THE EFFECT OF SELECTED PESTICIDES ON THE GROWTH AND NITROGEN FIXATION IN CYANOBACTERIA

by

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To my family

ABSTRACT

Algae, in general, are the primitive form of higher plants and they are found in water bodies and in moist locations such as tree trunks, walls, rocks and damp soil. Many species are found in all parts of the world, from the tropics to the polar regions. Water , light, temperature and the chemical composition of the medium are the factors that may stimulate or prevent the growth. The elements essential for the growth of algae are the same as those necessary for the growth of higher plants.

Algae are used for the removal of pesticides from the aquatic environment, therefore their functions and responses to external effects resulting from the use of chemicals are of concern. The choice of algal species for bioassays is partly based on algae being the primary producers in aquatic ecosystems. Since pesticides are pollutants, it is better to analyze them on these living organisms, which can give an information about other higher plants.

Contamination of aquatic and soil environments by pesticides results from agricultural origin. Pesticides enter the environment by direct or indirect routes. These agents can be sprayed on crops or dispersed in the field as a granular substance. Such pesticides are introduced directly into the aquatic environment. The cleaning of industrial mixing equipment, disposal of waste and accidents

increase the amount of pesticides found in soil and water. Rain and other precipitation can carry pollutants to the fresh water systems, so causing harmful effects for the algae. The application of pesticides for plant protection and their persistent residues has direct effect on the fresh water algae and consequently the soil fertility. These pesticides applied directly or indirectly to soil may upset the ecological balance of the soil. This situation clearly explains the importance of studying the effects of pesticides on the different physiological and biological processes.

Algal studies are recently well documented. Recent studies involve the use of some pesticides on algal cultures in order to examine their effects on growth and nitrogen fixation. Effects of chemical agents could quickly be investigated with blue-green algae before testing pesticides on higher plants.

The aim of this study was to examine the toxicity effects of a herbicide 2,4-dichelorophenoxyacetic acid (2,4-D) and an insecticide methyl parathion on two algal species, one being a heterocystous filamentous cyanobacteria *Anabaena cylindrica* and the other, unicellular cyanobacteria *Gloeocapsa*. Toxicity determinations were based on the growth measurements of these species. Moreover, a specific property of blue-green algae, nitrogen fixing ability was determined by using acetylene reduction technique. During the experiments light intensity, temperature and pH were under the control.

ÖZET

Genel olarak alglar, gelişmiş bitkilerin ilkel formlarıdır. Bunlar sular, ağaç gövdeleri, duvarlar, kayalar ve ıslak topraklar gibi nemli ortamlarda bulunurlar. Birçok cinsleri tropiklerden kutuplara kadar olan dünyanın her bölümünde varlık gösterirler. Su, ışık, sıcaklık ve ortamın kimyasal bileşeni büyümeyi hızlandıran ya da engelleyen faktörlerdir. Algların büyümeleri için gerekli elementler, gelişmiş, bitkilerin büyümeleri için gerekli olanlarla aynıdır.

Alglar, su çevresinin pestisitlerden arındırılmasında kullanılır. Bu nedenle kullanılan kimyasallardan oluşan dış etkilere karşı tepkileri ilgi noktasıdır. Algların biyoindikatör organizma olarak seçilmelerinin nedeni su ekosistemlerinde birinci derecede üretici olmalarıdır. Kirletici olan pestisitleri gelişmiş bitkiler hakkında bilgi verebilecek olan bu organizmalar üzerinde analiz etmek doğru olacaktır.

Su ve toprak çevrelerinin pestisitler tarafından kirlenişi tarıma dayalıdır.Pestisitler, doğru ya da dolaylı yollardan çevreye girerler. Bu maddeler ürünler üzerine sıkılır veya granüler tanecikler şeklinde tarlaya dağıtılır. Bu tip pestisitler doğrudan suya karışırlar. Endüstriyel karıştırıcıların temizlenmesi, çöplerin tanzimi ve kazalar, toprak ve suda bulunan pestisitlerin miktarını arttırır. Yağmur ve diğer yağışlar kirleticileri tatlı su sistemlerine taşıyabilirler, böylelikle alglar için zararlı olan etkilere yol açarlar. Pestisitlerin bitki korunumu için

kullanımının ve onların kalıcı artıklarının tatlı su algları üzerinde doğrudan etkileri vardır. Toprağa doğru ya da dolaylı yoldan etki eden bu pestisitler toprağın ekoloji dengesini bozabilir. Bu durum, pestisitlerin değişik fizyolojik ve biyolojik prosesler üzerindeki etkilerinin incelenmesi önemini açıkça ortaya koyar.

Bu çalışmanın amacı , bir herbisit olan 2,4-diklorofenoksiasetik asit (2,4-D) ve bir insektisit olan metil parationun iki alg türü, biri heterosistli filamentli *Anabaena cylindrica,* diğeri tek hücreli *Gloeocapsa* türleri üzerinde toksisite etkilerinin sınanmasıydı. Toksisite tespitleri bu türlerin büyüme ölçümleri üzerine yapıldı. Ayrıca, mavi-yeşil algların özelliği olan azot bağlama kapasitesi asetilen indirgenme tekniği kullanılarak tespit edildi. Deneyler sırasında ışık şiddeti, sıcaklık ve pH kontrol altında tutuldu.

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I. ALGAE

1.1. NATURAL OCCURRENCE, DISTRIBUTION AND CLASSIFICATION

Hardly any body of water or moist spot on the face of earth is devoid of algae; they are as widely distributed as bacteria. The variety of form and of color exhibited by algae are seemingly endless. If it were possible for fresh water algae to grow as large as some other plants and to live upon land, they would be considered highly attractive and would be much cultivated as ornamentals.

Algae attract attention for many reasons, partly because of their bright colors or because of significant growth in ponds, streams, lakes and along ocean shores. The varied shapes of both marine and fresh-water algae, coupled with their many colors have made them the subject of observation for a long time. It is not aesthetic quality of fresh water algae alone, they have many economic importances and considerable biological significance. Purely scientific problems, such as the role of algae in organic evolution, the biology of their reproduction and life histories and their ecology are common subjects of investigation. Although much is still to be learned from them, the solution of many problems in general biology and physiology have been obtained from studies of algae.

The organisms which are commonly known as "algae" are extremely diverse in form, color and in their habitats. Actually there are eight separate phyla or divisions of the plant kingdom included under "algae". The three major phyla of algae (those which are the most common) are the Chlorophyta (green), Cyanophyta (blue-green) and Chrysophyta (yellow or brown-green).

The phyla of fresh-water algae recognized are as follows (1):

1) Chlorophyta (Green Algae)
2) Cyanophyta (Blue-green Algae)
3) Chysophyta (Yellow-green Algae
4) Euglenophyta
5) Pyrrhophyta
6) Rhodophyta
7) Chloromonadophyta
8) Phaeophyta (Brown Algae)

Blue-green algae are a distinctive group sharply differed form other algae in a number of respects. They fall into three major groups according to their structures. They can be unicellular, heterocystous or non-heterocystous filamentous. They lack chloroplasts, the pigments are in solution and give colors to the entire protoplast, often they are found in the peripheral region of the cell. The pigments are;

1) Chlorophyll-a
2) Carotenes
3) Xanthophylls
4) Phycoerythrin
5) Phycocyanin

Their cell walls are thin, the membrane has a gelatinous outer sheath.

Some authors designate blue-green algae as a major group of bacteria (2). Bacteria are distinct living entities whose structural organization differs from that of all other microorganisms except blue-green algae. The organizational unit of bacteria and blue-green algae is the procaryotic cell (the word "procaryotic" implies an organism not showing a classical nuclear organization, and suggests a

more primitive condition). Microorganisms such as the other algae (brown, red and green) have eukaryotic cell forms, that is a "true" nuclear organization.

Bacteria and blue-green algae have some similarities. These similarities lie on the some type of ribosomes, the similar cell wall structure, mode of cell division, the ability to survive at high temperatures and the ability of some to fix dinitrogen. These common facts enable the blue-green algae to be renamed as "cyanobacteria".

Blue-green algae are very ancient organisms. There has been fossil evidence indicating that they had developed $3X10^9$ years ago (3) and that an oxygenic atmosphere may have become established at about this time. Thus, blue-green algae have been on Earth for over three billion years and it is very likely that during the course of evolution they have been the major providers of oxygen to the atmosphere (4).

The algae differ from the higher plants in that, they do not possess true roots, stems and leaves. The fresh-water algae are found widely in moist situations (as tree trunks, walls, woodwork, rocks, damp soil, lakes, ponds, rivers, etc.). The green algae occur in the sea , in fresh water and on land. The brown and the red algae are all confined to sea. Blue-green algae are found in almost all environments including soil, fresh and salt water, the open ocean as well as coastal habitats.

The successful introduction of algae into new localities depend upon their suitable habitats. Most blue-green algae are photoautotrophs which means that the algae are dependent on light for energy source and on CO_2 as principal carbon source (5). Water is essential for growth of the algae and the other factors are light, temperature, chemical composition and pH of the medium.

Blue-green algae have nitrogen fixing representatives. The ability to fix

dinitrogen (N_2) is widely distributed among the class of cyanobacteria. Biological systems able to fix dinitrogen are called diazotrophs. *Anabaena, Gloeocapsa, Nostoc* and *Dermocarpa* species are some examples of diazotrophs.

1.2. NITROGEN CYCLE AND DINITROGEN FIXATION

The major steps in the cyclic transformation of nitrogen are shown in Figure 1.1. (6). Plants and microbes make their component proteins from nitrates by way of nitrites and ammonia. Animals make their protein directly or indirectly from plants or microbes. Whether living or dead, such organic nitrogen is inaccessible to plants; it is sometimes called "immobilized nitrogen". Death, decomposition and putrefaction eventually lead to release of protein nitrogen as NH_3, a process called "ammonification" or mineralization. Some bacteria need NH_4^+ in addition to amino acids. Often amino acids are incorporated directly into bacterial protein without alteration, but under some conditions they are extensively metabolized by bacteria. Deaminations result in the liberation of *ammonium ion*. The overall process (ammonification) consist of the hydrolysis of protein by enzymes and the deamination of the resulting amino acids. Most of the ammonia is rapidly oxidized back to nitrate by nitrifying bacteria. Bacterial oxidation from ammonia to nitrate is called nitrification. Nitrification has been considered beneficial or even essential in agriculture. It has been used as index of soil fertility, as "richer" soils show greater numbers of nitrifying bacteria. Ammonium ions tend to remain complexed to soil components, but nitrate is more easily leached away by rain or irrigation water. Bacterial nitrification is promoted by application of ammonical fertilizers, but this often results in significant overall losses of nitrogen. The nitrate leached from such fields becomes a pollutant in the streams receiving the run-off and in the ground water as well. The possibility of inhibitory effects on nitrifying bacteria is considered in connection with applications of agricultural chemicals, such as persistent pesticides.

Losses of nitrate nitrogen occur not only because of leaching, but also because of bacterial action in the process of "denitrification". Under anaerobic conditions a number of bacteria, such as *Pseudomanas Stutzeri* , *Bacillus licheniformis* are able to use nitrate in place of O_2 as the terminal oxidant for their energy metabolism.

Nitrogen exists in the atmosphere almost exclusively as N_2. The entry of N_2 into the biosphere is made possible only by certain microorganisms, which are able to reduce the N_2 molecule to the level of ammonia. This reduction process, referred to as nitrogen fixation, is carried on by a number of different types of aerobic and anaerobic bacteria and also by certain blue-green algae and yeasts (6). Nitrogen fixation is not exclusively biological. Lightning, atmospheric pollution generates oxides of nitrogen; rain washes these oxides of nitrogen into the soil as nitrates. The fertilizer industry also provides locally very important quantities of chemically-fixed nitrogen.

Nitrification is an important step in the nitrogen cycle for two reasons.

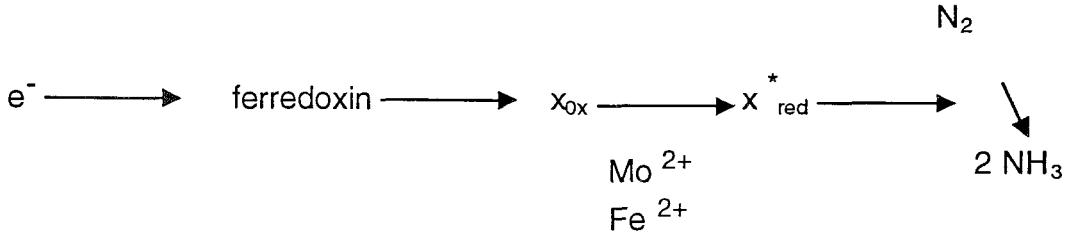
1) It provides the plants with fixed nitrogen in a form they seem to prefer.

