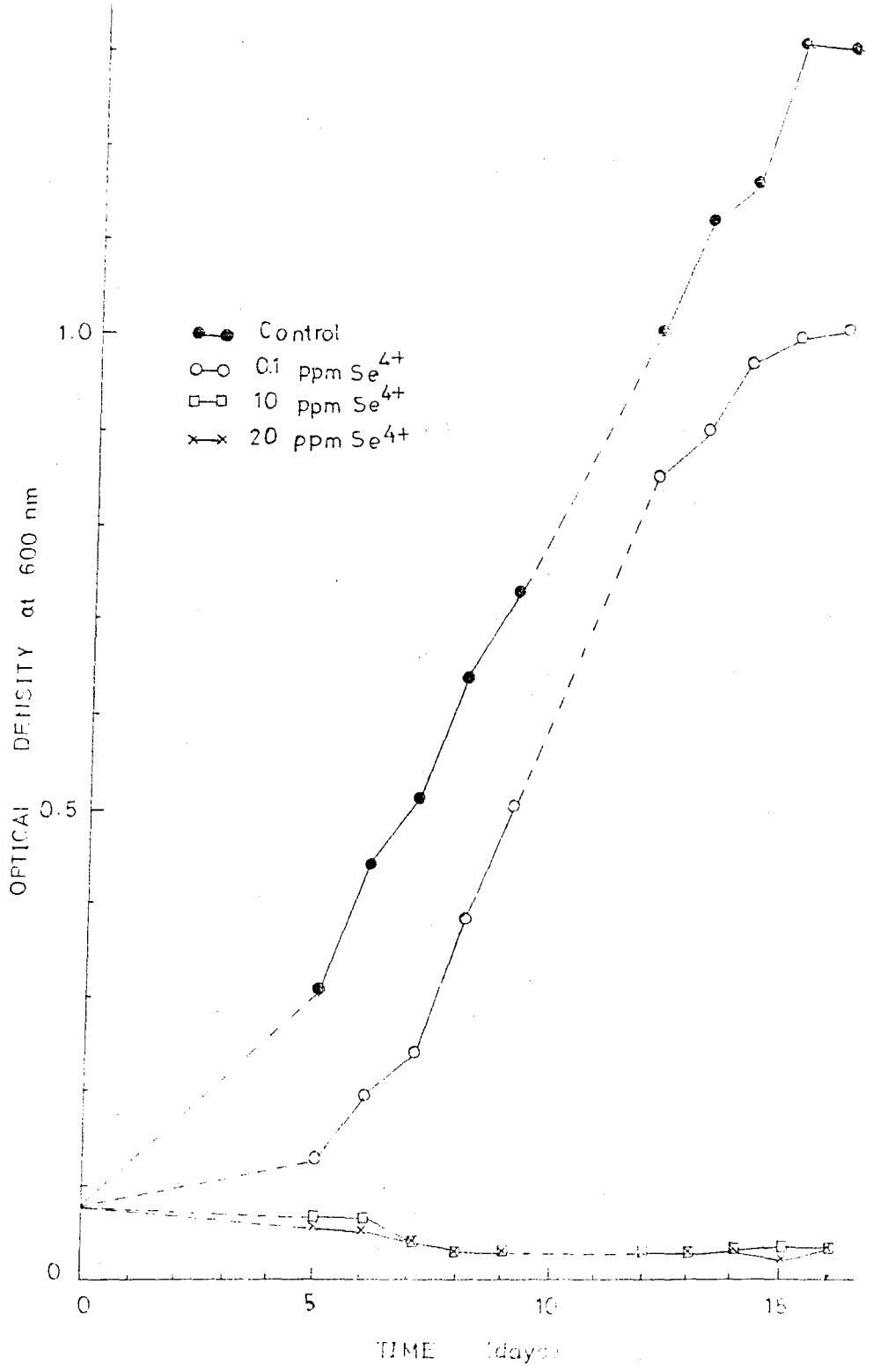


FIGURE 4.2.3.1. Effect of selenium on the growth of Anabaena flos-aquae

Table 4.2.3.1. Growth rates for Anabaena flos-aquae culture at the tested selenium concentrations

Time (Days)	SPECIFIC GROWTH RATES (μ) - Day^{-1}			
	CONTROL	0.1 ppm Se^{4+}	10 ppm Se^{4+}	20 ppm Se^{4+}
5 6	0.3511(*)	0.3927	-0.0155	-0.0678
6 7	0.1538	0.2252	-0.5213	-0.4055
7 8	0.2247	0.4576(*)	-0.1411	-0.1718
8 9	0.1327	0.2719	0.0298	0.0308
9 12	0.1281	0.1754	-0.0099	0.0099
12 13	0.0455	0.0547	0.0000	-0.0923
13 14	0.0349	0.0779	0.0870(*)	0.1214(*)
14 15	0.1444	0.0471	-0.0282	-0.1880
15 16	-0.0068	0.0736	0.0556	0.2162

(*) denotes maximum values

the first days.

Maximum specific growth rates were observed between 7 and 8^{th} days for 0.1 ppm selenium contaminated culture whereas it was between 5 and 6^{th} days for control.

During the experiment the growth rates of 0.1 ppm selenium contaminated culture appeared to be higher than the control. This again made the auther to think that having growth curves below the control's is the result of the decrease in populations in the first five days.

Growth rates showed alsot that cultures in 0.1 ppm selenium polluted medium reached a stationary condition on 14^{th} day, which is before the control, which starts it on 15^{th} day. This again showed a delayed toxicity as in the case of cadmium.

4.2.4. Experiments With Nickel

Culture is grown in 0.02, 0.05, and 0.2 ppm nickel solutions, This range of nickel is chosen because it is more representative nickel concentrations in freshwater.

Fig.4.2.4.1. shows growth during nickel experiment.

Up to the eighth day 0.02 and 0.05 ppm nickel polluted cultures could follow the growth of the control cultures with a smaller growth. After the nineth day while the differences between them start to increase, 0.05 ppm nickel contaminated culture showed a higher growth than 0.02 ppm nickel concentration.

