BIOCHEMICAL AND BIOTECHNOLOGICAL STUDIES ON THE CYANOBACTERIUM *SYNECHOCYSTIS SALINA* WISLOUCH

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By

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Dedicated to
the Glory of Almighty

CERTIFICATE

This is to certify that the thesis entitled "**Biochemical and Biotechnological Studies on the Cyanobacterium *Synechocystis salina* Wislouch**" submitted herewith by Mr.M.S.Francis is an authentic record of research work carried out by him in the Division of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Kochi - 16, under my supervision and guidance in partial fulfilment of the requirements for the award of Ph.D. degree of Cochin University of Science and Technology and that no part there of has been presented before for any other degree in any University.

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DECLARATION

I here by declare that the thesis entitled **"Biochemical and Biotechnological studies on the Cyanobacterium *Synechocystis salina* Wislouch"** is an authentic record of research work carried out by me under the supervision and guidance of **Prof. Dr. Babu Philip**, in partial fulfilment of the requirements for the award of the Ph.D. degree in the Faculty of Marine Sciences, Cochin University of Science and Technology and that no part of it has previously formed the basis for the award of any degree, diploma or associateship in any University.

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CONTENTS

CHAPTER 1

1. GENERAL INTRODUCTION

1.1 SCIENTIFIC BACKGROUND

Algae are photosynthetic nonvascular plants which contain chlorophyll-a and have simple reproductive structures. The group comprises some 1800 genera and 21,000 species and includes unicellular forms (such as Chlorella), small colonial forms (such as Volvox) and large multicellular forms (such as sea weeds). The prokaryotic blue greens (Cyanobacteria) are often included in the group of algae though these are now recognized to be more closely allied to the bacteria than to the rest of the eukaryotic algae (Robinson et al., 1986).

The prospects and potentials of phycology in the changing scenario are reviewed by Michanek (1978) and Becker (1994). Marine algae are good sources of different metabolites and bioactive compounds (Blunden, 1988). They also have an important role in pharmaceutical sciences (Hoppe et al., 1979). Significance of immobilized algae in biotechnology is reviewed by Robinson et al. (1986). Many algal forms have been successfully tried as food and food additives and for effluent treatment (Venkataraman and Becker, 1985; Becker, 1994).

1.2 BIOTECHNOLOGICAL APPLICATIONS OF CYANOBACTERIA

The group cyanobacteria includes a large number of organisms characterized by a low state of cellular organization. Their cells

lack a well defined nucleus. Cell division is by division of protoplast by an in growth of the septum. These organisms are characterized generally by a blue green coloration of the cell, the chief pigments being chlorophyll-a, carotenes, xanthophylls, C phycocyanin and C phycoerythrin. The product of photosynthesis is glycogen. These organisms lack flagellate reproductive bodies and there is a total lack of sexual reproduction (Desikachary, 1959). They are also unique because of the presence of murein in the place of cellulose (cell wall) and the absence of chloroplast, mitochondria and endoplasmic reticulum (Venkataraman and Becker, 1985).

Their distribution in different environmental conditions and association with other organisms are quite surprising. Cyanobacteria are of world wide distribution and in general, the more inhospitable the habitat is, the more likely it is that blue green algae will be important components (Fogg et al., 1973). They are reported from temperate areas (Lund, 1967) and from arid soils of tropical and subtropical regions (Fogg et al., 1973; Watanabe, 1959). Cyanophycean members are found in close association with Bacteria, Protozoa, Fungi, Bryophytes, Pteridophytes, Gymnosperms, Angiosperms and multicellular animals (Carr and Whitton, 1973). Affinity for sapropelic habitat and parasitic mode of nutrition is also noticed in some forms (Desikachary, 1959). Cyanobacteria are now successfully employed in different applied fields. They are:

1. **As a biofertilizer**

Many Cyanobacteria are so unique in having the ability to fix atmospheric nitrogen. The importance of nitrogen fixing blue green algae was first recognized by De (1936, 1939). The literature available in this field is appraised by Goyal (1987) and Metting (1992).

2. **As a source of Single Cell Protein (SCP) and Single Cell Oil (SCO)**

Cyanobacteria are very good sources of SCP and SCO. The protein and lipid ratio is very high in these organisms. The probable use of algal cells as a source of SCO is reviewed by Fatma (1989), Borowitzka (1992 a), and Roessler (1990). The use of algae as SCP is reviewed by Becker (1992, 1994).

3. **As a pollution indicator and in effluent treatment**

Different Cyanobacterial strains can be isolated and maintained for effluent treatment specific to the industries (Venkataraman, 1983; Shubert, 1984; Palmer, 1980; Mannion, 1992; Oswald, 1992; Becker, 1994). Their ability to grow at higher pH makes them potential indicators of pollution.

4. **As a source of Biogas, Biomass and animal feed**

The biomass developed by treating the effluent can be used for the production of biogas, animal feed etc. (Becker 1994). Addition of wet algal mass (3-5%) to biogas digesters with cowdung or poultry

dropping has been reported to enhance methanogenesis considerably which results in increased biogas production (Venkataraman and Becker, 1985). The effluent from algal tank as well as wet algal slurry can be used as a feed in aquaculture. Different cyanobacterial strains have been tried as poultry feed (Venkataraman and Becker, 1985). According to Morse et al. (1984), the amenability of the Cyanobacteria to large scale cultivation, and to physiological and genetic manipulation makes them useful for production of metamorphic inducers of marine invertebrate larvae and for further studies of the synthesis, structure and mechanism of action of such inducing molecules. Production of algal biomass and their various uses have been summarised by Shelef & Soeder (1980).

5. **As sources of pharmacological principles**

Different cyanobacterial members have been screened for their therapeutic properties. Rats fed on spirulina showed reduced tissue cholesterol levels when compared to control animals (Venkataraman and Becker, 1985). Some cyanobacteria have been found to exhibit antibacterial (Cannell et al., 1988) and antifungal (Kellem et al., 1988) activities. A number of Cyanobacterial extracts are found to be active against AIDS virus (Becker, 1994). The use of blue green algae for mosquito control is also suggested (Purdy, 1925; Thiery et al., 1991).