2) It circulates fixed nitrogen, because nitrate tends to wash out of soil whereas ammonia tends to remain bound.

Environmental problems arise when ammonia (added as fertilizer or formed by decomposition processes) becomes washed by rain or flow of ground water into rivers or lakes as nitrates.

The reduction of nitrogen is carried out by a multienzyme complex referred to as "nitrogenase" which includes low potential electron carriers, along with nonheme iron, labile sulfide and molybdenum. This reaction appears to consist of the reduction of an electron carrier, such as ferredoxin, by electrons from cellular

energy metabolism and then transfer of the electrons to some member (x) of the nitrogenase complex along with activation of this component by use of ATP. The activated reductant formed (x *red) can donate electrons to the N_2 molecule and yield ammonia.



Ferredoxin is found in the anaerobic nitrogen-fixing bacteria, such as *Clostridium*, but the e^- carriers in aerobes such as *Azotobacter* are not known with certainty. Some other electron acceptors can be reduced by the nitrogenase reaction, including acetylene, cyanide and azide. The reduction of acetylene to ethylene has proven useful as a simplified index of the N_2- fixing capacity of organisms in nature. (6)

1.3. NITROGEN FIXATION BY PHOTOAUTOTROPHS

Unlike the photosynthetic bacteria which have a rather limited natural distribution, the N_2- fixing blue-green algae are found in many different ecosystems and furthermore, make a substantial contribution to the nitrogen balance of their environment. This wider distribution is undoubtedly related to the fact that species of blue-green algae can fix N_2 aerobically. On the other hand, some species closely resemble the photosynthetic bacteria, being able to fix N_2 under anaerobic or microaerophilic conditions.

N_2- fixing blue-green algae fall into three main groups; the filamentous blue-green algae which possess characteristically differentiated cells, called heterocysts; filamentous blue-green algae which lack heterocysts and unicellular blue-green algae. (Table 1.1.)

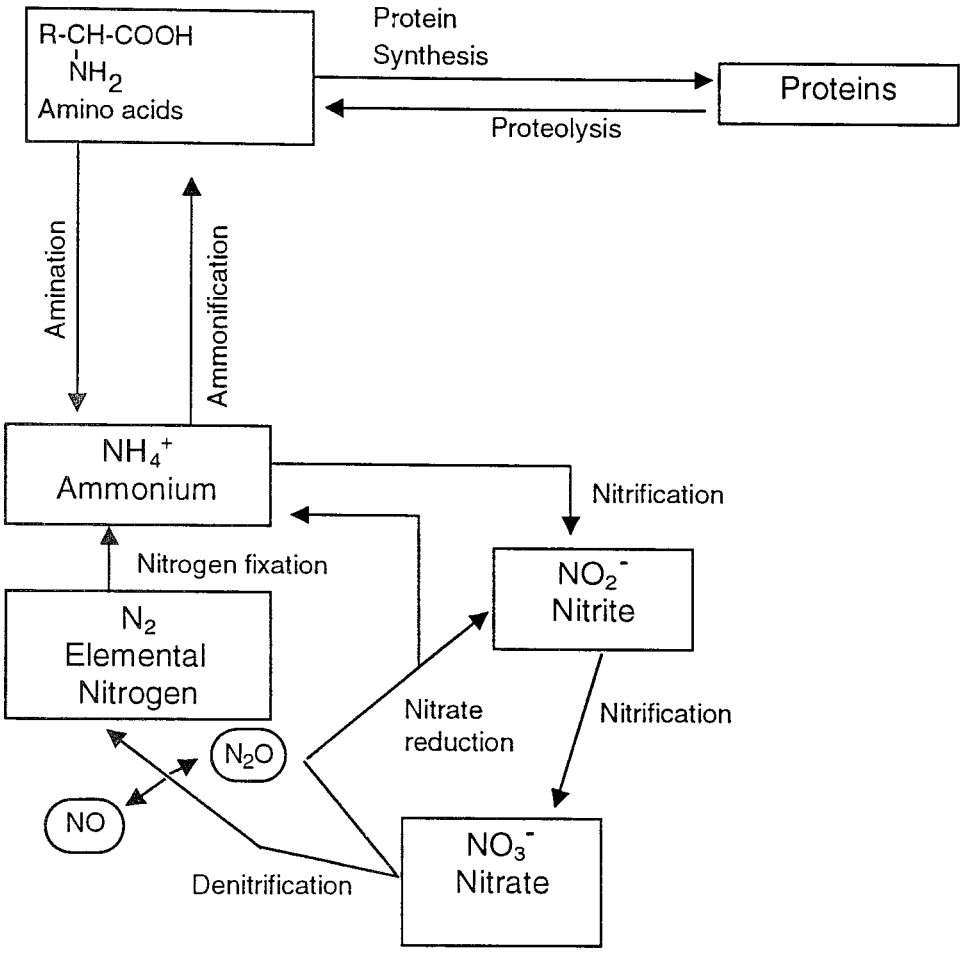


FIGURE 1.1. Nitrogen cycle

TABLE 1.1. Some nitrogen-fixing blue-green algae

1.	HETEROCYSTOUS, FILAMENTOUS
	E.g *Anabaena Cylindrica*
	Nostoc muscorum
	Aphanizomenon flos-aquae
2.	NON-HETEROCYSTOUS, FILAMENTOUS
	E.g *Plectonema boryanum*
	Trichodesmium sp
3.	UNICELLULAR
	E.g *Gloeocapsa* sp. *(Gloeothece)*
	Aphanothece pallida

1.3.1.HETEROCYSTOUS NITROGEN FIXING BLUE-GREEN ALGAE

N_2 fixation by heterocystous blue-green algae has been largely studied using organisms from only two genera, *Anabaena* and *Nostoc*. There has always been a tendency to extrapolate findings from these organisms to other species of blue-green algae in order to draw general conlusions. Several N_2-fixing blue-green algae, including heterocystous species, grown in batch culture behave differently according to the age of the culture when studied (7,8,9,10) therefore great care should be taken in extrapolating results from one organism to another.

1.3.1.1.LOCATION OF NITROGEN FIXATION

Most scientists consider that the heterocyst itself is the sole site of N_2 fixation under aerobic conditions of growth (11,12). These cells have a reducing environment, do not evolve oxygen, and are consequently an ideal site for the oxygen sensitive nitrogenase. Furthermore, these algae usually possess

heterocysts only under N_2- fixing conditions. However, detailed studies have been made on few species of blue-green alga and it is not impossible that other species may possess an active nitrogenase in their vegetative cells during aerobic growth.

Recent studies showed that one organism *Anabaena variabilis* grew aerobically on nitrogen-free medium and reduced acetylene, eventhough very few of the algal filaments possessed heterocysts (13).

Heterocysts originate by differentiation of vegetative cells. Vegetative cells have the genetic capability to fix N_2. During the differentation of vegetative cells to form heterocysts, precursor cells known as proheterocysts are formed. These cells which are microscopically distinquishable from both vegetative cells and heterocysts, synthesize the nitrogenase proteins (14) but cannot protect this enzyme from damage by oxygen. Consequently, proheterocysts cannot fix N_2. aerobically, but may do so under anaerobic or microaerophilic conditions. The blue - green algae, *Anabaena cylindrica* fix nitrogen under microaerophilic conditions. Nitrogenase activity was not confined to the proheterocysts, however; it was apparently located in all of the vegetative cells. Another blue-green alga, *Anabaena variabilis* , which could not form heterocysts or fix N_2 aerobically was also able to fix N_2 in the absence of oxygen.

Although, the heterocyst may be the sole site of aerobic N_2 fixation, it appears that under anaerobic or microaerophilic conditions vegetative cells also fix N_2 .However, since vegetative cells cannot protect their nitrogenase from inactivation by oxygen, they cannot fix N_2 aerobically.

1.3.1.2. REDUCTANT AND ATP

Heterocysts are strongly reducing environments. Heterocysts of *Anabaena cylindrica* lack the manganese-containing component of the oxygen evolving system associated with Photosystem II (the oxygen evolving system of the

photosynthetic apparatus) of photosynthesis (15). Consequently, heterocysts do not produce oxygen photosythetically and are unable to reduce ferredoxin, which is probably the natural electron donor to nitrogenase in *Anabaena cylindrica* (16), at the expense of electrons derived from water.

Apte *et al* (17) found that the ferredoxin -$NADP^+$ reductase of heterocysts of *Anabaena cylindrica* was not inactivated by light, unlike its vegetative cell counterpart.

Hydrogen is another possible source of reductant for N_2 fixation by heterocystous blue-green algae,(18). In light, hydrogen may generate reduced ferredoxin by donating electrons to the photosynthetic electron transport chain prior to Photosystem I (19) which is the anaerobic component of photosynthesis.

In heterocysts of *Anabaena cylindrica* , cyclic photophosphorylation is the major source of ATP in the light and can support maximum rates of acetylene reduction providing that an adequate supply of fixed carbon (20) is available. Heterocysts are incapable of non-cyclic photophosphorylation because they lack a functional Photosystem II. (Figure 1.2.) (21).

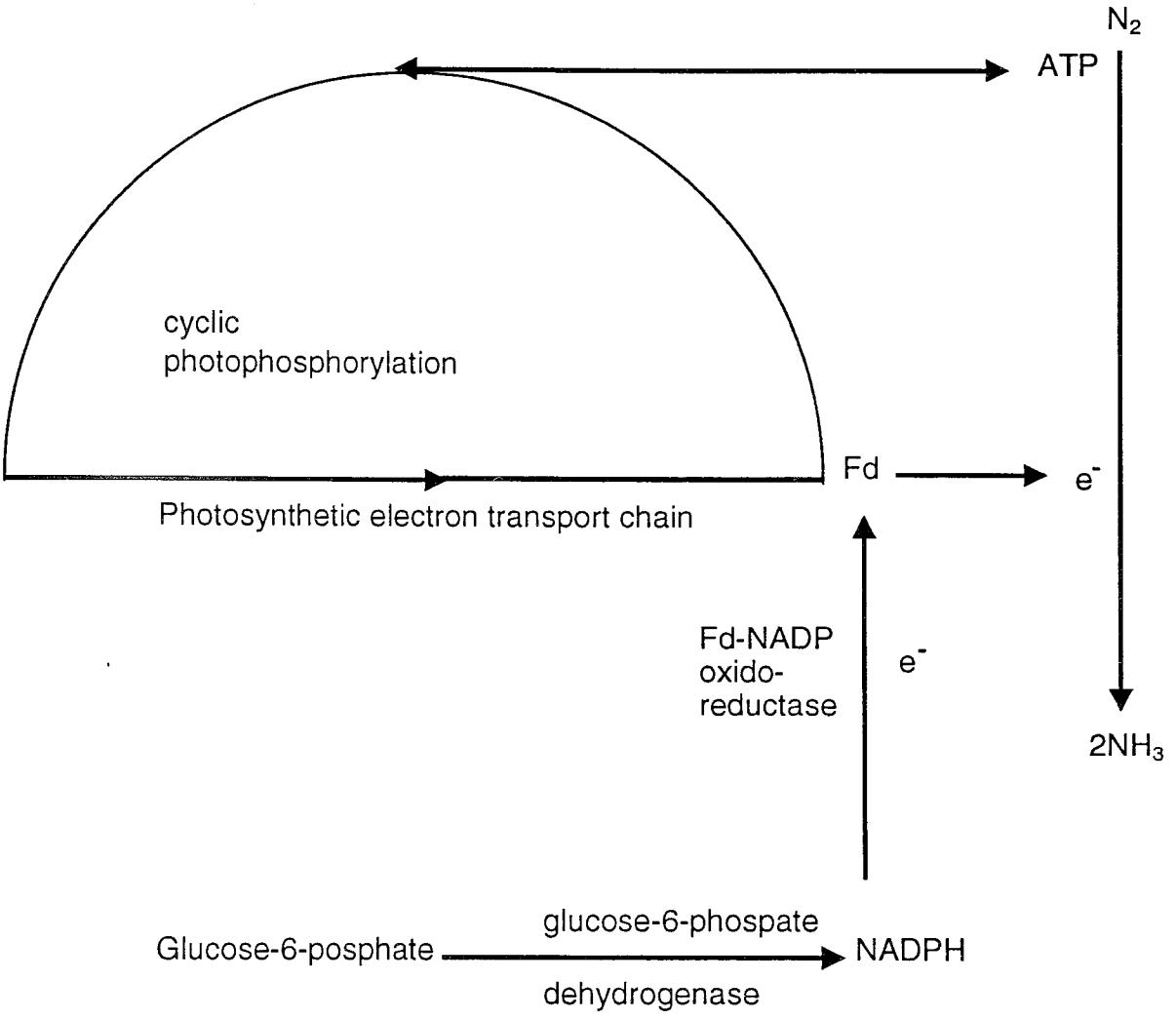


FIGURE 1.2 Generation of reduced ferredoxin for nitrogenase in heterocysts of *Anabaena cylindirica*

1.3.1.3 CONTROL OF NITROGEN FIXATION

i) INHIBITION OF NITROGENASE BY OXYGEN

All aerobic organisms require oxygen for their survival but it is known that oxygen is toxic to them at concentrations slightly higher than that in normal air.