Growth is stopped at 0.2 ppm nickel added medium till to the eight day. On nineth day a slight increases which was followed with a strong inhibition is observed. At this concentration a color change to yellowish green in the culture is also seen.

When the growth rates were observed (Table 4.2.4.1.) it is seen that maximum growth rates occur between 5 and 6^{th} days for control, 0.02 and 0.05 ppm nickel polluted culturesand on 8-9^{th} days for 0.2 ppm nickel polluted media.

As it is mentioned earlier, at 0.2 ppm nickel starting from the eighth day, culture population increased and reached the maximum quantity on nineth day and since readings on 10 and 11^{th} days could not be taken, it is not known whether this increase is continued or not. However, this growth rate increase is again appeared on 14-15 and 16^{th} days and although at this time growth rates are not as great as the maximum rate, these meant that an increase in growth may start by

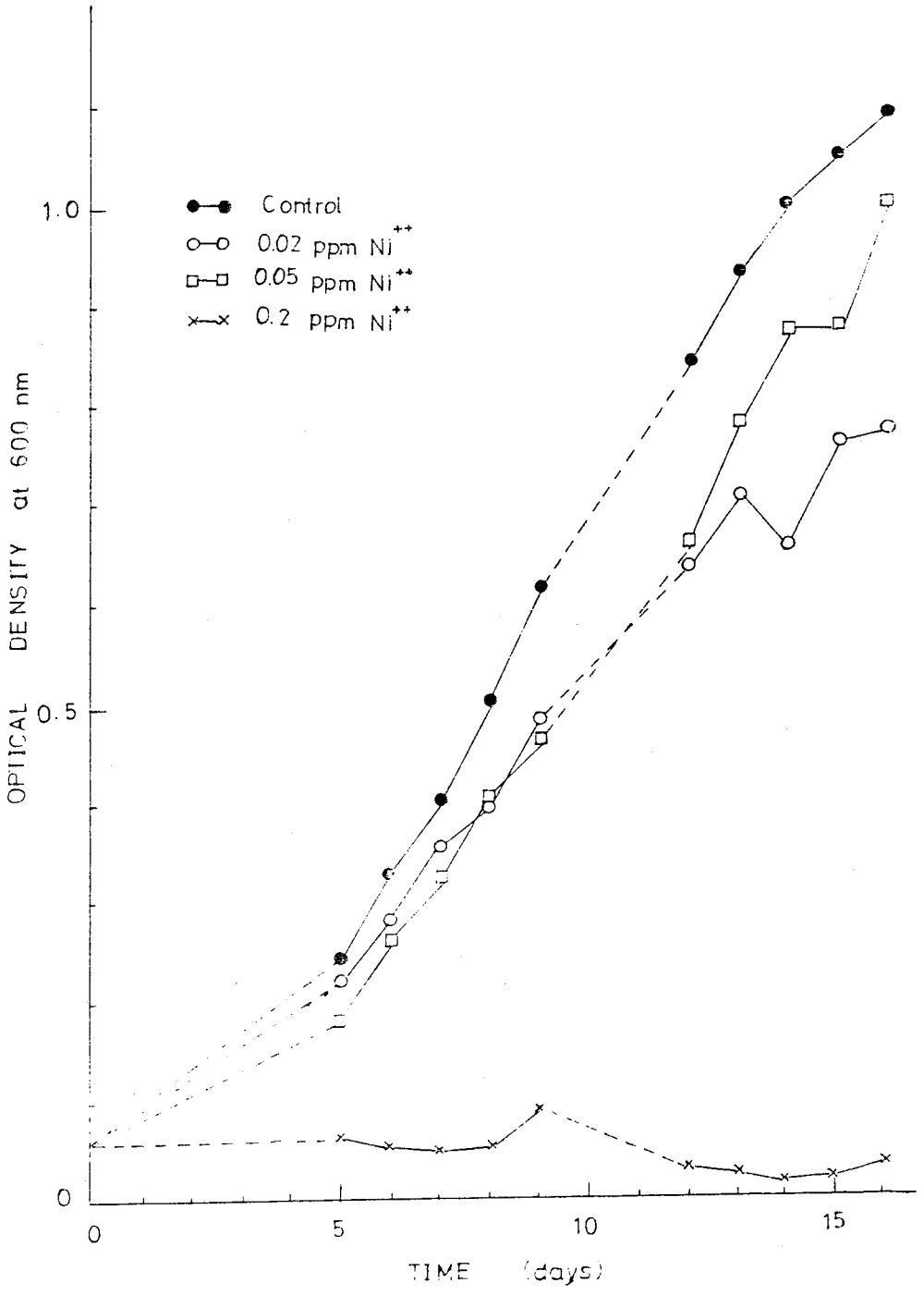


FIGURE 4.2.4.1. Effect of nickel on the growth of Anabaena flos-aquae

Table 4.2.4.1. Growth rates for Anabaenas flos-aquae culture at the tested nickel concentrations

Time (Days)	SPECIFIC GROWTH RATES (μ) -Day^{-1}			
	CONTROL	0.02 ppm Ni^{++}	0.05 ppm Ni^{++}	0.2 ppm Ni^{++}
5 6	0.3052(*)	0.2539(*)	0.3750(*)	-0.1686
6 7	0.1990	0.2196	0.2115	-0.0417
7 8	0.2296	0.1235	0.2306	0.0817
8 9	0.2014	0.1944	0.1388	0.5341(*)
9 12	0.1053	0.0926	0.1177	-0.3900
12 13	0.1026	0.0675	0.1673	0.0364
13 14	0.0713	-0.0720	0.1151	-0.2441
14 15	0.0496	0.1485	0.0023	0.2048
15 16	0.0409	0.0156	0.1522	0.2007

(*) denotes maximum values

perhaps some resistant cells or because of the disappearance of the effect of nickel ion after a time. For this concentration therefore, a longer period is needed for the experiment.