6. **As a source of growth promotors**

Recent studies have revealed that phycocyanin, extracted from Cyanobacteria can be used as a growth promoting agent in animal

tissue culture media for the production of monoclonal antibodies and interferons (Becker, 1994).

7. **As a source of various chemicals, colouring pigments, enzymes, vitamins etc.**

The utilization of various microalgae for the production of natural colouring pigments, vitamins, amino acids, enzymes and enzyme inhibitors is suggested by Bonotto (1988) and Borowitzka (1992 b).

8. **As organisms recovering valuable metals**

Cyanobacteria have been used to mobilize uranium from low grade ores in laboratory studies and this has encouraged investigations on the potential applications of cyanobacteria in processes designed to recover valuable metals by bioremediation (Lorenz and Krumbein, 1985; Garnham et al., 1993).

9. **As a tool for Immobilization technique**

Robinson et al. (1986) has reviewed the work done in this field.

Cyanobacteria have been successfully immobilised and the following processes are in progress using the technique:

i) Accumulation and removal of waste products in aqueous systems
ii) Production of ammonia
iii) Production of hydrogen
iv) Biosynthesis and biotransformation of different natural products etc. (Becker, 1994).

1.3 EXPERIMENTAL ORGANISM

Synechocytis salina Wislouch occur as small spherical cells of diameter 3 μ with bluish green colour. Rarely they occur as pairs. The species is characterised by jerky movement of the cells. (Desikachary, 1959). Sreesudha (1989) conducted ultrastructural studies on these organisms. She reported the presence of radial mucilaginous filaments projecting from cell surface. Within each cell the photosynthetic lamellae or thylakoids are arranged peripherally in 4-5 concentric layers running parallel to the envelope layer. On the outer surface of thylakoids regular rows of electron dense structures are closely attached. These granules carry the light harvesting phycobiliproteins - phycobilisomes. Cyanophycean granules are irregular in shape and larger in size especially in older cells. Apart from this are seen polyhedral and polyphosphate bodies. Small structural granules representing lipid inclusions are found scattered among thylakoids.

1.4 SCOPE OF THE PRESENT STUDY

Nanoplankters are essential food materials of almost all larval forms. The isolation, maintenance and mass culture of these micro algae is a prerequisite in the hatchery system throughout the world. They are likely to be of great importance as the chief food of molluscan larvae (particularly in the initial stages) which can ingest nothing larger than 10 microns (Gopinathan, 1984).

In the present work, the growth kinetics, heavy metal tolerance, tolerance mechanisms, antibacterial studies etc. on Synechocystis salina Wislouch is carried out for the potential biotechnological applications of this organism.

Being a nanoplanktonic euryhaline cyanobacterium, Synechocytis salina Wislouch possesses excellent biotechnological potentials. Thiery et al. (1991) suggested the significance of euryhaline nanoplanktonic cyanobacteria as probable recipients of bacterial genes that encode endotoxins against mosquito larvae. The use of nanoplankters in effluent treatment is another area that invites urgent attention. The ability of these micro organisms to be present in large numbers in limited volume exposing large surface area can be considered as a boon for effluent treatment, especially in removing and concentrating heavy metals. The biochemical mechanism by which the heavy metals are sequestered by the organism have been investigated in the present work.

The need for novel bioactive substances is increasing. Bacteria are becoming resistant against many antibiotics. Natural sources are being screened to identify and isolate effective novel antibiotics. In the present study, the antibacterial actions of bioactive substances derived from S. salina has been studied in detail.

CHAPTER 2

2. KINETICS OF GROWTH OF SYNECHOCYSTIS SALINA WISLOUCH

2.1 INTRODUCTION

Growth of a living organism is defined as an increase in mass or size accompanied by the synthesis of macromolecules, leading to the production of a new organised structure. Populations of organisms as in the case with unicellular algae may also be said to grow because increase in number of organisms by replication is accompanied by synthesis and a new organised structure is produced (Becker, 1994).

General populations of unicellular organisms may be measured in terms of either the number of individual cells or their mass. The former may be termed 'cell concentration' defined as the number of individual cells per unit volume, whereas the latter may be called 'cell mass' or 'cell density' defined as the weight of cells or biomass per unit volume. Under the typical regime of a simple homogenous batch culture, the algal growth passes through several different phases, such as

a. Adaptation (lag phase)
b. Accelerating growth phase
c. Exponential growth (log phase)
d. Decreasing log growth (linear growth)
e. Stationary phase
f. Accelerated death
g. Log death

The various phases represent the reaction of algal population to the change of the environmental conditions and depend on the inoculum, the actual cultivation method, nutrient concentration, light intensity, temperature etc. (Becker, 1994).

Although the basic knowledge on the role of planktonic micro algae in the economy of the sea is well recognized, information on the growth characteristics of the nanoplankters, especially in a culture system is still meagre (Gopinathan, 1984). According to him, the lack of knowledge on these organisms is largely due to the difficulty of making comparative observations and raising them on mass scale in a culture system. Realising the importance of these organisms as the essential food of almost all the larval forms, the isolation, maintenance and mass culture of these micro algae is a prerequisite in the hatchery systems, through out the world. They are likely to be of great importance as the chief food of the Molluscan larvae, particularly in the initial stages (Gopinathan, 1984) crustaceans and fish (Brown, 1991). The aminoacid and sugar composition of 16 species of micro algae used in mariculture was analysed by Brown (1991).

Previous work on the growth of marine microalgae has been summarised by Harvey (1955). In a subsequent paper Braarud (1945) has recorded the relative growth constant of many species grown in culture system under controlled conditions. Nair (1974) has reviewed the growth kinetics of several species of phytoplankters from the natural marine environment and Joseph and Nair (1975) have

studied the growth kinetics of three species of estuarine phytoplankters in a culture system. Droop (1983) made an extensive review on 25 years of algal growth kinetics. Algal growth characteristics are greatly influenced by physical and chemical factors such as light, temperature and the composition of the medium.

a. **Light**

Physiological acclimation to changes in light intensity and spectral quality is an important factor determining variations in photosynthetic responses and growth rates of algae in nature. (Falkowski, 1984 a; Richardson et al., 1983; Palmisano et al., 1985; Geider et al., 1986; Harrison and Platt, 1986; Cullen and Lewis, 1988; Cullen 1990). Morphologically it is accompanied by changes in cell volume, the number and density of thylakoid membranes (Post, 1985; Berner et al., 1989), the size of pyrenoids and other storage bodies within plastids (Sukenik et al., 1987 a), and sometimes by changes in the number of plastids per cell. On a cellular level, there are changes in pigment and lipid content and composition (Prezelin and Alberte 1978; Flakowski and Ownes 1980; Perry et al., 1981; Sukenik et al., 1989). On a physiological level there are changes in the minimum quantum requirement for photosynthetic oxygen evolution (Dubinsky et al., 1986), respiration (Falkowski et al., 1985; Geider et al., 1986; Langdon, 1987), and growth rate (Laws and Bannister, 1980; Post et al., 1984; Falkowski et al., 1985).