Nitrogenases are notoriously sensitive to inactivation by oxygen. Blue-green nitrogenases have been found to be irreversibly inactivated *in vitro* by exposure to very low concentrations of oxygen (22). *In vivo*, however, the enzymes are able to function in the presence of the oxygen which is evolved during their photosynthesis and present in normal air. So, *in vivo*, these organisms must have some protective mechanism against oxygen inactivation.

Heterocystous filamentous blue-green algae fix their nitrogen in heterocysts (23,24,25) which, as mentioned above, do not themselves evolve oxygen photosynthetically and have a low intracellular oxygen concentration. In these organisms, the separation of oxygen evolution from nitrogenase and a barrier to the diffusion of atmospheric oxygen to the site of nitrogen fixation could be the method of protecting nitrogenase from oxygen inactivation.

Non-heterocystous filamentous blue-green algae such as *Plectonema* can fix nitrogen only under microaerobic conditions so *in vivo*, these organisms have very little, if any, protection against oxygen inactivation. However, in 1976 Carpenter and Price demonstrated (26) aerobic nitrogen fixation in a non-heterocystous alga, *Oscillatoria sp.* Nitrogen fixation took place in centrally located cells in the colony which do not themselves photosynthesize. They also demonstrated that this mode of protecting nitrogenase from oxygen inactivation

would be very fragile.

The unicellular blue-green alga *Gloeocapsa*, can grow and fix nitrogen aerobically (27,28) eventhough it does not possess heterocysts and its photosynthetic apparatus is in the same undifferentiated cell as nitrogenase. Therefore this organism has same protective mechanism against the damaging effects of oxygen *in vivo*.

ii) INHIBITION OF NITROGENASE BY FIXED NITROGEN

Stewart *et al* have suggested that ammonia, the product of nitrogen fixation is an important regulator of the process. When the concentration of ammonia in the medium was high, nitrogenase synthesis was inhibited and in the absence of ammonia there was an increase in nitrogenase synthesis. The intracellular concentration of ammonia is the regulating factor rather than its concentration in the external medium (29).

Ammonia, itself, does not inhibit the production of nitrogenase and heterocysts; glutamine synthetase and/or a product of ammonia assimilation is involved in regulating the production of both (30).

1.3.2. NON-HETEROCYSTOUS NITROGEN-FIXING BLUE-GREEN ALGAE

Until 1969 it was commonly believed that only heterocystous blue-green algae could fix nitrogen. However, in that year came the demonstration that a unicellular (and therefore non-hetrocystous) blue-green alga, *Gloeocapsa* , could grow aerobically on medium free of combined nitrogen and could also reduce acetylene to ethylene. Anaerobic N_2 fixation by non-heterocystous blue-green

algae are also common. In all the cultures studied,nitrogenase activity was rapidly destroyed by exposure to air. The only non-heterocystous blue-green algae so far identified as capable of fixing N_2 aerobically are the five strains of *Gloeothece sp, Aphanothece pallida* (31), *Synechococcus sp, Trichodesmium sp* (32) and *Microcoleus chthonoplastes* (33). In contrast to anaerobic N_2 fixation, therefore, the ability to fix N_2 aerobically has only a limited distribution among non-heterocystous species of blue-green algae.

1.3.2.1 ANAEROBIC NITROGEN FIXATION

Most studies on anaerobic N_2 fixation by non-heterocystous blue-green algae have been made using *Plectonema boryanum*, although many other species are now known to fix N_2 anaerobically (34). *Plectonema boryanum* grows heterotrophically in the dark, in the presence of a suitable carbon source (35). However, the experiments were unable to demonstrate N_2 fixation under these conditions(36). On the other hand *Plectonema boryanum* can fix N_2 microaeorophilically in the light (37). In the dark, *Plectonema boryanum* could supply ATP and reductant to an active nitrogenase, it appears that the nitrogenase of this organism can be inactivated by atmospheric oxygen and it is protected from photosynthetically produced oxygen. This limited protection could be respiratory, or could be the result of a temporal separation of N_2 fixation and photosynthetic oxygen evolution (38).

1.3.2.2. AEROBİC NITROGEN FIXATION

Almost all of the studies on aerobic N_2 fixation by non-heterocystous blue-green algae have been made using the unicellular organism *Gloeocapsa.*

Gloeocapsa has associated rod-like bacteria which live in the extensive slime capsule surrounding the algal cells. These bacteria cannot fix N_2 , and under N_2 - fixing conditions they are present in very low numbers. *Gloeocapsa* has a shorter doubling time, a slightly higher specific activity of nitrogenase, and can tolerate slightly better to high light intensities and elevated oxygen levels than its axenic strain. *Gloeocapsa* is less tolerant of high and low pH than is *Anabaena cylindrica.* For example, *Anabaena cylindrica* exhibits 85 % or more of its maximum rate of acetylene reduction in the pH range 6.8-10, for *Gloeocapsa* the equivalent range is pH 7.6-9.2.

Gloeocapsa, like all the other blue-green algae, possesses a photosynthetic mechanism similar to that of higher plants in which water is the ultimate electron donor and oxygen is evolved. Since *Gloeocapsa* contains its nitrogenase in the same undifferentiated cell as well as its photosynthetic apparatus, it seems that ATP and reducing power necessary to support nitrogen fixation might be supplied by photosynthesis. The natural source of reducing power for *Gloeocapsa* nitrogenase is probably ferredoxin (39), as it is for the nitrogenases from certain other blue-green algae.

Although *Gloeocapsa* fixes N_2 aerobically, the optimum oxygen concentration for growth and N_2 fixation is 0.1 atm (40). In addition carbon dioxide at concentrations greater than 0.01 atm. markedly inhibits acetylene reduction by this organism.

Since there are no obviously differentiated cells, such as heterocysts, in *Gloeocapsa*, nitrogen fixation apparently takes place in the same cell as

photosynthesis which produces oxygen. Oxygen rapidly inactivated *Gloeocapsa* nitrogenase *in vitro* (41) and *in vivo* (8). In order to overcome this inactivation by oxygen, *Gloeocapsa* must have some sort of protective mechanisms.There is a temporal seperation of maximum nitrogenase activity from maximum photosynthetic oxygen evolution (40). This could be the means of protecting the enzyme but there is still nitrogenase activity present, although at a low level, during the period of maximum oxygen evolution and oxygen is still evolved during the period of maximum nitrogenase activity. Consequently, there are possible mechanisms which might function to protect nitrogenase from inactivation by oxygen.

1.3.3. NITROGEN FIXATION DETERMINATION BY ACETYLENE REDUCTION ASSAY

The facility and sensitivity of the C_2H_2 -C_2H_4 assay make the assay of choice for many measurement of N_2 fixation.

The biochemical basis of this assay depends on the activity of nitrogenase. Reduction of acetylene to ethylene appears to be uniquely universal with respect to nitrogenase *in vitro* and *in vivo*. N_2 - fixing organisms have been found to reduce significant amounts of acetylene to ethylene. The stoichiometric relationship between C_2H_2 reduced and N_2 fixed is important for conversion measurements. A theoretical conversion factor (C_2H_2 reduced : N_2 fixed) has been used in most reported conversions. The average ratio for blue-green algae is 3.2 (42).

The relationship between the amounts of acetylene reduced and nitrogen fixed can be emphasized with the given reactions (43):

$$2e^- + 2H^+ + C_2H_2 \xrightarrow{N_2\text{-ase}} C_2H_4 \ (C_2H_2 \text{ reduction})$$

$$6e^- + 6H^+ + N_2 \xrightarrow{N_2\text{-ase}} 2\,NH_3 \ (N_2 \text{ fixation})$$

The two reactions are specific for nitrogenase and no other biological system can conduct this reaction. Ethylene formation from acetylene provides a rapid and valuable test for nitrogenase both for pure research and field studies.Ethylene does not inhibit N_2- fixation and is not reduced anymore to ethane.

II. PESTICIDES

2.1. CLASSIFICATION AND THE USES OF PESTICIDES

Nitrogen fixing blue-green algae occur abundantly in rice-fields of tropical countries. Their agricultural technologies involve extensive application of pesticides, for selective elimination of pests and weeds of rice crops.

The use of chemicals to control pests dates back to classical Greece and Rome. They dealt with the insects by using sulphurous fumes and arsenicals. The middle of the nineteeth century marked the beginning of the first systematic scientific studies into the use of crop protection chemicals. Chemical crop protection is too desirable also for the future.

Pesticide science is multi-functional field. It includes the chemistry of synthesis and analysis, the physics of formulation and the mechanics of application. Pesticides are formed from several minor groups of compounds. These are; insecticides which are used for the destruction of insects, fungicides for fungi and herbicides for weeds.

2.1.1. HERBICIDES

The global weed control problem is somewhat different from the relating to the control of insects and pathogens. Weeds seriously reduce yield or quality of product when they compete with the crop for available moisture, nutrients and light.

Herbicides are used in agriculture to remove weeds that would compete with the crop.

Herbicides can be classified in several ways. They are initially grouped according to whether they are normally applied to leaves or to soil (Table 2.1.) Some examples are given in the Figure 2.1. (44)

Each of these groups is then further divided according to the chemical structure of the herbicides. Some foliage-applied herbicides are translocated within the plant and others kill by action at or near the region where they contact with the leaves.

Such a classification loses some its usefulness if it is adapted too rigidly.

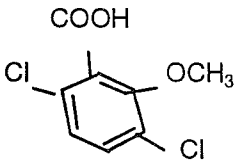
2.1.1.1 HERBICIDES APPLIED TO THE FOLIAGE

i) PHENOXYALKANOIC ACIDS : STRUCTURE, FORMULATION AND USES

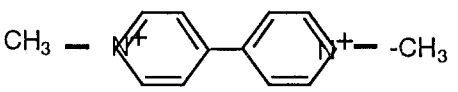
Phenoxy alkanoic acids are the most extensively used of weedkillers. They are used to control broadleaved weeds in cereal crops and grassland.

All members of the group have a chlorine atom attached to carbon-4 of the benzene ring and either a chlorine atom or a methyl group on carbon-2. Sometimes an additional chlorine atom is present on carbon-5- (Figure 2.2.)

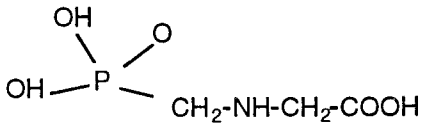
Formulation is usually either as esters emulsified in oil or as water-soluble amine or other salts. Amine salts are very soluble in water and they are convenient for low volume application. Ester formulations are insoluble in water and more toxic to weeds than the ionized forms.