In addition to these, a smaller maximum specific growth rate as well as other rates are observed at 0.02 ppm nickel medium than the control as expected, but the higher value in 0.05 ppm shows again a stimulation on daily growth rate. As well as this maximum specific growth rate other rates are also higher in this culture than control. Therefore it is again concluded thet having a growth curve below the control with 0.05 ppm nickel is resulted because of the duration in the first days.

4.2.5. Experiments With Chromium

In these experiments, chromium was supplied as potassium chromate. Concentrations are chosen as 0.5,10 and 20 ppm which are again naturally observed figures.

The growth responses of the algae versus time are shown in Fig. 4.2.5.1.

It seems that at three concentrations growth was progressively reduced. Again growth was slowed down in first five days in 0.5 and 20 ppm chromium contaminated media. In fact although 10 ppm is the 20 times of the 0.5 ppm chromium concentration, somehow a great difference in growth could not be observed.

Following the graph it is observed that the difference between control and the polluted media increases with time due to the degree

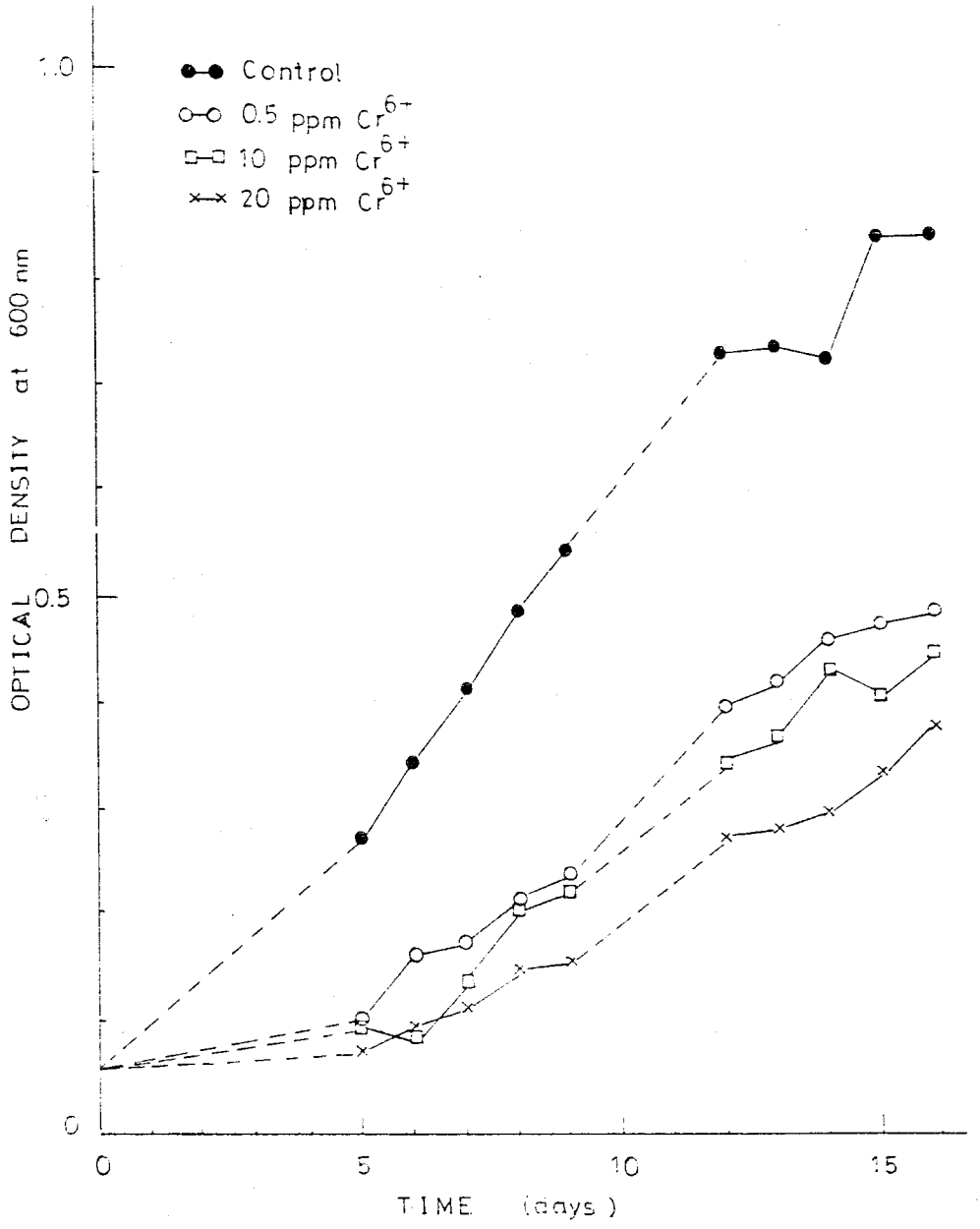


FIGURE 4.2.5.1. Effect of chromium on the growth of Anabaena flos-aquae

Table 4.2.5.1. Growth rates for Anabaena flos-aquae culture at the tested chromium concentrations

Time (Days)	SPECIFIC GROWTH RATES (μ) - Day^{-1}			
	CONTROL	0.5 ppm Cr^{6+}	10 ppm Cr^{6+}	20 ppm Cr^{6+}
5 6	0.2360(*)	0.4545(*)	-0.0914	0.2542(*)
6 7	0.1837	0.0694	0.4195(*)	0.2108
7 8	0.1552	0.2153	0.3890	0.2541
8 9	0.1110	0.0986	0.0906	0.0439
9 12	0.0968	0.1692	0.1385	0.1803
12 13	0.0108	0.0503	0.0663	0.0351
13 14	0.0148	0.0851	0.1625	0.0537
14 15	0.1442	0.0358	-0.0609	0.1170
15 16	0.0082	0.0286	0.0988	0.1204

(*) denotes maximum values

of pollution.

At 15^{th} day control curve comes to the stationary position and it seems that as in the selenium and cadmium cases, here also 0.5 ppm chromium contaminated culture stabilizes before the control nearly on the 14^{th} day. However, 10 and 20 ppm chromium added cultures continued to grow even at 16^{th} day although with a smaller growth rate.