To some extent, all algae are capable of photoacclimation, including symbiotic dinoflagellates (Falkowski and Dubinsky, 1980), marine macrophytes (Henley and Ramus, 1989a; Gerard, 1988), free living dinoflagellates (Prezelin and Alberte, 1978), ice algae (Cota, 1985; Palminaso et al., 1985; Cota and Sullivan, 1990), diatoms (Perry et al., 1981; Falkowski et al., 1985), unicellular chlorophytes (Sukenik et al., 1987 a, b) and cyanobacteria (Raps et al., 1983; Kana and Gilbert, 1987). The process occurs on a time scale shorter than or comparable to a cells generation (Falkowski, 1984b; Post et al., 1985; Cullen and Lewis, 1988).

It is generally considered that cyanobacteria exhibit a growth rate that is proportional to the duration of the effective light period and intermittent illumination doesnot give better yields than continuous illumination (Foy et al., 1976; Fogg et al., 1973; Philips et al., 1989). There are also reports saying that light quality and quantity affect protein/carbohydrate ratio (Lorenzen & Hesse, 1974), growth, photosynthesis, nitrogen fixation and carbohydrate production (Philips et al., 1989), lipid composition (Roessler, 1990) and Lipid, carbohydrate, protein ratio (Falkowski and La Roche, 1991).

b. **Temperature**

This is a major factor controlling the rate of photosynthesis in all plants. The algae are an ideal group to study the

fundamental responses of photosynthesis to temperature free from complications such as stomatal CO_2 transport, inherent in more complex plants (Davison, 1991). In addition photosynthetic algae occur in the hottest and coldest environments in which autotrophic plants can be found. Arctic and antarctic ice algae achieve net photosynthesis at a constant temperature of $-2^{o}C$ (Palmisano et al., 1987; Michel et al., 1989) and antarctic soil algae continue to photosynthesize at temperatures as low as $-7^{o}C$ (Davey, 1989), whereas thermophilic hot spring cyanobacteria photosynthesize up to approximately $75^{o}C$ (Castenholz, 1969). Davison (1991) made an indepth review on the short term effects, phenotypic changes and genetic differences on photosynthetic response with respect to temperature. Many aspects of photosynthetic response to temperature in unicellular algae as well as the more general effect of temperature on algal growth have been reviewed by Li (1980), Geider (1987) and Raven & Geider (1988). Generally photosynthesis continues to increase up to an optimum temperature, beyond which it declines rapidly. But in many macro algae the maximum photosynthetic rates occur over a range of several degrees (Davison, 1991). Sheridan and Ulik (1976) found that temperature acclimation in Synchococcus lividus involves increased rate of electron transport in low temperature grown plants parallel with increase in photosynthetic activity. In the blue green algae, Anacystis nidulans and Synechococcus lividus, differences in the fatty acid composition of thylakoid membrane occur between plants grown at

different temperatures (Fork et al., 1979; Ono and Murata, 1979). According to Davison (1991), low temperature acclimated algae have high cellular activities of Calvin cycle enzymes and low contents of photosynthetic pigments, whereas the reverse is true in low-temperature grown plants. Algae from different temperature regimes exhibit differences in the kinetic properties of their photosynthetic enzymes. Descolas Gros and deBilly (1987) found that the maximum substrate affinity (minimum k_m) for Rubisco occurred at 4.5°C in the enzyme from antarctic diatoms and at 20°C in the enzyme from temperate species.

c. **Composition of the Medium**

Several factors like abundance of nitrogen and phosphorous, availability of elements like Mn, Mg, Cu and Fe, pH and salinity of the medium, dissolved CO_2 etc. influence the growth of the algae in the medium.

According to Vanlerberghe et al., (1990), as much as 50% of the algal carbon is integrally coupled with nitrogen metabolism. As the assimilation of N into protein requires both energy and organic carbon skeletons, it is not surprising that there are major interactions between N-assimilation and photosynthetic metabolism (Turpin et al., 1988). The most obvious effect of algal N-deficiency is the decline in nitrogenous photosynthetic pigments - chlorophylls and phycobilins (Plumley and Schmidt, 1989). Carotenoid/chl-a ratios increase dramatically under N-

limitation (Plumley et al., 1989). N-limitation also causes a reduction in thylakoid stacking and absorptivity (Plumley et al., 1989). In the cyanobacteria, N-deficiency causes little change in chl a/cell and major reduction in phycoblilin content (de Loura et al., 1987). This implies maintenance of PS II density and a decrease in the size of the antenna (Turpin, 1991).

Uptake of phosphate occurs via a rather specific transport system but arsenate can act as a competitive inhibitor (Nalewajko & Lean, 1980). Phosphate uptake is an energy dependent reaction and in algae either respiration or photosynthesis can supply the energy (Healey, 1973 a). Phosphorus is present in alagal cells in several chemical forms. It may be present in vacuoles or in cytoplasm as polyphosphates (Nalewajko & Lean, 1980). There are four main organic phosphorus fractions in cells: RNA, DNA, lipids and esterphosphorus. Two physiological changes seem specifically to accompany phosphate depletion and potentially could be useful for diagnostic purposes: the development of cellular alkaline phosphatase activity and initial rate of phosphate uptake on exposure to phosphate (Nalewajko & Lean, 1980). Most of the available data indicate that in phosphorus limited cultures and natural populations, phosphate uptake follows Michaelis Menten Kinetics (Titman & Kilham, 1976). Since trace metals often act as enzyme activators and inhibitors, it is not surprising that their most

marked effect is on growth rate rather than total yield (Anderson et al., 1978). Many trace metals are important in plant and animal nutrition, where as micronutrients they play an essential role in tissue metabolism and growth. The essential trace metals include cobalt, copper, chromium, iron, manganese nickel, molybdenum, selenium, tin and zinc (Leland and Kuwabara, 1985). Quantitatively the most important trace metal for phytoplankton is iron. Iron is required by the cell for numerous redox reactions as well as for chlorophyll synthesis. The enhanced growth of plankters in the chelated form of iron is presumed to be due to their ability to solubilize iron, making it more available for algal uptake (Huntsman and Sunda, 1980).