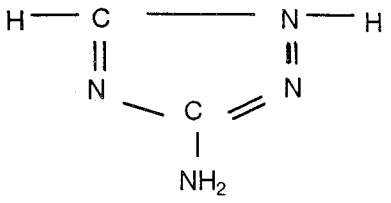
Dicamba



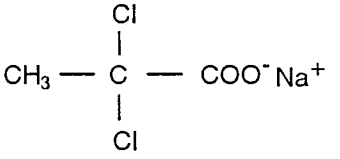
Paraquat cation



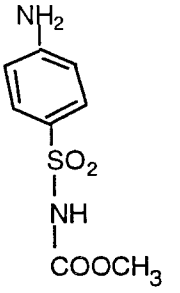
Glyphosate



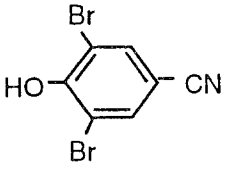
Aminotriazole



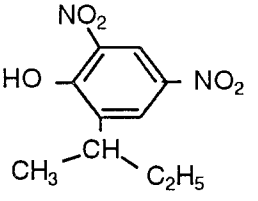
Dalapon-Na



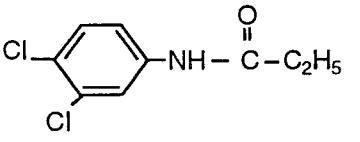
Asulam



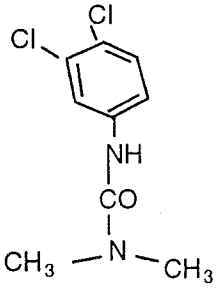
Bromoxynil



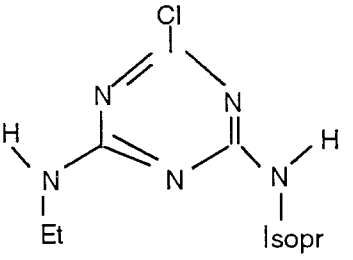
Dinoseb



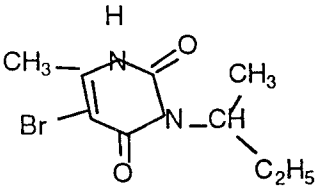
Propanil



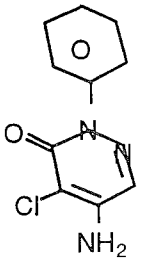
Diuron



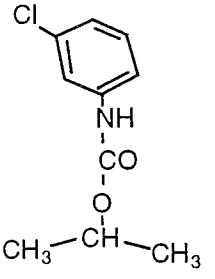
Atrazine



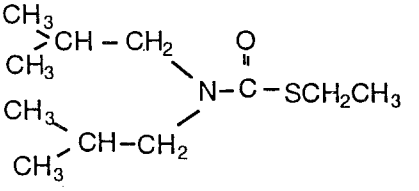
Bromacil



Pyrazone

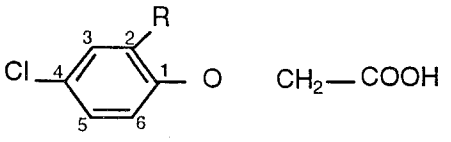


Chlorpropham



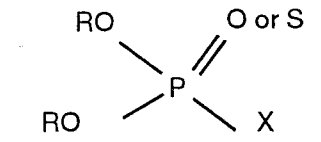
Butylate

FIGURE 2.1 Some examples of herbicides



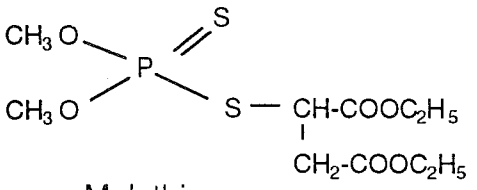
Phenoxyalkanoic Acid

FIGURE 2.2

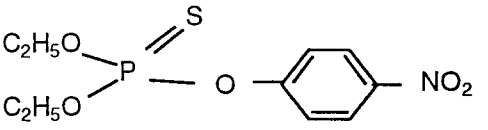


Organophosphorus Insecticide

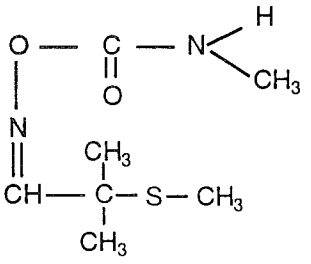
FIGURE 2.4



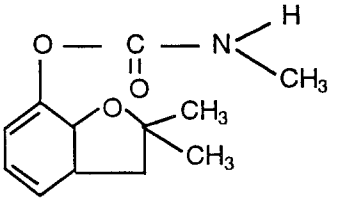
Malathion



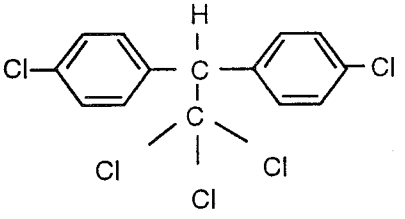
Parathion



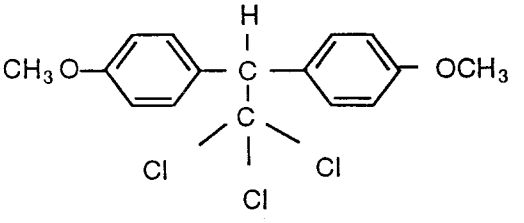
Aldicarb



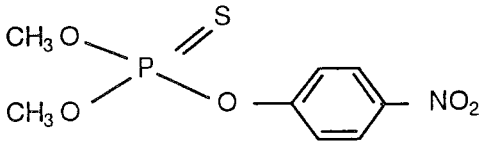
Carbofuran



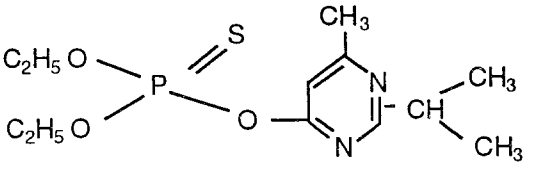
p_1 p'- DDT



Methoxychlor



Methyl parathion



Diazinon

FIGURE 2.3 Some examples of insecticides

Table 2.1. Herbicide Classification

I. Foliar Application
   A. Systemic or translocated herbicides
      1. Phenoxyalkanoic acids (E.g 2.4.D, MCPB)
      2. Growth regulators based an benzoic acid (E.g dicamba)
      3. Quaternary ammonium coupounds (E.g paraquat)
      4. Amino acid derivatives (E.g glyphosate)
      5. Triazoles (E.g aminotriazole)
      6. Chlarinated aliphatic acids (E.g dalapon)
      7. Translocated carbamates (E.g barban, asulam)
   B. Contact herbicides
      1. Petroleum oils (mixture of saturated hydrocarbons)
      2. Hydroxyaryl nitriles (E.g bromoxynil)
      3. Dinitrophenols (E.g dinoseb)
      4. Miscellaneous (E.g pentaclor, propanil)

II. Soil Application
   1. Substituted ureas (E.g diuron, linuron)
   2. Triazines (E.g atrazine)
   3. Uracil (pyrimidine) derivatives (E.g bromacil)
   4. Pyridazine derivatives (E.g pyrazone)
   5. Phenylcarbamates (E.g chlorpropham)
   6. Thiolcarbamates (E.g butylate)
   7. Nitroanilines (E.g dinitroamine)
   8. Nitrophenyl ether
   9. Miscellaneous (E.g dichlobenil)

The alkali metal and amine salts of MCPA and 2,4-D are used on wheat, barley, oats, rye, rice, maize and sorghum. At lower concentrations salts of 2,4-D are used as growth regulators to reduce fruit drop. 2,4-D as one of the most frequently used herbicides nowadays, is commonly investigated either for its toxic properties or for biodegradability. 2,4-D is observed to be reasonably biodegradable, because the removal of herbicides from the environment and aquatic system is a very difficult problem for scientists. Algae are used for the removal of herbicides from the aquatic environment and herbicides are known to be metabolized by various aquatic plants and algae. Rice field herbicides, while protecting the rice seedlings, selectively destroy the weeds and indirectly increase the yield. Therefore, under the water-logged conditions of rice fields, both nitrogen-fixing cyanobacteria and rice field herbicides are found to interact resulting in the destruction of cyanobacteria (45).

2.1.1.2. SOIL ACTING HERBICIDES

Soil acting herbicides are structurally different from those applied to foliage. However, in most cases the same substance can be used in either mode. Herbicides applied directly to the soil are frequently used to attack germinating weed seeds or very young plants. Five different circumstances are appropriate for the soil application of the herbicide. Briefly, these five include three non-crop situations and two in which a crop is present.

2.1.2. INSECTICIDES

Insecticides are mainly divided into three groups:

a) organophosphorus insecticides (E.g. malathion, methyl parathion)

b) carbamate insecticides (E.g aldicarb, carbofuran)

c) organochlorine insecticides (E.g DDT, methoxychlor)

Some structural examples are given in Figure 2.3.

2.1.2.1. ORGANOPHOSPHORUS INSECTICIDES

Most organophosphorus insecticides have the general structure as shown in Figure 2.4. The two-R groups are usually methyl or ethyl and are the same in any one molecule, while-x is frequently a rather complex aliphatic, homocyclic or heterocyclic group. Therefore the structural variability of organophosphorus compounds is reflected not only in their physical properties but also in a diversity of mechanisms to be attacked by enzymes.

In modern agriculture, insecticides are widely used to increase plant productivity by controlling pests but the insecticides may adversely affect plant growth and metabolism.

The majority of organophosphorus compounds are effective in the control of aphids and similar soft-bodied small insects.

It is possible to classify organophosphorus insecticides according to their practical uses. Compounds of low chemical stability and soluble in water but more or less rapidly hydrolyzed by it are used as "contact" insecticides. E.g mevinphos, tetraethyl pyrophoshate. Compounds of moderate to high chemical stability, with low solubility in water are used as "persistent contact " insecticides. These compounds soak into leaves but do not travel around the plant. E.g malathion, methyl parathion. "Systemic" compounds have moderate to high chemical stability and they are activated before reaching their site of action in insects. E.g.

formothion, disulfaton. Compounds with sufficiently high vapor pressure and low chemical stability are used as "fumigants". E.g dichlorvos. In addition to these compounds there are substances also suitable for formulation as "granules" for soil application. Among all these insecticides the persistent contact orgonophosphorus insecticides are very largely used. This group is subdivided into three principal families. These are the malathion family, the parathion family and the family of the compounds whose members contain a heterocyclic leaving group.

Parathion, methyl parathion and fenitrothion have similar agricultural uses. Parathion is more persistent than methyl parathion and much more persistent than fenitrothion. Malathion appears to be safe to algae. Parathion is extremely toxic to algae even to humans and its toxicity is much more than both malathion and fenitrothion.

2.1.2.2. CARBAMATE INSECTICIDES

Carbamate insecticides are frequently employed to control insects which do not readily respond to organophosphorus compounds. These are particularly useful for dealing with aphids and other pests which have developed resistance to organophosphorus compounds or which are difficult to control for other reasons. These insecticides are powerful inhibitor of insect nerve cholinesterase.

2.1.2.3. ORGANOCHLORINE INSECTICIDES

The acute toxicity of many members of the group is acceptably low. Some are notoriously persistent in the environment and so they can be a real hazard to wildlife and a potential hazard to man. The organophoshorus and carbamate insecticides have largely replaced them for many aspects of crop protection in numerous countries.

2.1.3. FUNGICIDES

Problems associated with the chemical control of fungi are in some ways different from those that arise in relation to the control of insects. Most fungicides are primarily protective in action.

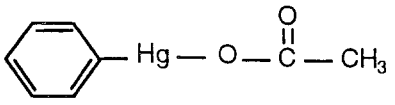
A fungicide which kills on contact can check the growth of mycelium and limit the production of reproductive structures. Such action delays or prevents the disease from spreading from infected plants to healthy ones nearby, but the majority of successful non-systemic fungicides are most effective when applied prior to the arrival of the infection. Non-systemic fungicides must therefore possess some degree of persistence and the majority of such fungicides are, in fact, insoluble in water. (Table 2.2).

In addition to airborne pathogen, fungi may attack plants through the soil or be transmitted on or in the seeds. Such fungi are frequently controlled by soil or seed treatment, often by the use of fungicides specially developed for these purposes. Clearly, a knowledge of the habits and life cycles of such fungi is an essential pre-requisite for effective chemical control. Some structural examples are given in Figure 2.5.

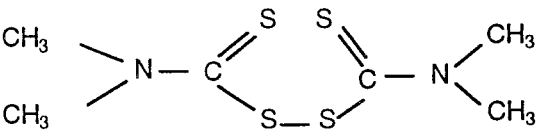
To conclude, the use of pesticides needs great care, because they are toxic to cyanobacteria at higher concentrations. Since cyanobacteria are found in the roots of the plants and they fix nitrogen, misuse of pesticides may affect the nitrogen fixation ability.