Maximum specific growth rates are again observed between the $5\text{-}6^{th}$ days for control, 0.5 ppm and 20 ppm chromium solutions, and on $6\text{-}7^{th}$ days for the medium with 10 ppm chromium.

In general all of the polluted samples gave higher growth rates than control. Thus, it is again concluded that the population obtained in the first five days are very important for chromium toxicity, because once population starts to increase, polluted cultures reach higher growth rate than the control.

4.2.6 Experiments With Arsenic

In this research sodium arsenate is used as the arsenic supplier for 0.1,5, and 10 ppm arsenic concentrations.

Throughout the experiment a significant inhibition can not be observed (Fig. 4.2.6.1.). 0.1 and 5 ppm arsenic contaminated cultures followed each other very closely during the tests, and 10 ppm arsenic polluted culture continued to grow.

5 ppm arsenic added medium follows 0.1 ppm arsenic polluted medium very closely although it is 50 fold of the other. Therefore, this is an unexpected situation. Especially when the population of

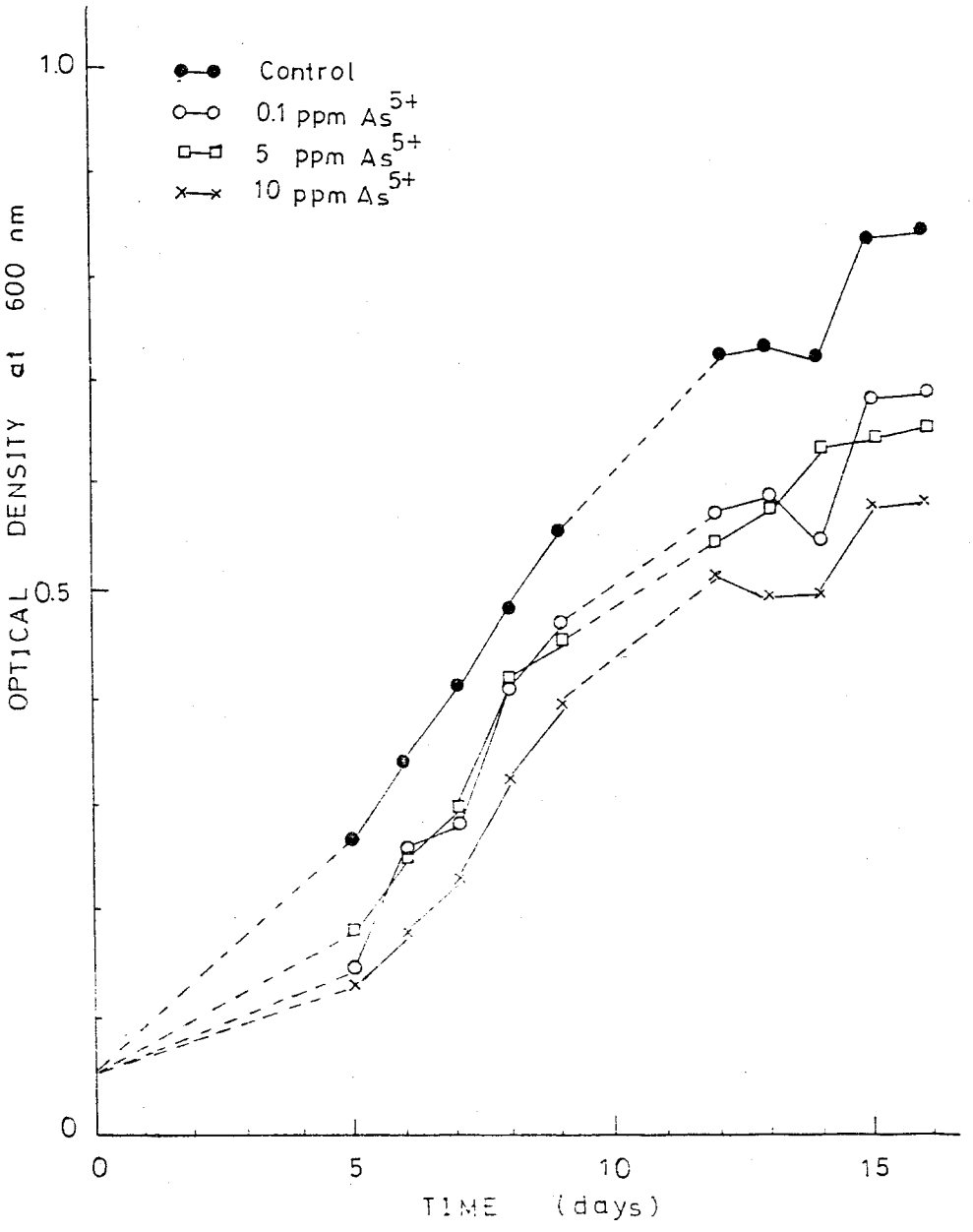


FIGURE 4.2.6.1. Effect of arsenic on the growth of Anabaena flos-aquae

Table 4.2.6.1. Growth rates for Anabaena flos-aquae culture at the tested arsenic concentrations

Time (Days)	SPECIFIC GROWTH RATES (μ) - Day^{-1}			
	CONTROL	0.1 ppm As^{5+}	5 ppm As^{5+}	10 ppm As^{5+}
5 6	0.2360(*)	0.5513(*)	0.3032	0.2755
6 7	0.1837	0.0855	0.1727	0.2242
7 8	0.1552	0.3577	0.3140(*)	0.3436(*)
8 9	0.1110	0.1421	0.0989	0.1838
9 12	0.0968	0.0639	0.0605	0.0871
12 13	0.0108	0.0253	0.0555	-0.0407
13 14	-0.0148	-0.0699	0.0919	0.0098
14 15	0.1442	0.2160	0.0153	0.1525
15 16	0.0082	0.0100	0.0165	0.0117

(*) denotes maximum values

the two cultures are nearly same on 13th day. 5 ppm arsenic culture showed a greater growth rate (Table 4.2.6.1. and came above the 0.1 ppm arsenic polluted growth curve on 14th day. However, between the 14 and 15th days 0.1 ppm arsenic contaminated medium showed a greater growth rate and again came above the other.