Second to iron, Mn and Zn are approximately equal in their cellular concentrations (Riley & Roth, 1971). Both metals are required in small amounts to activate non photosynthetic and photosynthetic enzymes and frequently they may be replaced by other metals such as iron or magnesium.

Copper is an essential micronutirent for both algae and higher plants, being a constituent of plastocyanin, a protein involved in photosynthetic electron transport (Katoh et al., 1962) as well as a cofactor of several enzymes.

The pH of the medium may affect the metal availability through changes in chemical speciation (Huntsman & Sunda, 1980). The concentrations of the major nutrients (phosphate and nitrate) appear to affect the uptake and metabolic effects of metals

(Hannan and Patouillet, 1972; Hannan et al., 1973). Salinity and temperature may also strongly affect metal uptake. In the case of salinity, part of the effect may be due to shifting of chemical equilibria (Sunda et al., 1978). The availability of inorganic carbon may influence photosynthesis, particularly in marine macro algae in which ambient Ci levels are subsaturating (Surif and Raven 1989; Madsen and Maberly, 1990). The role of vitamins on phytoplankton growth was reviewed by Swift (1980).

2.2 MATERIALS AND METHODS

Unialgal cultures of Synechocystis salina were used for the experiment. Inocula for the experiment were taken after thorough shaking from 15 days old cultures, incubated at room temperature (8:16 light, dark at 3000 lux). The inocula were discharged @ 2.5ml/100 ml of the medium. S. salina cells were grown in 5 different media (Miquel, 1892; Allen and Nelson, 1910; Kilian, 1911; Ketchum & Redfield, 1938; Matudaira, 1942) under 3 different sets of conditions. In condition I temperature ranges between 28°C-32°C and the light intensity was 1000 lux with a period of exposure 12h light: 12h dark. In condition II, temperature ranges between 34°C - and 36°C, the light intensity was 3000 lux with a period of exposure 8 h light: 16 h dark. In condition III, temperature was kept at 25 ± 2°C and the light intensity was 2000 lux with a period of exposure of 8 h light: 16 h dark. In all the 3 cases during night the ambient temperature fluctuated and reached a lower limit of 25°C.

Allen and Nelson's medium with different concentrations of nitrate was prepared to study the effect of concentration of nitrate on the growth of S. salina. Similarly inorder to study the effect of initial pH on the growth of S. salina, the pH was appropriated with 0.1 N NaOH or HCl to get a range of pH.

Synechocystis salina culture was sufficiently diluted and values of optical density at different wave lengths were noted. A graph was plotted taking cell count on Y axis and optical density on X axis. All the growth measurements were taken by appropriating the optical density at 665 nm.

20 ml of the algal sample was centrifuged and extracted with acetone following the conventional acetone extraction method. Similarly relationship between cell count and dry weight was also plotted.

Effect of salinity on growth of S. salina was studied by measuring the growth over a range of salinities. Growth constant of S. salina was calculated in condition II in Allen & Nelson's medium by substituting on the formula $K = \frac{0.7}{t_g}$ (Strickland, 1960)

2.3 RESULTS AND DISCUSSION

Wave length scan of the cultures revealed that, there is an absorbance peak around 665 nm. (Fig. 1). So this wavelength is used for finding out the optical density of the cultures. Optical density of the cultures as well as the chlorophyll extract obey Beer

Lambert's Law (Fig. 2). Similarly optical density and cell count are better correlated (Fig.3) than dry weight and cell count (Fig. 4).

A comparison of the growth at 3 different sets of conditions using 5 different media revealed the superiority of Allen and Nelson's medium under condition II (Fig. 5-12 & Table 2). However Kilian's medium on 5th day (Fig. 5) and all the media on 10th day (Fig. 6) favoured the Ist set of conditions. On the 5th day maximum growth of S. salina was noticed in Ketchum & Redfield's medium under condition III. Generally, condition II favoured growth ($34^{o}C$-$36^{o}C$ temperature, 3000 lux light intensity at an exposure period 8h light: 16h dark).

It is well known that light intensity and spectral quality are important factors determining variations in photosynthetic responses and growth rates (Falkowski, 1984a; Richardsons et al., 1983; Palmisano et al., 1985; Geider et al., 1986; Harrison and Platt, 1986; Cullen and Lewis, 1988; Cullen, 1990). Photoacclimation occurs in response to changes in photonflux density and spectral distribution (Falkowski and La Roche, 1991). In the aquatic environment, changes in intensity are inextricably linked to changes in spectral distribution (Kirk, 1983). There is an optimum light intensity for each species (Falkowski and La Roche, 1991). As cells acclimate to higher irradiances, there is an increased danger of photodamage to reaction centres (Neale, 1987). Many algae produce β carotene type carotenoids that absorb light but do not transfer the excitation energy to reaction centres. Thus cells acclimated to high

light accumulate carotenoids and may have reduced quantum yields (Dubinsky et al., 1986). However in the present study, in condition II there is an increased growth rate of S. salina.