Table 2.2. Classification of organic fungicides

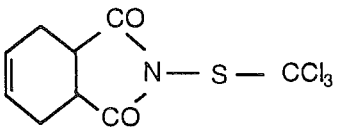
A. Organometallic compounds
    1. Organomercury (e.g phenylmercury acetate)
    2. Organotin (e.g fentin acetate)
B. Organosulfur compounds
    1. Dithiocarbamates (e.g thiram)
    2. Phthalimides (e.g captan)
C. Dinitrophenol derivatives (e.g dinocap)
D. Chlorinated derivatives of benzene and naphthalene
    1. Chloronitro benzenes, chloronitroaniline (e.g dicloran)
    2. Chloronitriles (e.g chlorothalonil)
    3. Chloroquinones (e.g dichlone)
    4. Chlorophenols (e.g pentachlorophenol)
E. Cationic surfactants
    1. Guanidine derivatives (e.g dodine acetate)
    2. Imidazoline derivatives (e.g glyodin acetate)
F. Other non-systemic conpounds
    1. Dicarboximides (e.g iprodione)
    2. Dithianon
G. Systemic antifungal compounds



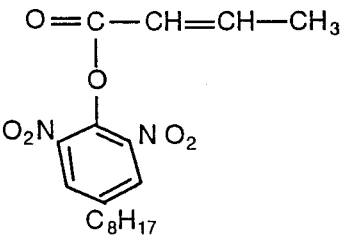
Phenylmercury acetate



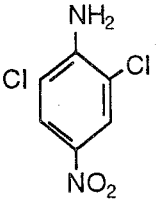
Thiram



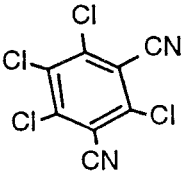
Captan



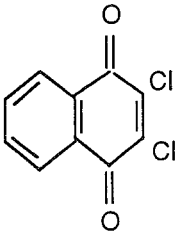
Dinocap-4



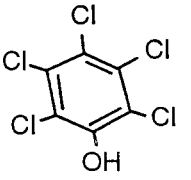
Dichloran
(Soil and foliage application)



Chlorothalonil
(foliage application)



Dichlone
(seed dressing)



Pentachlorophenol

FIGURE 2.5 Some examples of fungicides

2.2. EFFECTS OF PESTICIDES ON SOME STRAINS OF ALGAE IN RECENT STUDIES

The latest studies involve the use of some pesticides on some specific algae in order to determine their effects on growth, photosynthesis and nitrogen fixation.

Pesticides in microgram amounts can have an effect on the growth of cyanobacteria. Since the photosystems of the cyanobacteria are similar to those of higher plants chemical agents that are harmful to the cyanobacteria should also be toxic to plants. Effects of chemical agents could quickly be investigated with cyanobacteria before testing pesticides on higher plants.

S.M. Mallison tried the pesticides DCMU, ametryn, and malathion on cyanobacteria *Plectonema boryanum* and found that DCMU and ametryn killed the cyanobacteria whereas malathion stimulated the growth (46).

The importance of nitrogen fixation by blue-green algae relating to the fertility of rice fields is generally well recognized. Substantial increases in rice yield following inoculation of rice fields with nitrogen fixing blue-green algae have been reported (47).

Great information is avaliable on the interaction of insecticides with green algae, however little attention has been given to the effects of pesticides on blue-green algae. Thus with a view to the importance of the nitrogen-fixing blue-green algae in rice agriculture, the effects of three insecticides DDT, fenitrothion and chlorpyrifos on growth, photosynthesis and nitrogen fixation in blue-green algae *Anabaena* and *Aulosira fertilissima* were studied by Lal and Lal (47). The experiments gave the following results : All concentrations of DDT (10,50 and 100 ppm for both algae) inhibited the growth of *Anabaena* and the inhibition was dose dependent. In contrast to *Anabaena, Aulosira* showed a different type of response to DDT. The rate of growth was faster than the controls until 25 days of treatment. Fenitrothion at 1, 5 and 10 ppm. was more toxic to *Anabaena* than

DDT. Inhibition of growth was concentration dependent and a maximum reduction was noticed with 10 ppm. Fenitrothion, although toxic to *Aulosira* at 5 and 10 ppm,was stimulatory at 1 ppm.Chlorpyrifos was less toxic than fenitrothion but more toxic than DDT. The growth of *Anabaena* was inhibited at 5 and 10 ppm, whereas the inhibition was not sifnificant with 1 ppm. The growth of *Aulosira* was stimulated by 1 ppm but inhibited by 5 and 10 ppm. Photosynthesis was inhibited by all three insecticides at all concentrations. DDT was stimulatory at 50 and 100 ppm to *Anabaena* in nitrogen fixation, whereas the others were inhibitor for nitrogenase. These results gave no positive relationship between the effect on nitrogen fixation and growth.

M. Megharaj (48) also determined the influence of four insecticides (monocrotophos, quinalphos, cypermethrin and fenvalerate) and four phenolics (p-nitrophenol, m-nitrophenol, 2,4 dinitrophenol and catechol) on nitrogen-fixing capacity of *N.linckia*. As a result they established that the effect of insecticides on nitrogen fixation by cyanobacteria is independent of growth. The ability of the cyanobacterium to fix atmospheric nitrogen was affected to varying degrees under the influence of the selected insecticides and phenolic compounds.

Another pesticide, atrazine has been used on blue-green algae and as a result stimulation on nitrogen fixation with variable degrees in all treated cultures was determined (49).

Triazine herbicides are toxic to algae by distrupting photosynthesis (50). S-triazine herbicides are often the most algicidal of any of the herbicide groups when tested on fresh water algae (51). Atrazine, one of the S-triazine herbicides is used very commonly in this research area.

Blue-green algae are more tolerant to atrazine than the other type of algae (52,53,54). Although fewer data are available regarding the effects of other

triazine herbicides to fresh water algae they appear to be comparable in toxicity to atrazine (55,56). H.Abou-Waly observed a reduction in growth of algae with an increase of atrazine concentration (57).

The increasing use of herbicides, particularly 2,4-dichlorophenoxyacetic acid (2,4-D) is likely to create microbial imbalance by affecting their growth and development.

2,4 D is commonly investigated either for its toxicity or its biodegradability. A.K. Mishra and D.N. Tiwari tried 2,4 D on the nitrogen-fixing cyanobacterium *Nostoc linckia* . The 2,4 D which is a synthetic growth hormone analogue was stimulator of growth and heterocyst formation in the cyanobacterium. They reported an increase in growth of *N. linckia* in the presence of lower concentration of 2,4 D (58).

A.K. Mishra and A.B. Pandey studied the toxicity of three herbicides 2,4 D, machete and saturn on some nitrogen fixing cyanobacteria (45). The 2,4 D stimulated the growth and nitrogen fixation up to 100 µg/ml concentration. However, with machete and saturn this type of stimulation was not observed even at lower concentrations. The lethal dose of 2,4 D was found to be 1500 to 2000 µg / ml for different species whereas the lethal dose of machete and saturn was found to be 6-8 µg / ml for different species. Similar to the growth of the cyanobacterium, nitrogen fixation was also inhibited by increasing concentrations of different herbicides, but 2,4 D (100 µg/ml) stimulated the acetylene reduction. The 2,4 D at 1500 µg / ml was found to inhibit the nitrogen fixation more or less completely. It is presumed that inhibition of growth and nitrogen fixation at higher concentrations of 2,4 D may be due to inhibition of photosynthesis.

The present observations on 2,4 D stimulation of cyanobacterial nitrogen fixation growth, provide evidence to suggest that 2,4 D at field doses (10-40 ppm) may be serving doubly advantageous eradicating weeds as well as in stimulating nitrogen fixing growth of paddy soil cyanobacteria, which are used as sources of biofertilizers in paddy fields nowadays. Growth inhibition of the cyanobacterium at higher doses of 2,4 D which are many fold higher than the field doses is useful in understanding the interactions of herbicides with cyanobacteria and with other chemicals available in nature (45).

III. MATERIALS AND METHOD

3.1. MATERIALS

3.1.1. CULTURES

Two algal species *Anabaena cylindrica* and *Gloeocapsa* were obtained from Gottingen Institute, Germany.

3.1.2. CHEMICALS

2,4- dichlorophenoxy acetic acid (Koruma A.Ş.) and methyl parathion (Polyscience Corporation "Analytical Standarts" of U.S.A.) were a kind gift of Prof. Dr. Yüksel İnel.

All other chemicals used in the preparation of the medium were purchased from Merck, Darmstadt, Germany; Fisher Scientific Company, New Jersey, U.S.A; Riedel-De Höen AG, Germany; Proses Kimya Sanayi ve Tic. A.Ş. İstanbul, Turkey.

All gases were obtained from HABAŞ, Topkapı, İstanbul.

3.2. METHODS

3.2.1. GROWTH OF CULTURES

Gloeocapsa sp. and *Anabaena cylindrica* were grown in 3,75 l. of sterile

medium free of combined nitrogen. The medium was slightly modified ASM-1 medium (10) (Table 3.1). The pH of the medium containing the required chemicals was adjusted to 7.5 - 7.6 by the addition of solid $NaHCO_3$.

Cells were grown under continuous illumination of 2000 lx at 25 ± 2°C. The cells were bubbled contiuously with filtered air.

3.2.2. PREPARATION OF TEST CULTURES

One herbicide (2,4-D) and one insecticide (m-parathion) were chosen as test chemicals. The application of these pesticides into the algal species varied according to their solubility properties. The methyl parathion stock solution was prepared in acetone whereas 2,4-D was used both as a solid and as a solution prepared in acetone. The different concentrations of these pesticides were studied by their addition to 750 ml. algal cultures, keeping one as a control (Table 3.2).

3.2.3. NITROGENASE ASSAY WITH INTACT CELLS OF *GLOEOCAPSA* AND *ANABAENA CYLINDRICA*

Nitrogen fixation by algal cultures was measured by the acetylene reduction technique. Unless otherwise stated, assays were performed aerobically at 25 °C under illumination of light in an orbital shaker.

During the exponential phase of growth, cells containing the different amount of pesticides were harvested and 25 ml portions were transferred to 125 ml. Erlenmeyer flasks. The flasks were then sealed with suba-seals, acetylene (1% v/v) was added, then after a further 60 minutes of incubation, a 1 ml sample was removed and ethylene formed was measured by gas chromatography. The Schimadzu GC-8A Gas Chromotograph was equipped with a hydrogen flame

Table 3.1. Components of the algal medium

	Compound	Amount (μ mol / L)
Macronutrients	NaCl	2000
	$MgSO_4$	150
	$MgCl_2$	145
	$CaCl_2$	190
	K_2HPO_4	100
	Na_2HPO_4	100
Micronutrients		
Soln A :		
	H_3BO_3	6
	$FeCl_3$	9
	$MnCl_2$	6
	$ZnCl_2$	3
	Na_2EDTA	20
Stock solutions :		
	MoO_3	0.1
	$CuCl_2$	0.0008
	$CoCl_2$	0.08

Table 3.2. Final concentrations of pesticides used on *algae* (ppm)

2,4-D	Methyl Parathion
100	1
125	5
150	10
175	50
200	100

Table 3.3. GC Operation Conditions

Detector Temparature	180 °C
Column Temperature	110 °C
Nitrogen inlet pressure	1 kg/cm^2
Hydrogen inlet pressure	0,6 kg/cm^2
Air inlet pressure	0,9 kg/cm^2

ionization detector and Porapak N Column. The operating conditions are given in Table 3.3.

Ethylene peaks on the recorder tracings were identified by retention time and quantified by the area calculation method.

3.2.4. GROWTH MEASUREMENTS

Growth of algal cultures was measured spectrophotometrically at 600nm. using a Schimadzu UV Spectrophotometer 120-01. Effects of the pesticides on the growth were studied in terms of a change in the optical density. The optical density of each sample was measured three times and the average value was considered.