In addition to these, it seems all the cultures with and without arsenic came to the stationary conditions on the same days, nearly on 15-16th days, which can be understood both from the graph and the growth rates.

4.2.7. Comparison

During the experiments different concentration for each metal ion is used since the aim was to study the naturally observable and inhibition starter concentrations, and these vary from one metal to another widely. Nevertheless, similar concentrations were used in some cases permitting comparative examinations.

In cadmium and nickel experiments, it has been seen that 0.02 and 0.05 ppm cadmium was more inhibitory than the same concentrations of nickel.

0.1 ppm concentration is found to reduce growth more in selenium than arsenic and cadmium at the beginning. However, although the cultures with selenium and arsenic could continue to grow following the control, algae in cadmium polluted medium couldn't show this increase and growth stopped and continued after a temporary reduction with

a slower growth rate. Therefore, it can be said that in longterm 0.1 ppm cadmium concentration is more inhibitory than the same concentration of selenium.

When 10 and 20 ppm concentrations of selenium and chromium are compared with eachother, it is concluded that selenium is markedly more inhibitory than chromium, at this concentration of selenium algae can not survive, whereas in chromium contaminated medium, they show a growth although it is very below of the control.

It is also found that 10 ppm chromium was more toxic than 10 ppm arsenic.

Finally bearing these in mind and considering that smallest growth reducing concentrations in the tests were found in mercury, a decreasing order of toxicity for Anabaena flos-aquae among these metals can be made as:

Greater inhibition					Least inhibition
Mercury	> Cadmium >	Nickel	> Selenium >	Chromium	> Arsenic

V. CONCLUSIONS

In general results are in accordance with those cited in the literature.

Any difference that may be observed between the sets of results may be due to the usage of different culture media and, mostly due to different types of algae.

The conclusion of this study can be summarized as follows.

(a) All the six metals were toxic to the algae under study,

(b) Mercury inhibited the growth at relatively low concentrations causing a lag in the first days,

(c) Cultures in 50 and 100 pbb cadmium, 0.1 ppm selenium and 0.5 ppm chromium concentrations showed a delayed toxicity

(d) Cadmium concentrations greater than 100 ppb would have had a significant inhibition on the growth.

(e) Cultures growtn at 10-and-20-ppm-selenium-polluted-media completely failed within a week,

(f) Cells grown at 0.2 ppm nickel showed a mostly inhibited inconsistent growth,

(g) Growth was progressively reduced, due to the degree of pollution, in chromium added medium,

(h) A significant difference could not be observed between 0.5 and 10 ppm chromium and 0.1 and 5 ppm arsenic added cultures although they were 20 and 50 fold of eachother respectively.

VI. RECOMMENDATION FOR FUTURE WORK

After presentation and evaluation of the data, a few recommendations for further work in the field are appropriate.

In a future research, measurement of metal concentrations in the media and in the algae along with that of growth of algae during the experiment is needed,to eliminate (or at least to detect) any loss of the metal from the system.

Together with this, a continous supply of the metal at the required concentration or a buffer system keeping amount of the metal at the desired level throughout the experiment may be of further help.

Finally, examination of the data presented reveals that, for a more sound evaluation measurements should be carried out, during all phases of experimentation, especially in the first five days, which have been neglected in the research for the algae to reach a certain concentration that enables convenient observation.

APPENDIX I

OPTICAL DENSITY AT VARIOUS DILUTIONS OF ALGAL SUSPENSION

The linearity between the optical density and the concentration has been verified at 600 nm on the spectrophotometer and it has been found that the relationship between the optical density and the dilutions of the algal suspension can be expressed as:

$$D = 3.8 \gamma$$

where D is optical density and γ is dilution.