Higher temperature available in condition II also might have helped in attaining a higher growth rate. Generally photosynthesis continues to increase upto an optimum temperature (Davison, 1991). Temperature affects the activity of enzymes and physical processes such as diffusion and cellular pH. (Raven and Smith, 1978; Raven and Geider, 1988). PS II is also believed to be the most thermolabile aspect of the photosynthetic apparatus, causing the reduction in photosynthesis at temperatures above the temperature optima (Fork et al., 1979). In chilling sensitive cyanobacteria, high temperature inhibition of photosynthesis is associated with disruption of energy transfer between phycobilisomes and PS II (Schreiber, 1980). Although the initial photochemical reactions are independent of temperature, many associated aspects of photosynthesis such as enzymes of phosphorylation, electron transport and plastoquinone are temperature dependent (Oquist, 1983; Raven and Geider, 1988). In many cases temperature acclimation of photosynthesis is associated with changes in the cellular activity of Rubisco and other Calvin cycle enzymes, which increase in the low temperature grown plants (Li and Morris, 1982; Davison and Davison, 1987) to compensate for the reduction in the activity of individual enzyme molecules (Hochachka and Somero, 1984). There has been an overall tendency to regard the cyanobacteria as organisms favoured by high temperature conditions (Foy et al., 1976). This assumption has been evidenced by

their association with hot springs, where they are the only oxygen evolving photosynthetic organisms to occur at temperatures above 56°C and the tendency for cyanobacteria to be more abundant in tropical rather than temperate regions in habitat (Whitton and Sinclair, 1975). In Allen & Nelson's medium, the growth constant, K was calculated as 0.047 with a mean generation time of 15 hrs.

A comparison of the composition of the five media (Table 1) revealed that highest concentration of nitrate is present in Allen & Nelsons medium. Higher growth rate in Allen and Nelsons medium may be attributed to the higher nitrate content. Studies on the effect of nitrate concentration on growth of S. salina disclosed that normal nitrate concentration proposed by Allen & Nelson is apt for the growth of S. salina, even if fluctuations are noticed in the initial period of growth (Fig. 13-15 & Table 3).

Nitrate concentration is an important parameter that determines the growth of the cyanobacteria. Vanlerberghe et al., (1990) reported that as much as 50% of algal carbon is integrally coupled with N metabolism. Nitrogen concentration in the medium affects synthesis of chlorophyll and phycobilins (Plumley & Schmidt, 1989). N-limitation also causes a reduction in thylakoid stacking and absorptivity (Plumley et al., 1989). N-limitation also affects the enzymes of photosynthetic carbon metabolism and a decline in Rubisco per cell is well established (Falkowski et al., 1989; Plumley and Schmidt, 1989; Beardall, 1991). This decreased ability to dissipate light energy in the reduction of CO_2 may be related to the increased

susceptibility of N-limited algae to photoinhibition (Prezelin et al., 1986; Rheil et al., 1986; Kolber et al., 1988).

According to Turpin et al., (1988) photosynthesis and nitrogen metabolism are integrally coupled. The assimilation of N into amino acids occurs primarily via the Glutamine synthetase/glutamine: 2 oxoglutarate aminotransferase (GS/GOGAT) pathway resulting in the production of glutamate (Turpin and Harrison, 1979; Cullimore and Sims, 1981; Syrett, 1981; Zehr and Falkowski, 1989). If the cells are grown under nutrient replete conditions, levels of endogenous carbohydrate reserves decline, and the assimilation of inorganic nitrogen into amino acids is dependent upon recent photosynthate (Guerrero and Lara, 1987; Lara et al., 1987 a; Larsson and Larsson, 1987). Although there are some exceptions, N-sufficient cyanobacteria do not take up and reduce nitrate in the absence of CO_2 (Flores et al., 1983; Lara et al., 1987a). Therefore, cells that are unable to assimilate NH_3 into amino acids, due to the lack of recent photosynthate or stored carbohydrate do not carryout futile nitrate reduction. N-sufficient algae exhibit molar rates of photosynthetic carbon fixation 7-10 times those of N-assimilation. Under these conditions, photosynthetic carbon fixation is capable of supplying all the carbon required for amino acid synthesis. When algal cells are cultured under N-limitation, their capacity for N-assimilation increases dramatically relative to photosynthesis (Turpin, 1991).

The absence of growth in Allen & Nelson's medium devoid of nitrate revealed the inability of S. salina to fix atmospheric N_2. Similarly reduced concentrations and higher concentrations of nitrate reduced the growth rate of S. salina. (Fig. 13-15)

Initial pH of the medium affects the growth of S. salina (Fig. 16-18 & Table 4). At acidic pH, the salts might be undergoing full speciation and the abundance of these ions might be accounted for the better growth of S. salina at lower pH. Huntsman & Sunda (1980) reported that the pH of the medium may affect the metal availability through changes in chemical speciation.

However in all the cases the pH of the medium reached maximum by the 15th day (9.5) and then gradually reached a constancy around 9 (Fig. 22).

According to Fogg and Thake (1987), alteration of pH of the medium is as a result of preferential absorption of particular constituents of the medium. Absorption of nitrate ion results in an increase in pH, but this is buffered by the medium taking up more CO_2 so that it rarely affects growth to an appreciable extent. If CO_2 is limiting, the utilization of bicarbonate in photosynthesis may result in the pH of the media rising as high as 11 or more, which may bring growth to an end (Fogg and Thake, 1987). Utilization of organic acids without equivalent intake of cations may also result in the medium becoming too alkaline for growth (Hutchens 1948).

From the results it is clear that there is an increased uptake of CO_2 and the buffering activity of carbonic acid maintained the pH around 9. Salinity between 15‰ and 25‰ favours growth. A higher salinity 34‰ adversely affect the growth. (Fig. 19-21 & Table 5) At higher salinities there may be decreased intake of ions from the medium, owing to the increased binding of ions on cell wall, already present in the sea water. This might have reduced the intake of different ions needed for the growth. Similarly at higher pH the plant also might be spending a quantum of its energy for making the osmoticum required for keeping the osmotic potential of the cytoplasm and avoiding a collapse. This channelisation of energy might have a negative impact on the quantum yield of cyanobacterial cells.

Table - 1

Name of the salt	Name of the media				
	Miquel (1892)	Allen & Nelson(1910)	Kilian (1911)	Ketchum & Redfield (1938)	Matudaira (1942)
$MgSO_4$	0.2 gms			0.02 gms	
NaCl	0.2 gms				
Na_2SO_4	0.1 gms				
NH_4NO_3	0.02 gms		0.02 gms		0.01 gms
KNO_3	0.04 gms	0.404 gms	0.04 gms	0.1111 gms	0.02 gms
$NaNO_3$	0.04 gms		0.04 gms		0.02 gms
KBr	0.004 gms				0.002 gms
KI	0.004 gms				0.001 gms
Na_2HPO_4 $12^2H_2O^4$	0.05 gms	0.05 gms	0.05 gms	0.02 gms	0.04 gm
$CaCl_2$ $6H_2O$	0.05 gms	0.05 gms	0.05 gms	0.02 gms	0.04 gm
HCl	0.025 ml	0.025 ml	0.025 ml	0.01 ml	0.01 ml
$FeCl_3$	0.025 ml	0.025 ml	0.025 ml	0.01 ml	

Concentration of various salts in 1 litre of different media used for the study.