IV. EXPERIMENTAL RESULTS AND DISCUSSION

4.1. EXPERIMENTS WITH 2,4-D ON *ANABAENA CYLINDRICA*

The test material 2,4-D was used as a solid since its water solubility was acceptable to some extent. Different amounts of the herbicide, 2,4-D, were added into the already prepared algal medium and the effects of 2,4-D on growth and nitrogen fixation were examined.

i) GROWTH

Fig. 4.1 shows the effect of different concentrations of 2,4-D on the growth of *Anabaena cylindrica* in different days of incubation. 2,4-D was used at concentrations of 50, 100, 150, 200 and 500 ppm in *Anabaena cylindrica.*

50 ppm 2,4-D appeared to be slightly effective on the algal growth during the period of incubation. The inhibition of growth reached to maximum value (60%) on the third day, but it seemed to reduce approximately to 35% in the following days. Interestingly, on the eighth day, 50 ppm 2,4-D concentration showed stimulative effect on the growth of *Anabaena cylindrica* by 11%.

100 ppm 2,4-D was found to inhibit the algal growth initially (73% inhibition during the first three days), but the inhibition was short lived. After the day seven, an increase in optical density values was observed. The algal growth inhibition was found to be reduced from 64% to nearly 30% after the seventh day. As can

be seen from the Figure 4.1, absorbance values of 100 ppm exceeded the values that of 50 ppm after the nineth day, for example on the tenth day, the inhibition for 50 ppm was 44%, whereas only 34% growth inhibition was observed for 100 ppm.As a result, *Anabaena cylindrica* was found to be more resistant to 2,4-D at 100 ppm than 50 ppm.

An increase in the concentration of 2,4-D in the medium was found to inhibit the growth. 150 ppm 2,4-D inhibited the growth by 85% at the beginning , however the inhibition decreased to nearly 55% till the seventh day. The effect of 150 ppm 2,4-D was comparable with 50 ppm 2,4-D on the nineth day (33% inhibition for 150 ppm, 35% inhibition for 50 ppm was calculated), whereas for 50 ppm and 150 ppm the growth was inhibited by 16% and 40% respectively on the 13^{th} day.

The increase in concentration to 200 ppm and 500 ppm further decreased the growth. Maximum inhibition of 94% and 91% was observed on the 13^{th} day by 200 ppm and 500 ppm 2,4-D respectively. Figure 4.1 clearly indicates that these concentrations of 2,4-D were found to be effective in complete growth inhibition of *Anabaena cylindrica* .

ii) NITROGENASE ACTIVITY

2,4-D at 50 ppm inhibited nitrogenase activity of the algal cultures by about 50% during the seven days of assay (Table 4.1). Maximum inhibition by 57% was observed on the seventh day, however, it was reduced to 24% on the eighth day. At the end of the experiment, inhibition of nitrogenase by 36% was noticed on the 16^{th} day of incubation.

100 and 150 ppm 2,4-D concentrations were found to inhibit the nitrogenase activity about 70% on the third day, but the value decreased to about

30% on the sixth day. However, inhibition reached to approximate value of 54% for 100 ppm and 55% for 150 ppm on the seventh day of treatment. In contrast to the growth measurements, nitrogenase was found to be more sensitive to 100 ppm rather than 50 ppm, since percent inhibition of nitrogenase appeared to be higher for 100 ppm. Acetylene Reduction Assay results proved that the nitrogenase activity was dose dependent, because the increase in concentration from 50 to 100 and to 150 ppm 2,4-D caused a remarkable inhibition of the nitrogenase activity.

Inhibition by 200 and 500 ppm 2,4-D was pronounced from the beginning of inoculation. As the table 4.1 indicates, complete inhibition of nitrogenase activity by 2,4-D was very obvious for 200 and 500 ppm.

Although nitrogen fixation is regarded as a growth-linked process, no significant correlation of growth and nitrogen fixation was observed for 50 and 100 ppm, 2,4-D concentrations, since the algae were found to be more resistant on growth to 100 ppm than 50 ppm but adversely, nitrogenase was affected from 100 ppm much more than 50 ppm. However, the true correlation of growth and nitrogen fixation was acceptable for the other concentrations (150, 200 and 500 ppm).

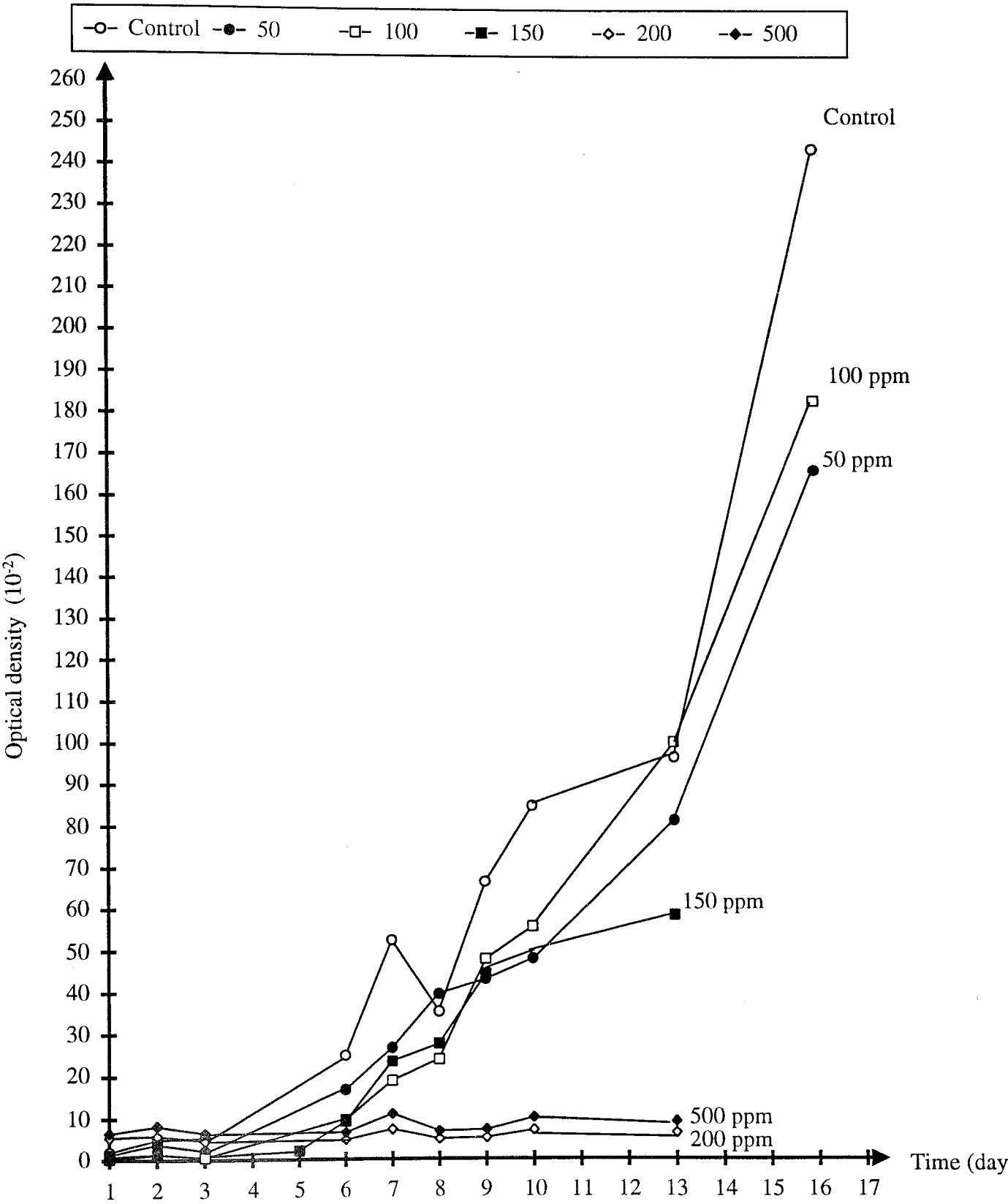


FIGURE 4.1. Effect of 2,4-D on the growth of *Anabaena cylindrica*

TABLE 4.1. Percent of ethylene produced by *Anabaena cylindrica* in the presence of 2,4-D

(The below values represent percentages based on the ethylene peak areas. Percentages are calculated by taking the control peak area as 100 %)

Time (days)	Concentrations (ppm)				
	50	**100**	**150**	**200**	**500**
3	51.16	30.23	29.95	11.62	10.46
6	69.84	74.6	68.18	3.70	2.82
7	43.26	46.15	45.40	2.24	1.70
8	76.11	65.62	57.58	5.55	4.16
16	63.60	46.91	40.82	~0	~0

4.2 EXPERIMENTS WITH 2,4-D ON *GLOEOCAPSA*

i) GROWTH

The Figure 4.2 clearly indicates the effect of solid 2,4-D introduced to cultures at different concentrations such as 100, 125, 150, 175 and 200 ppm.

100 ppm 2,4-D inhibited the growth of *Gloeocapsa* by around 40% during the assay period. At the day of inoculation (zeroth day) the optical density of 100 ppm was found to be higher than that of the control (0.0473 for the control, 0.0490 for 100 ppm). The minimum inhibition by 25% was observed on the fourth day and the maximum growth inhibition by 54% was pronounced on the 11^{th} day of treatment.

125 ppm 2,4-D was less effective than 100 ppm 2,4-D on the growth of algal culture. The average inhibition was found to be 28% and the maximum inhibition was calculated as 40% on the 11^{th} day.

As the Figure 4.2 indicates, 150 ppm 2,4-D appeared to be ineffective on the growth of *Gloeocapsa*. The average inhibition was found to be 11 % during the time of assay.

The effect of 175 ppm 2,4-D on the growth of *Gloeocapsa* was similar to that of 150 ppm. The average inhibition by 12% was calculated from the optical density results. Maximum inhibition was found to be 34% on the sixth day of assay.

In addition, as can be seen from the Figure 4.2, 200 ppm 2,4-D had markedly inhibited the growth. Maximum inhibition by 71% was pronounced on the 13^{th} day of treatment. In fact, on the seventh day bleaching of the cells was observed. This observation and the absorbance results indicated that, 200 ppm 2,4-D was toxic for the growth of *Gloeocapsa*.

ii) NITROGENASE ACTIVITY

Nitrogen fixation is measured by acetylene reduction technique and the results obtained from the chosen concentrations of 2,4-D are tabulated in Table 4.2.

At 100, 125 and 150 ppm 2,4-D concentrations, nitrogenase activity was neither stimulated nor inhibited. In contrast to the growth of *Gloeocapsa* at 100 and 125 ppm 2,4-D , nitrogen fixation was not affected significantly. As the table indicates, the amounts of ethylene produced were similar to, or above, control readings.

The inhibition of nitrogenase activity was remarkable with 175 ppm and 200 ppm 2,4-D . Nitrogen - fixing capacity of the cyanobacteria was halved until the seventh day of incubation.

One can conclude that, algal growth has completely stopped after seven days (Figure 4.2) and bleaching was observed with 200 ppm 2,4-D; however complete inhibition of nitrogenase activity was not pronounced during the time of assay.