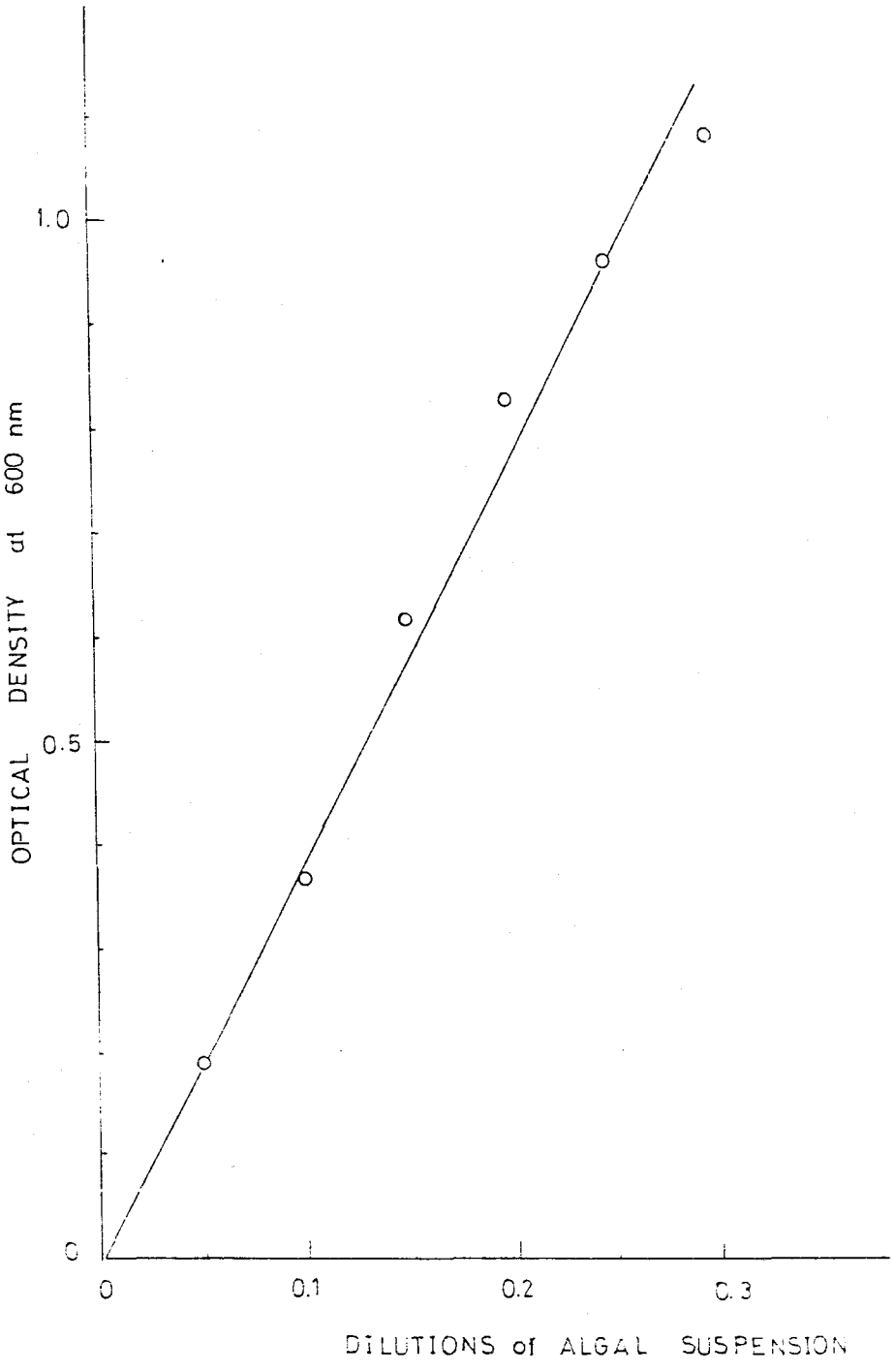
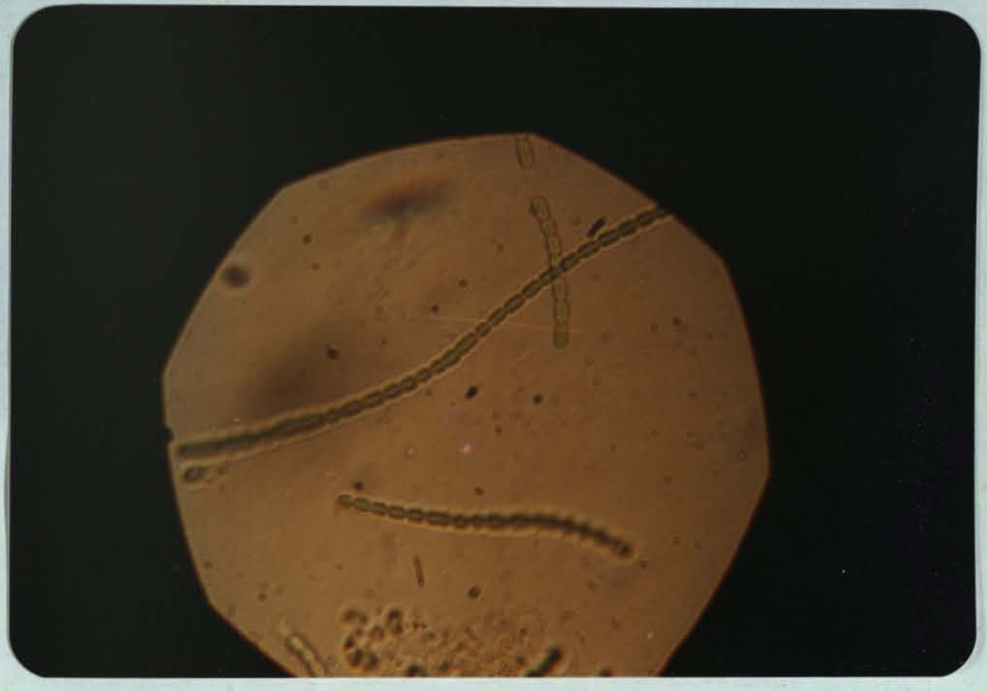


FIGURE A.I.1 The relationship between the optical density and the dilutions of algal suspension

APPENDIX II

PHOTOGRAPHS OF ANABAENA FLOS-AQUAE CULTURE

CONTROL



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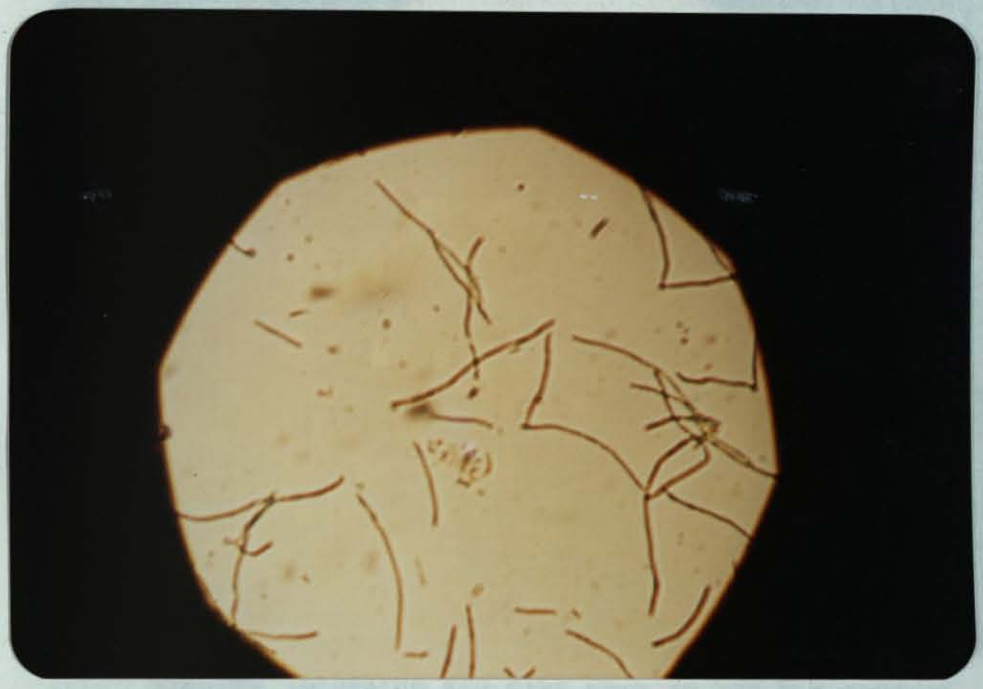