Table 2

Age of Culture (Days)	Set of condition	Culture Media				
		Ki	A&N	Mi	Ket & Red	Mat
		x 10^3 cells/ml				
05	1	70	30	30	10	30
	2	50	80	70	50	50
	3	0	10	00	110	10
10	1	220	120	220	120	240
	2	200	100	140	80	200
	3	10	30	70	50	30
15	1	430	200	430	450	430
	2	490	410	540	710	600
	3	200	160	220	300	160
20	1	680	600	710	680	540
	2	680	570	810	1000	790
	3	580	430	430	410	330
25	1	800	650	920	770	650
	2	950	810	1140	1300	1090
	3	1030	810	730	430	950
34	1	920	1170	1280	1060	760
	2	1520	980	1440	1420	950
	3	1360	1330	1220	760	490
41	1	1470	1060	1330	1110	570
	2	1240	1190	1550	1410	950
	3	1520	1220	1220	790	480
46	1	1190	1410	1360	1090	710
	2	1280	1560	1550	1360	900
	3	1200	1400	1300	810	520

Growth of Synchocystis salina in five different media under three different set of conditions.

Table 3

Age of Culture (Days)	Concentration of NO_3				
	N/4	N/2	N	2N	4N
	x 10^3 cells/ml				
05	380	290	330	220	190
10	1060	1190	1170	380	350
15	1170	1240	1220	650	760
20	1550	1630	1380	1030	1060
25	1280	1520	1630	2010	1490
30	1410	1220	1420	1340	1360
35	1300	1240	2120	1500	1400
40	1000	1570	2390	1650	1970
45	1170	1510	2390	1680	2070

Effect of NO_3^- concentration on the growth of Synechocystis salina.

Table 4

Age of Culture (Days)	pH			
	6.25	6.80	7.5	8
	x 10^3 Cells/ml			
05	420	190	160	230
10	1550	460	380	410
15	2250	1110	1110	920
20	2360	1320	1320	1360
25	2700	1760	1760	1190
30	3250	2190	2440	1470
35	2910	2240	2740	1380
40	4440	2740	2960	2390
45	4440	2990	2960	2220

Effect of pH on the growth of Synechocystis salina.

Table 5

Age of Culture (Days)	Salinity			
	15‰	20‰	25‰	34‰
	x 10^3 Cells/ml			
05	120	160	180	210
10	220	510	490	418
15	920	1190	1330	928
20	1280	1420	1540	1360
25	2020	1850	2050	1194
30	2520	2490	2370	1480
35	2810	2740	2810	1388
40	3400	3250	3850	2390
45	3250	3400	3250	2200

Effect of salinity on the growth of Synechocystis salina.

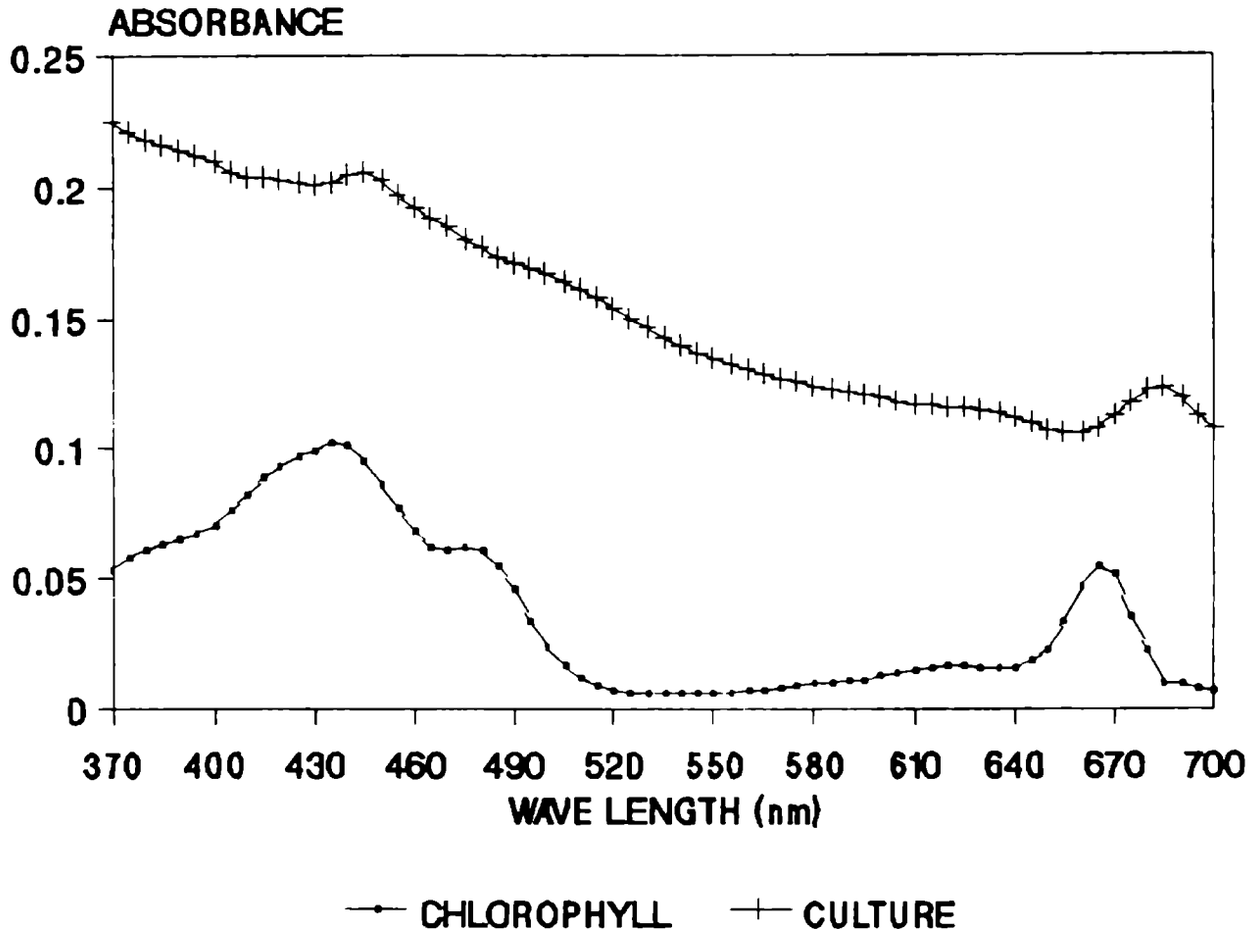


Fig. 1. Wavelength scan of S. salina culture and chlorophyll extract.