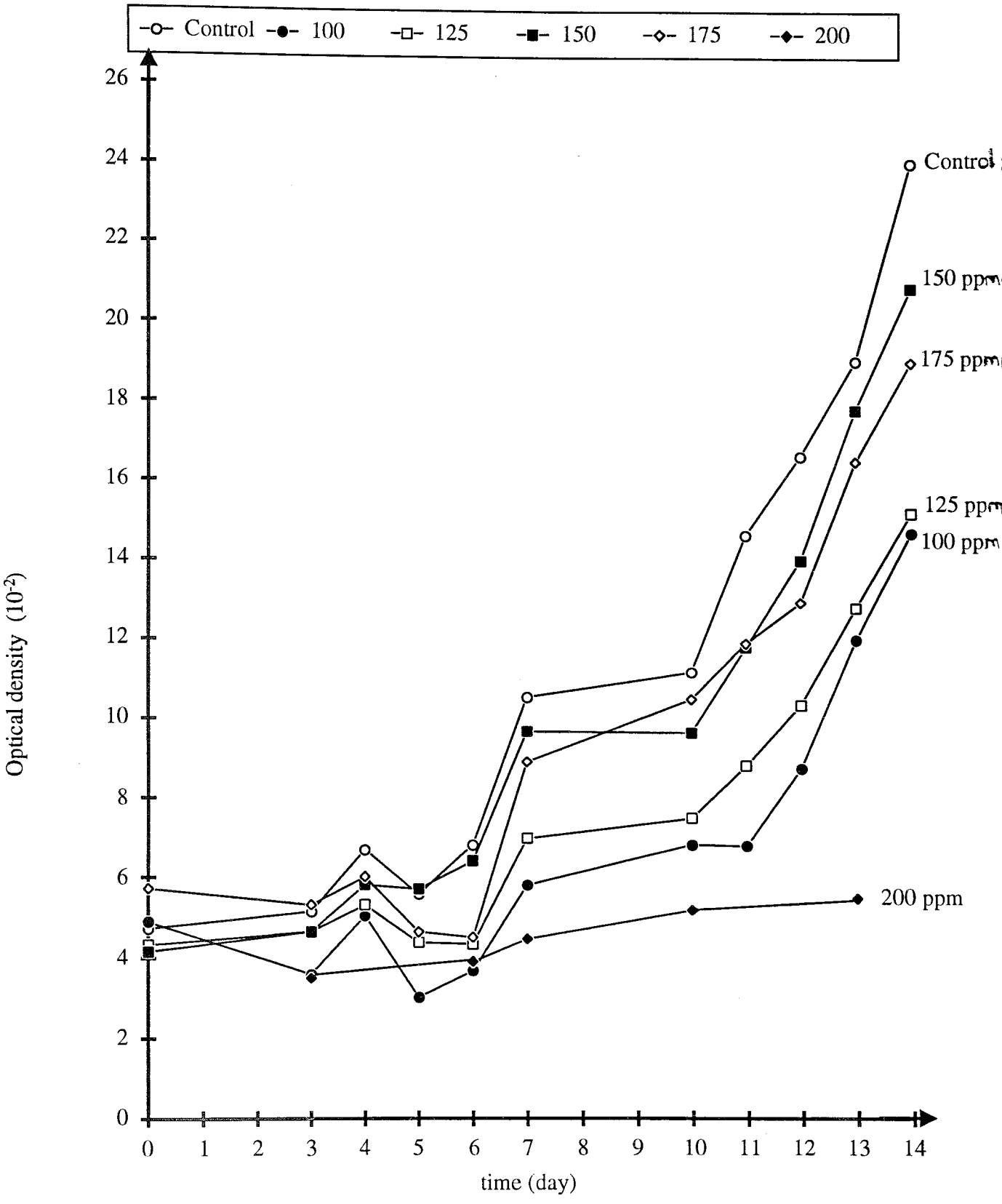


FIGURE 4.2. Effect of solid 2,4-D on the growth of *Gloeocapsa*

Table 4.2 Percent of ethylene produced by *Gloeocapsa* in the presence of 2,4-D

(The below values represent percentages based on the ethylene peak areas. Percentages are calculated by taking the control peak area as 100 %)

Time (days)	Concentrations (ppm)				
	100 ppm	125 ppm	150 ppm	175 ppm	200 ppm
1	97.82	80.43	73.91	70.92	28.53
2	100.30	105.20	80.50	51.08	66.87
3	91.73	82.30	90.15	58.46	71.95
6	90.00	76.47	56.47	41.76	43.88
7	81.42	88.70	68.05	64.28	83.64
8	94.40	99.69	94.40	44.40	83.30
9	80.00	100.00	115.00	-	90.00
10	98.05	95.09	103.62	86.36	84.21
11	120.00	112.50	-	93.11	89.28
12	89.83	110.69	96.25	86.63	89.83
13	100.00	96.00	-	114.00	-
14	95.00	-	-	-	-

4.3 EXPERIMENTS WITH 2,4-D IN ACETONE ON *GLOEOCAPSA*:

In this set of experiments, 2,4-D is dissolved in acetone and the concentrations are adjusted to 100, 125, 150, 175 and 200 ppm.

i) GROWTH

The response of *Gloeocapsa* to different concentrations of 2,4-D is shown in Figure 4.3.

Growth of the algae exposed to 100, 125,and 150 ppm was not affected significantly. Following the graph, it is observed that 150 ppm 2,4-D concentration was less effective than 100 and 125 ppm 2,4-D. The average growth inhibitions calculated for 100, 125 and 150 ppm 2,4-D concentrations were 17%, 27%, and 11% respectively. Optical density measurements for 150 ppm were higher than those of 100 ppm and 125 ppm.

The results for 175 and 200 ppm 2,4-D concentrations appeared on the Figure 4.3, indicate that the growth of *Gloeocapsa* is generally more sensitive to these concentrations than the others. The growth inhibition by 50% for 175 and 200 ppm 2,4-D was noticed till the end of the eighth day. After that day growth of the cultures were completely inhibited. The inhibition was 75% for 175 ppm on the 12^{th} day. After the eighth day onwards, bleaching of the algal cells was observed with 175 and 200 ppm 2,4-D.

ii)NITROGENASE ACTIVITY

The results obtained from the acetylene reduction studies in these set of experiments are given in Table 4.3.

At the beginning of the assay period, nitrogenase activity was not affected significantly in the case of 100 ppm 2,4-D. After the fifth day onwards, inhibition reached to an average value of 30%. In contrast to the optical density measurement results for 100 ppm 2,4-D, nitrogenase activity appeared to be slightly inhibited, and never diminished during the time of assay.

In the case of 125 ppm 2,4-D the average inhibition of nitrogenase activity was found to be around 42%. During the first five days , nitrogenase was inhibited only by 20%.

As the table indicates, increase in concentration of 2,4-D caused an increase in the percent inhibition of nitrogenase. In the case of 150 ppm, inhibition was more remarkable than 100 and 125 ppm and found to be around 50% during the time of assay.

Similar to the growth measurements 175 and 200 ppm 2,4-D caused an inhibition on nitrogenase activity for the first six days, the inhibition was about 50% and after the seventh day onwards, inhibition of nitrogenase was more pronounced by reaching to about 90%. At that time, bleaching of the algal cells was also observed.

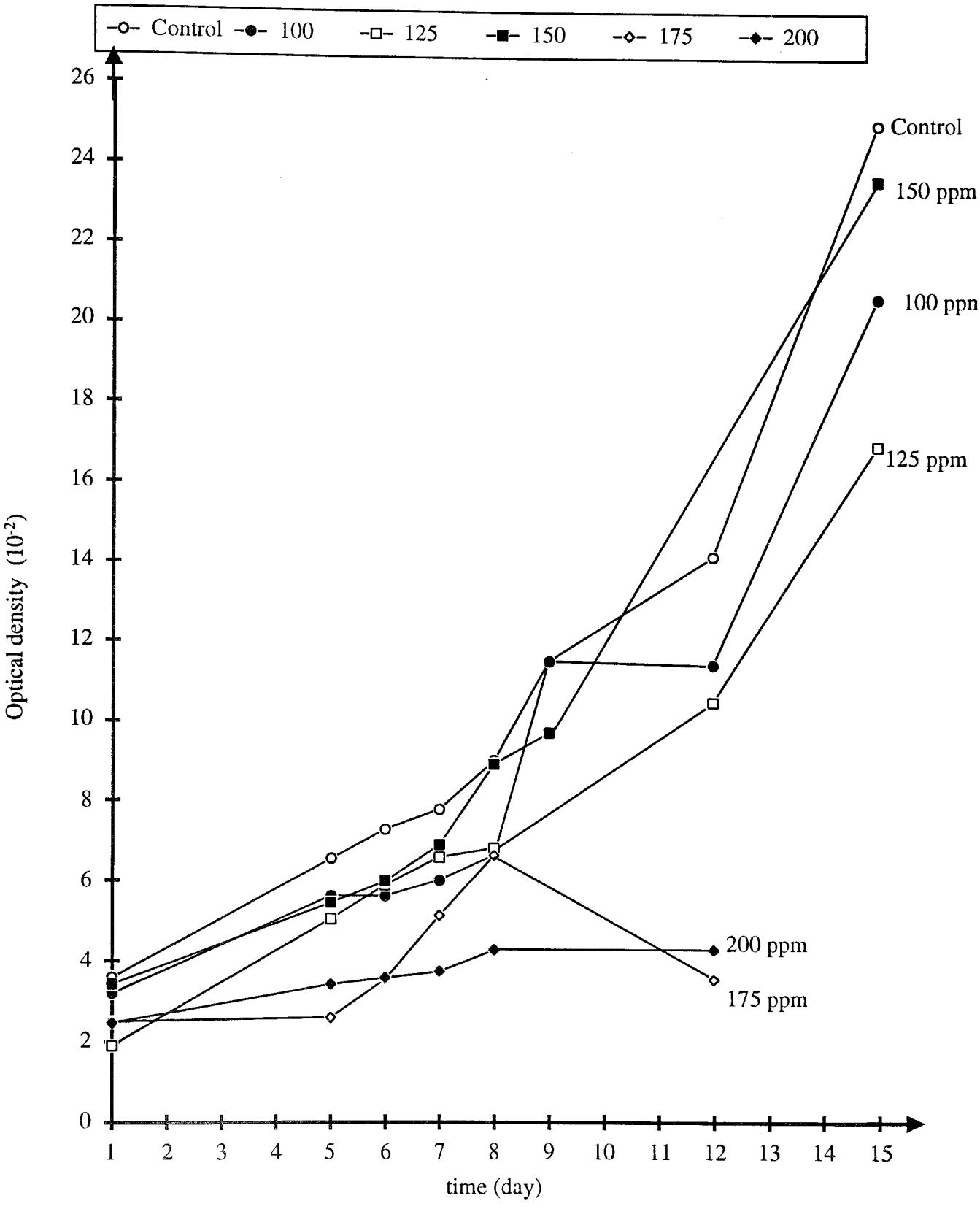


FIGURE 4.3. Effect of solid 2,4-D, dissolved in acetone, on the growth *Gloeocapsa*

Table 4.3. Percent of ethylene produced by *Gloeocapsa* in the presence of 2,4-D in acetone.

(The below values represent percentages based on the ethylene peak areas. Percentages are calculated by taking the control peak area as 100 %)

Time (days)	Concentrations (ppm)				
	100	**125**	**150**	**175**	**200**
1	90.00	85.00	67.50	62.30	47.80
5	108.30	75.00	62.30	50.00	40.73
6	77.70	61.25	61.10	40.55	37.72
7	66.60	52.94	38.80	30.28	28.93
8	85.29	47.00	36.90	28.75	16.54
9	56.66	41.25	30.00	-	-
12	74.05	49.13	73.50	17.09	8.55

4.4. EXPERIMENTS WITH METHYL PARATHION ON *GLOEOCAPSA*

Regarding to the concentrations mentioned in literature, and to the values that are observable in the environment 1,5,10,50 and 100 ppm methyl parathion concentrations have been chosen. The test material methyl parathion stock solution was prepared in acetone.

i) GROWTH

The response of *Gloeocapsa* to the concentrations chosen is shown in Figure 4.4.

Growth of the algae exposed to various concentrations of methyl parathion was not affected significantly as in the case of 2,4-D.

As can be seen from the figure, 1 and 5 ppm methyl parathion had no effect on the growth of the algae. Especially, 1 ppm appearad to be stimulating the growth. Optical density measurements were found to be very close to those of the control values at the beginning and started to rise after the eighth day reaching to high levels. The stimulation was around 5 % till the end of assay period. 5 ppm methyl parathion was slightly effective on the growth of *Gloeocapsa*. Average inhibition obtained from the absorbance results was found to be around 7 %.

In 10 ppm methyl parathion introduced culture medium, it was observed that, growth inhibition was not pronounced significantly. Increasing the concentration from 5 ppm caused an increase in inhibition by around 12 %.

As the figure indicates, 50 and 100 ppm methyl parathion concentrations were also ineffective on the inhibition of growth. During the time of assay, the

remarkable inhibition for 50 and 100 ppm methyl parathion concentrations were 23 % and 24 % respectively. On the seventh day, the maximum inhibition was pronounced for both concentrations by 51 % and 49 % respectively. From the eighth day onwards, color change in algal medium, from green to yellow, was noticed. However, optical density values for 50 and 100 ppm methyl parathion concentrations appeared to increase, showing that the growth of the cells never stopped.

ii) NITROGENASE ACTIVITY

The results obtained from the acetylene reduction studies were given in Table 4.4.

It is found that the blue-green algae *Gloeocapsa* was quite tolerant to 1 ppm methyl parathion. Similar to the growth measurements, (Figure 4.4.) nitrogenase activity seemed not to be inhibited at this concentration. The percent of ethylene produced was found to be around 96 in the presence of 1 ppm methyl parathion. Inhibition of nitrogenase was more pronounced after 13 days of inoculation reaching to a value about 30 %.