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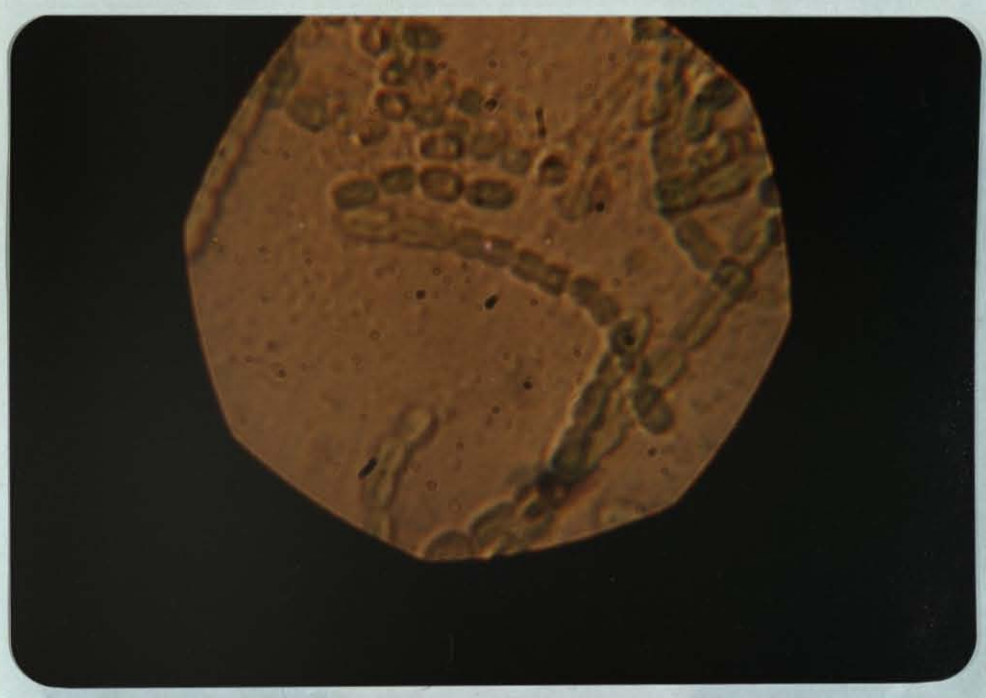
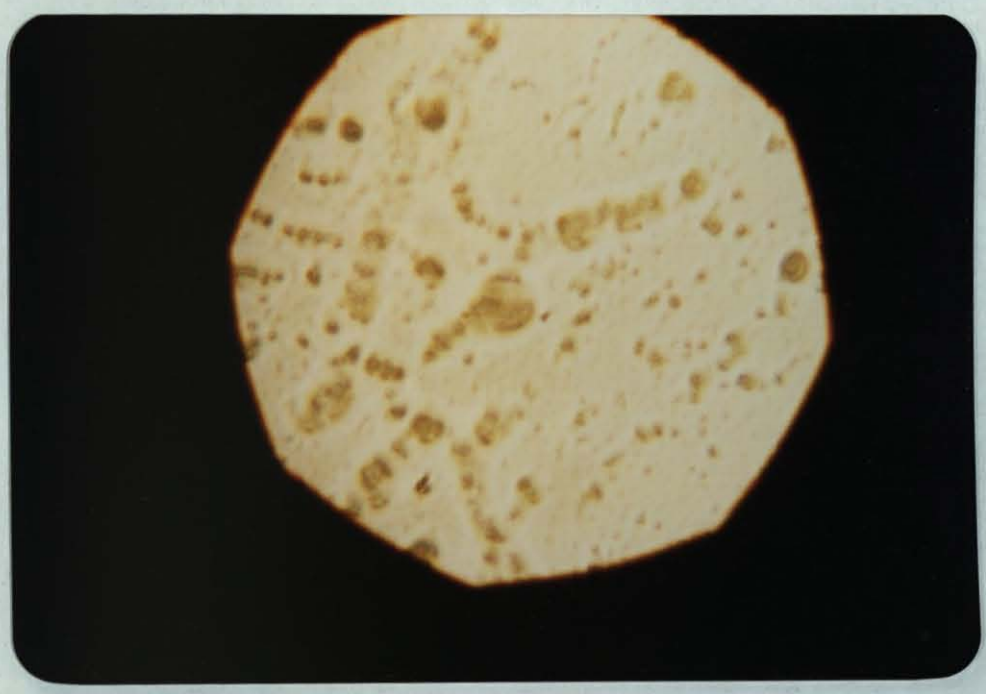
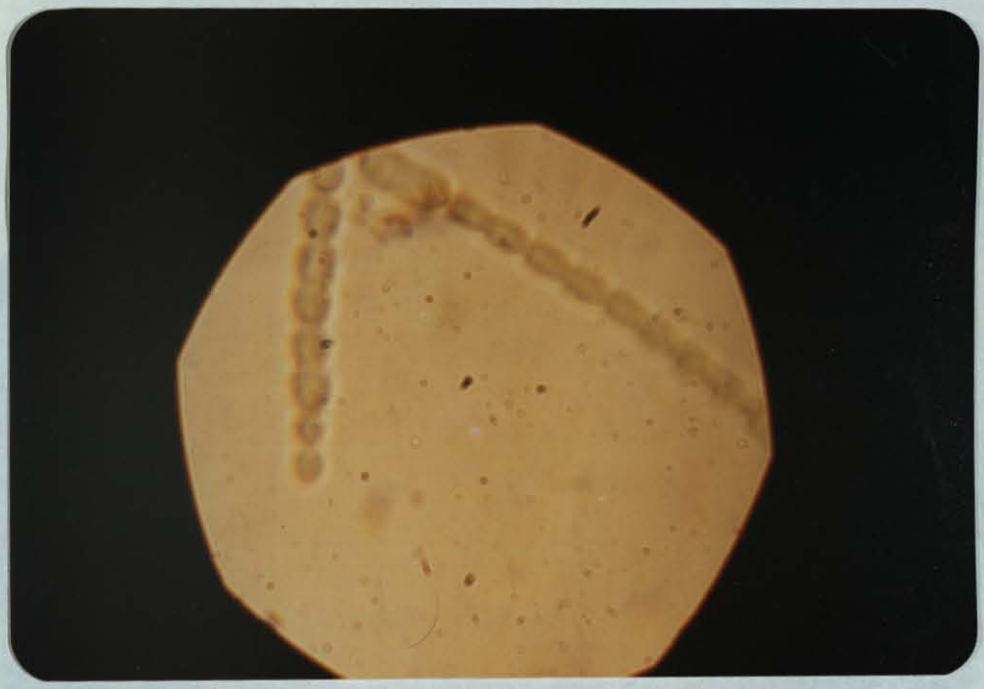