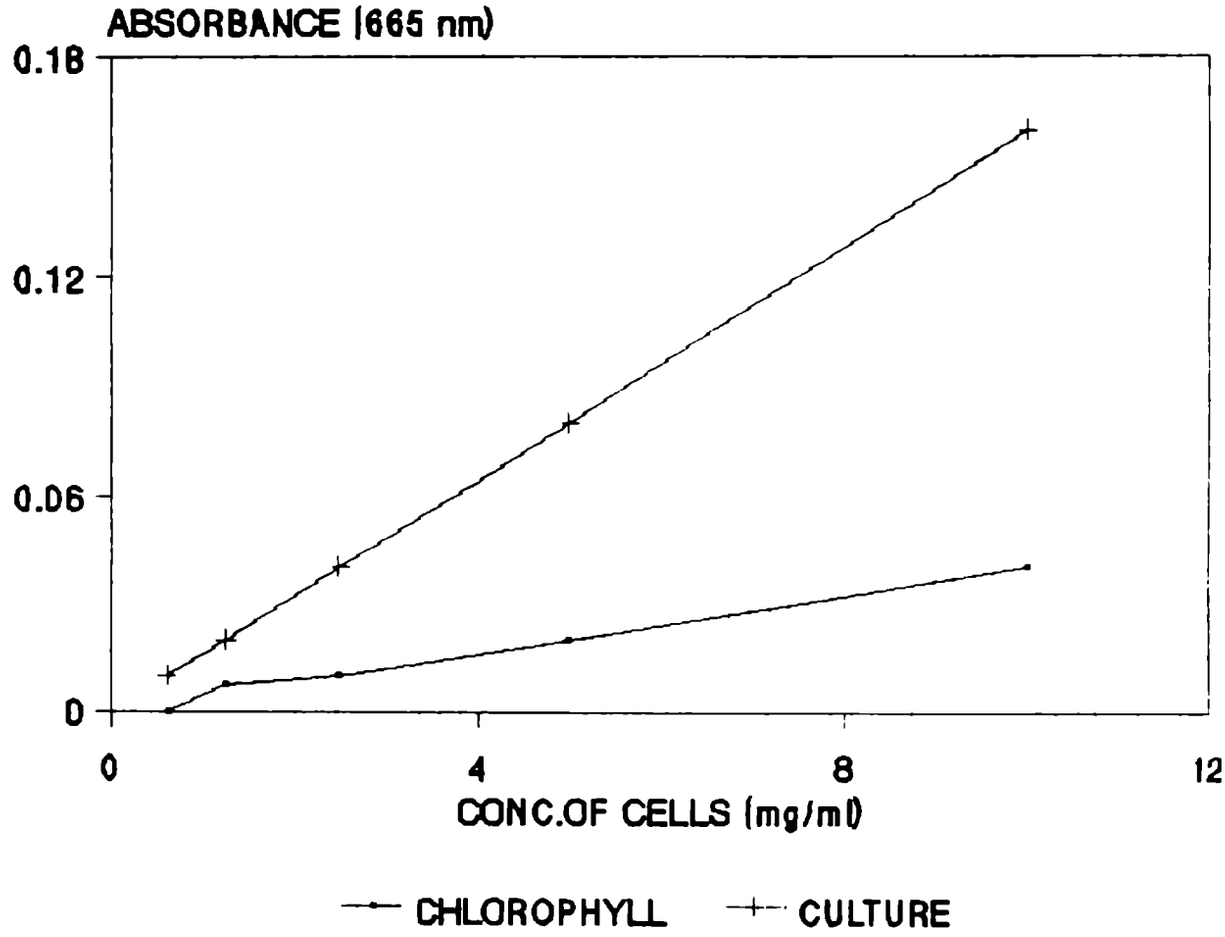


Fig. 2. Comparison between the absorbance of culture and its chlorophyll extract of S. salina at 665 nm.