5 ppm methyl parathion introduced algal cultures were found to be affected much more than that of the 10 ppm insecticide added cultures during the first five days. The inhibition of nitrogenase activity was around 50% for 50 ppm whereas 20 % inhibiton was pronounced for 10 ppm methyl parathion. Average inhibiton of nitrogenase activity for 5 ppm was found to be about 40 % during the time of assay. After the day sixth onwards, inhibition started to increase for 10 ppm reaching to 60% till the end of the tenth day.

Following the Table 4.4, it was observed that, *Gloeocapsa* in the presence of 50 and 100 ppm methyl parathion lost its nitrogenase activity till the end of the tenth day of assay. Those concentrations were effective at nearly same levels in the inhibition of nitrogenase. Average inhibiton by 71% was pronounced for 50 ppm and 74 % inhibition was due to 100 ppm methyl parathion. These values reached to 90 % inhibition at the end of assay,

In contrast to growth, 50 and 100 ppm methyl parathion appeared to have an inhibitory effect on the nitrogenase activity in *Gloeocapsa.*

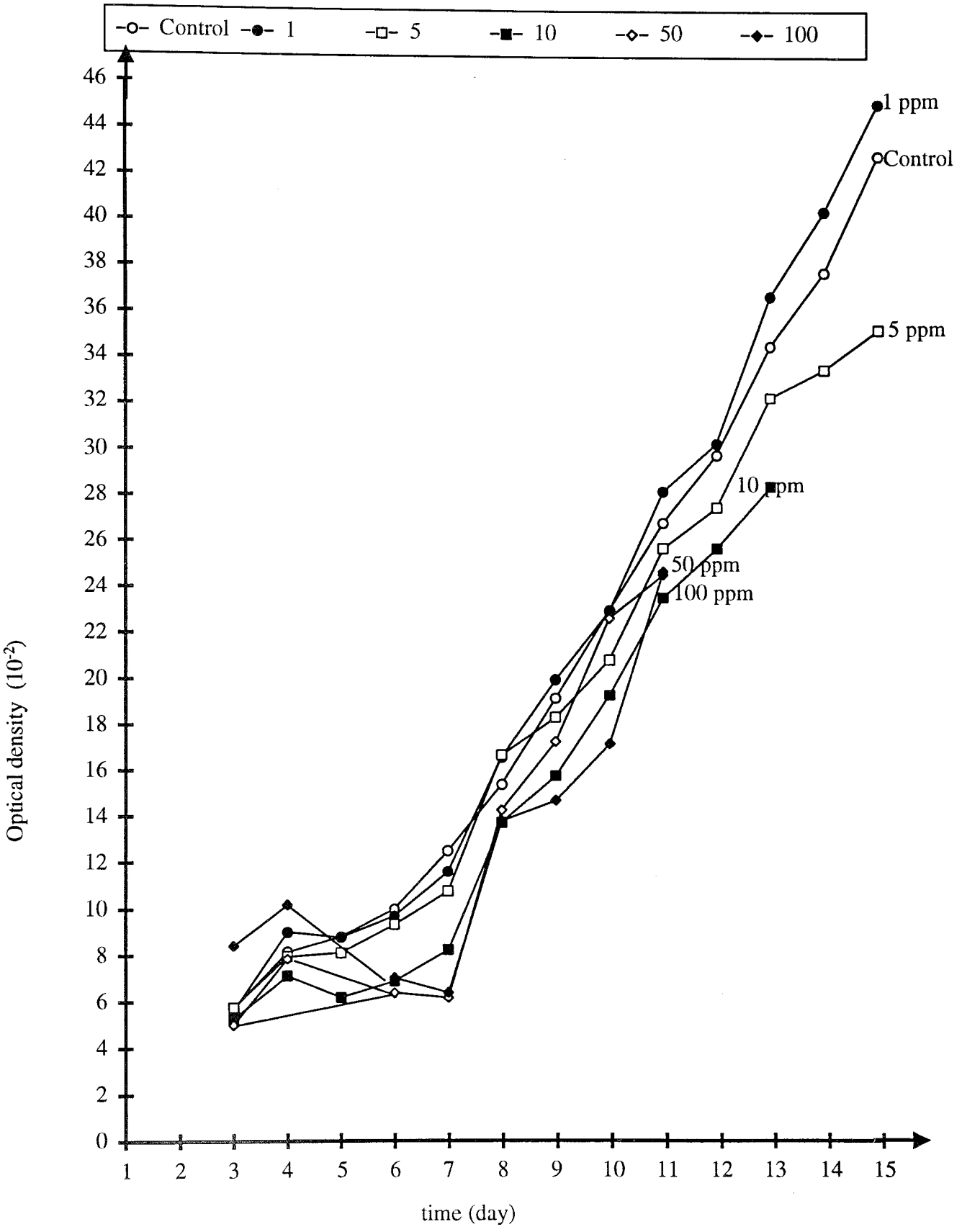


FIGURE 4.4. Effect of m-parathion on the growth of *Gloeocapsa*

TABLE 4.4. Percent of ethylene produced by *Gloeocapsa* in the presence of methyl parathion.

(The below values represent percentages based on thhe ethylene peak areas. Percentages are calculated by taking the control peak area as 100 %)

Time (days)	Concentrations (ppm)				
	1	5	10	50	100
3	97.00	31.79	81.52	14.67	19.56
4	75.21	50.00	84.61	61.53	35.89
6	153.00	90.00	68.99	39.23	41.09
7	80.56	56.16	49.61	33.57	34.85
8	91.10	42.35	28.88	27.19	21.17
9	88.80	70.83	54.30	22.20	22.20
10	89.10	73.80	36.90	9.00	7.20
11	137.50	133.30	~0	~0	~0
13	92.05	54.54	~0	~0	~0
14	86.72	78.36	~0	~0	~0
15	66.60	59.26	~0	~0	~0

V. CONCLUSION

The work presented in this thesis, attemps to find the effects of the two pesticides on the two algal species called *Anabaena cylindrica* and *Gloeocapsa*. Furthermore, the relationship between nitrogen fixation and growth of the blue-green algae is discussed.

Photosynthetic nitrogen-fixing cyanobacteria are known to maintain the fertility of the soils and water-logged paddy fields by fixing atmospheric nitrogen (59,60). Such ecosystems provide most suitable conditions for healthy growth of these cyanobacteria as principal components.

The increasing use of herbicides in paddy fields, particularly 2,4-dichlorophenoxyacetic acid (2,4-D), is likely to create microbial imbalance by affecting the growth and the development of algae and thus, causing a great drawback to the nitrogen status of the soil.

The test herbicide 2,4-D, which is a synthetic growth hormone analogue, is commonly investigated for its toxicity and found to be a stimulator of growth and heterocyst formation and also nitrogen fixation up to 100 ppm concentration (45).

In this thesis, the use of 2,4-D on *Anabaena cylindrica* and on *Gloeocapsa* exhibited certain effects on growth and nitrogenase activity. However, the results differed from most of the previous reports that are related to the toxicity of the herbicides to different cyanobacteria with respect to their growth and nitrogen fixation. In the first set of experiments, 2,4-D as a solid, was added to the medium containing *Anabaena cylindrica* cultures. The findings showed that all

concentrations from 50 ppm to 500 ppm were more or less growth inhibitors. In contrast to the previous reports no stimulation was observed in this work. Although the algae called *Nostoc linckia* was found to be tolerant to 2,4-D up to 1500 ppm (45), even 200 ppm 2,4-D inhibited the growth of *Anabaena cylindrica.*

The effect of 2,4-D on nitrogen fixation was more remarkable. Nitrogenase activity was inhibited by increasing concentrations of the herbicide. The 2,4-D at 200 ppm and 500 ppm was found to inhibit nitrogen fixation by *Anabaena cylindrica* almost completely. Therefore, the toxicity of 2,4-D on these algae was clearly observed. It is presumed that, inhibiton of growth and nitrogen fixation at higher concentrations of 2,4-D may be due to the inhibition of photosynthesis, since the 2,4-D has been reported to reduce the photosynthetic evolution of oxygen (61).

When the 2,4-D was used on another species of blue-green algae namely *Gloeocapsa* some differences in growth and nitrogen fixation results were observed. 150 ppm and 175 ppm 2,4-D had no serious effect on the growth of *Gloeocapsa*, whereas 100 ppm and 125 ppm 2,4-D were more effective. At 200 ppm 2,4-D the results were comparable with each other, since growth inhibiton at this concertration was remarkable for both *Anabaena cylindrica* and *Gloeocapsa.*

Nitrogen fixation was not affected seriously by 100, 125 and 150 ppm 2,4-D. At 175 and 200 ppm nitrogenase activity was halved in the beginning but after the seventh day onwards the decrease in the inhibition of nitrogenase was pronounced for these concentrations. Interestingly bleaching was also observed at 200 ppm, although nitrogenase activity seemed not to be inhibited completely. The bleaching that occurred at 200 ppm may be because of the interference of the herbicide 2,4-D with chlorophyll synthesis, which disturbs the ratio between

chlorophyll and phycobilins possibly explains the colour change in *Anabaena cylindrica*.

The present observations on 2,4-D provide evidence for suggesting that 2,4-D at field doses (10-40 ppm) (46) may be advanageous in eradicating weeds as well as stimulating nitrogen-fixing growth of paddy soil cyanobacteria which are nowadays used as sources of biofertilizers in paddy fields. Growth inhibition of the cyanobacterium at doses of 2,4-D which are higher than the field doses is useful in understanding the interactions of herbicides with other chemicals freely avaiable in nature.

The application of pesticides for plant protection and their persistent residues have direct effect on algae. Although information is avaiable about the toxic effects of insecticides on the growth of algae, their influence on metabolic activities, such as photosynthesis and nitrogen fixation has been little studied. The present study was carried out in order to find out the effects of methyl parathion on growth and nitrogen fixation by *Gloeocapsa*.

Experimental results indicated that methyl parathion was stimulative at 1 ppm and ineffective at the other concentrations. The maximum inhibition of growth was observed by about 50 % for 50 ppm and 100 ppm concentrations, throughout the incubation period. Complete colour change from green to yellow occurred in the cultures of *Gloeocapsa* at 50 and 100 ppm after the eighth day. Interestingly, optical density values appeared to increase. Knauf and Shulze (1973) (62) attributed changes of the algal medium in the presence of organophophorus insecticides to the presence of sulphur molecules. Therefore, the colour change from green to yellow in this study, may be due to sulphur which is present in methyl parathion (Figure 2,3).

Murray and Guthrie (1980)(63) observed that organophosphorus insecticides appear to inhibit algal growth initially, but the inhibition is usually short lived, with the algae eventually returning to control levels. The present findings were in accordance with this observation and methyl parathion appeared to be safe to *Gloeocapsa* when used at low concentrations.

From the point of view of nitrogen fixation, it was found that similar to the optical density results, 1 ppm methyl parathion had no effect. However, the inhibition of nitrogenase activity was dose dependent. In contrast to the growth of *Gloeocapsa,* nitrogenase activity was found to be affected seriously by 50 and 100 ppm methyl parathion concentrations.

Toxic effects of insecticides on the growth of algae depend on the type of algal species, insecticide concentration and time of exposure. The sensitivity of blue-green algae to insecticides depends on the type and the nature of the insecticide, the nature of the organisms and the experimental conditions. Although organophosphorus insecticides do not persist and are highly biodegradable, their persistence, even for a short period, can be injurious to algae.

In this study, with the experimental conditions being kept constant, the data on the toxicity of 2,4-D as a herbicide and methyl parathion as an insecticide indicate that the latter is more toxic for algae than the former. Though nitrogen fixation is regarded as a growth-linked process, no correlation of growth and nitrogen fixation was observed in some parts of the study, suggesting that different pesticides may effect specific metabolic activities (e.g nitrogen fixation), while adversely affecting the growth.

Pesticides even in microgram amounts, can have an effect on the growth of cyanobacteria. Since the photosystems of the cyanobacteria are similar to those of higher plants, chemical agents which are toxic to cyanobacteria should also be toxic to plants. Cyanobacteria are fairly inexpensive to obtain and to grow compared with higher plants. Therefore, effects of chemical agents could be investigated with cyanobacteria before testing pesticides on higher plants.

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