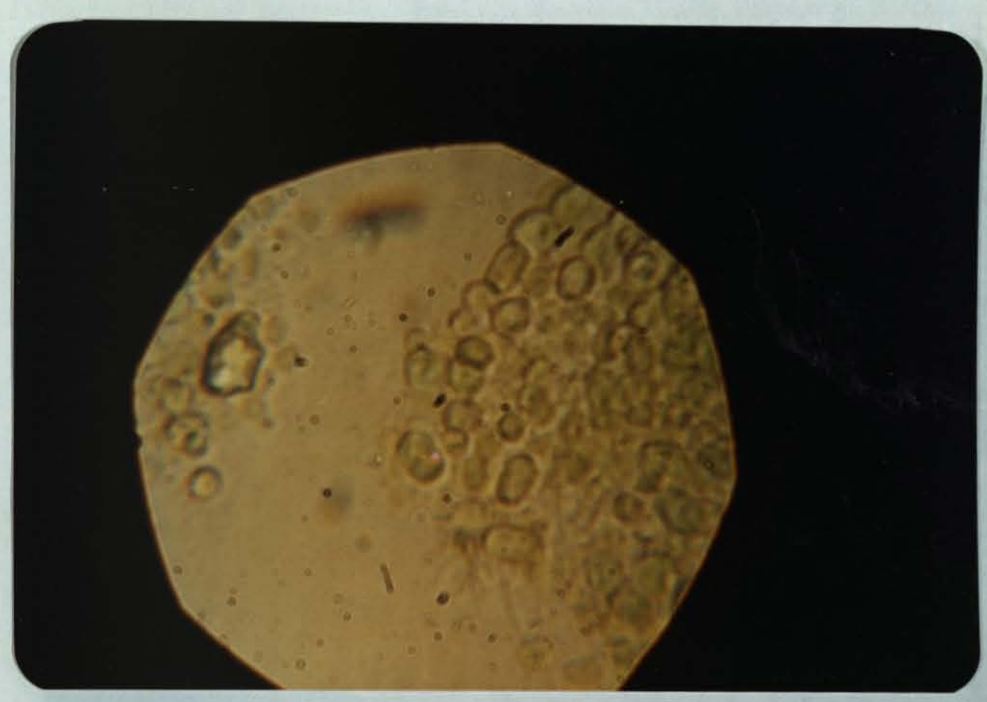
CULTURE INCUBATED FOR FIVE DAYS IN 8 PPB MERCURY



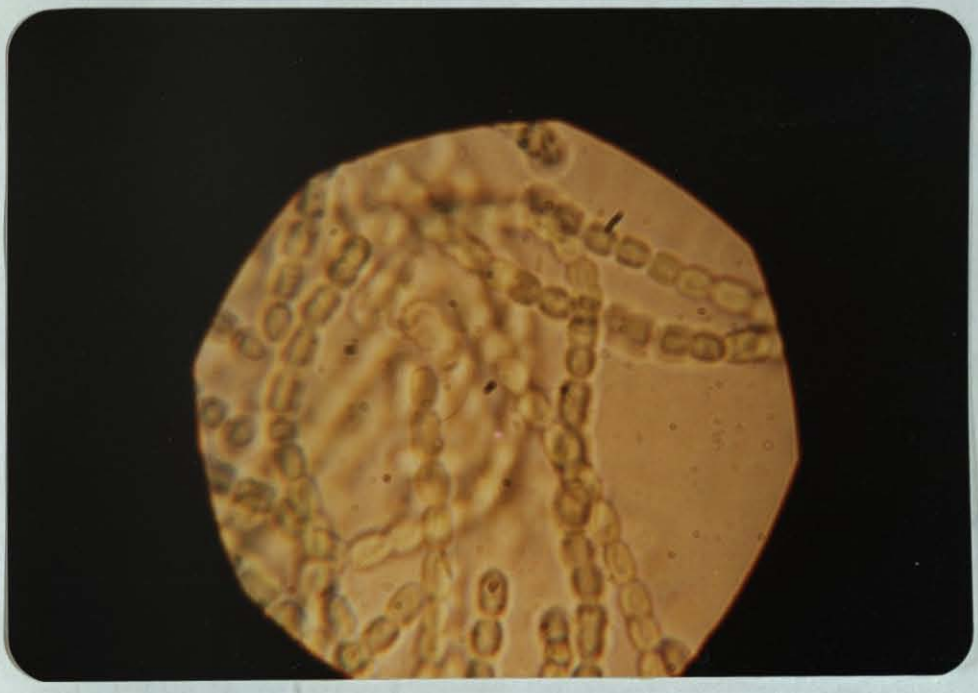
CULTURE INCUBATED FOR FIVE DAYS IN 100 PPB CADMIUM



CULTURE INCUBATED FOR FIVE DAYS IN 20 PPM SELENIUM



CULTURE INCUBATED FOR FIVE DAYS IN 0.2 PPM NICKEL



CULTURE INCUBATED FOR FIVE DAYS IN 20 PPM CHROMIUM

CULTURE INCUBATED FOR FIVE DAYS IN 10 PPM ARSENIC

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