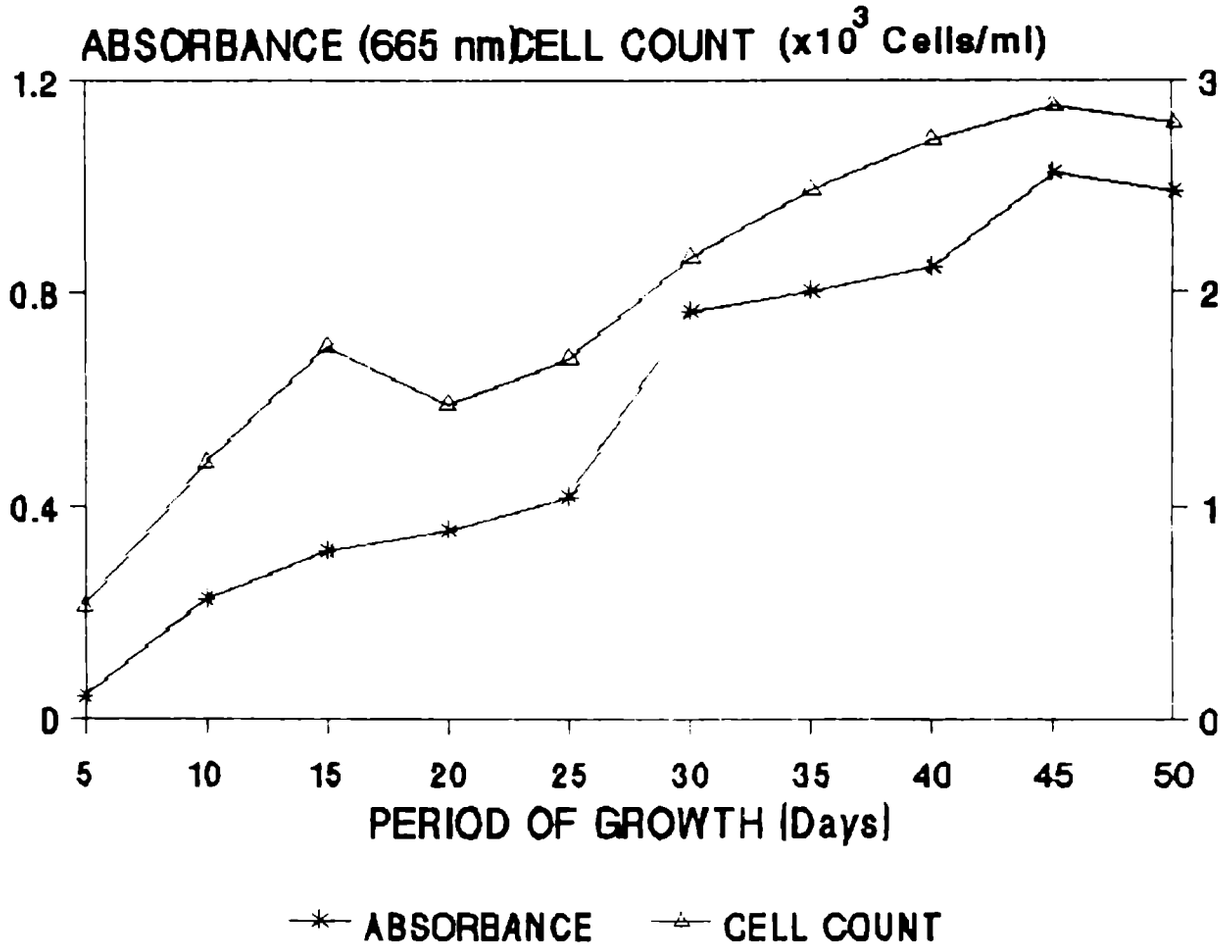


Fig. 3. Relationship between cell count and absorbance of culture at 665 nm at different growth periods.

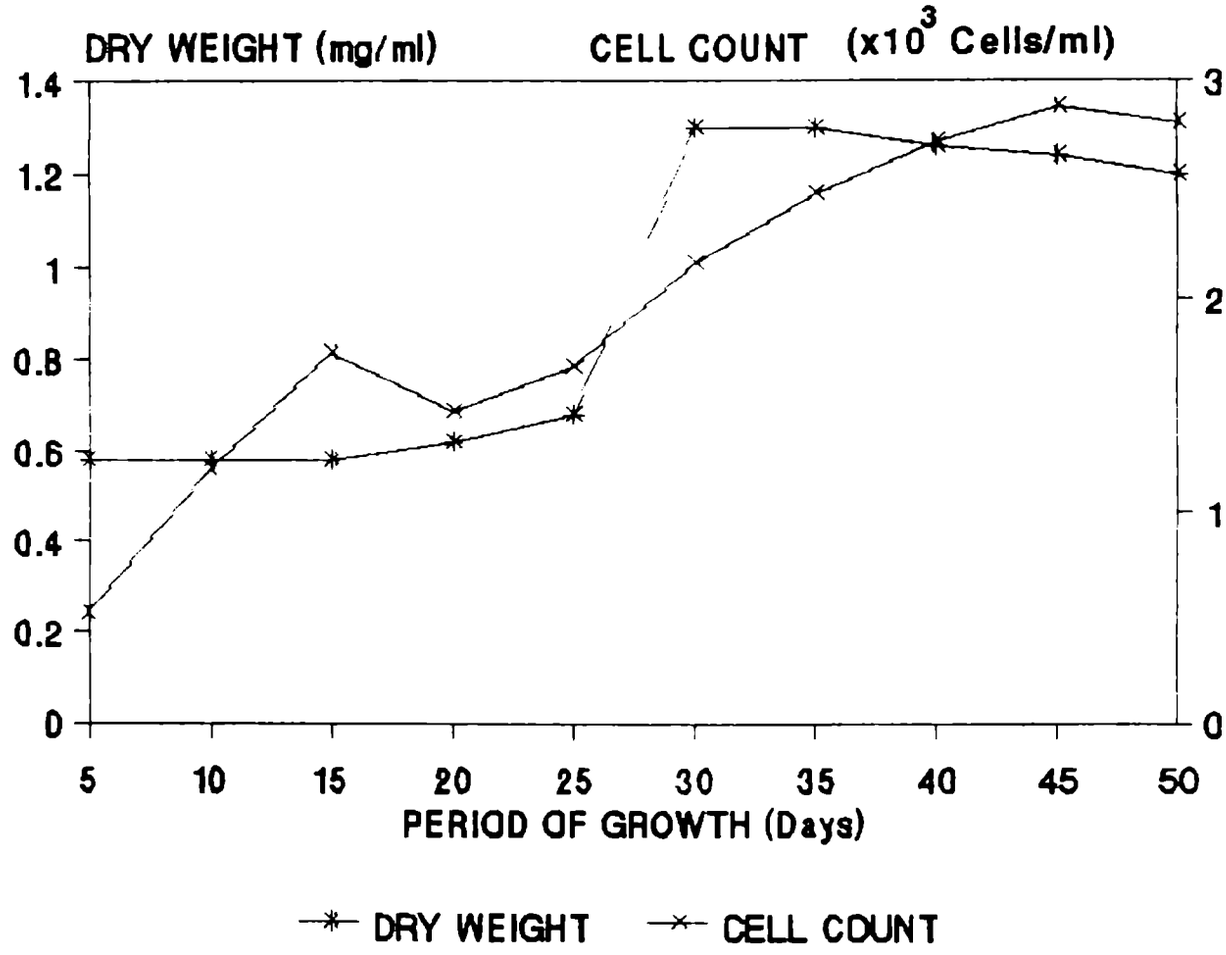


Fig. 4. Relationship between cell count and dry weight.

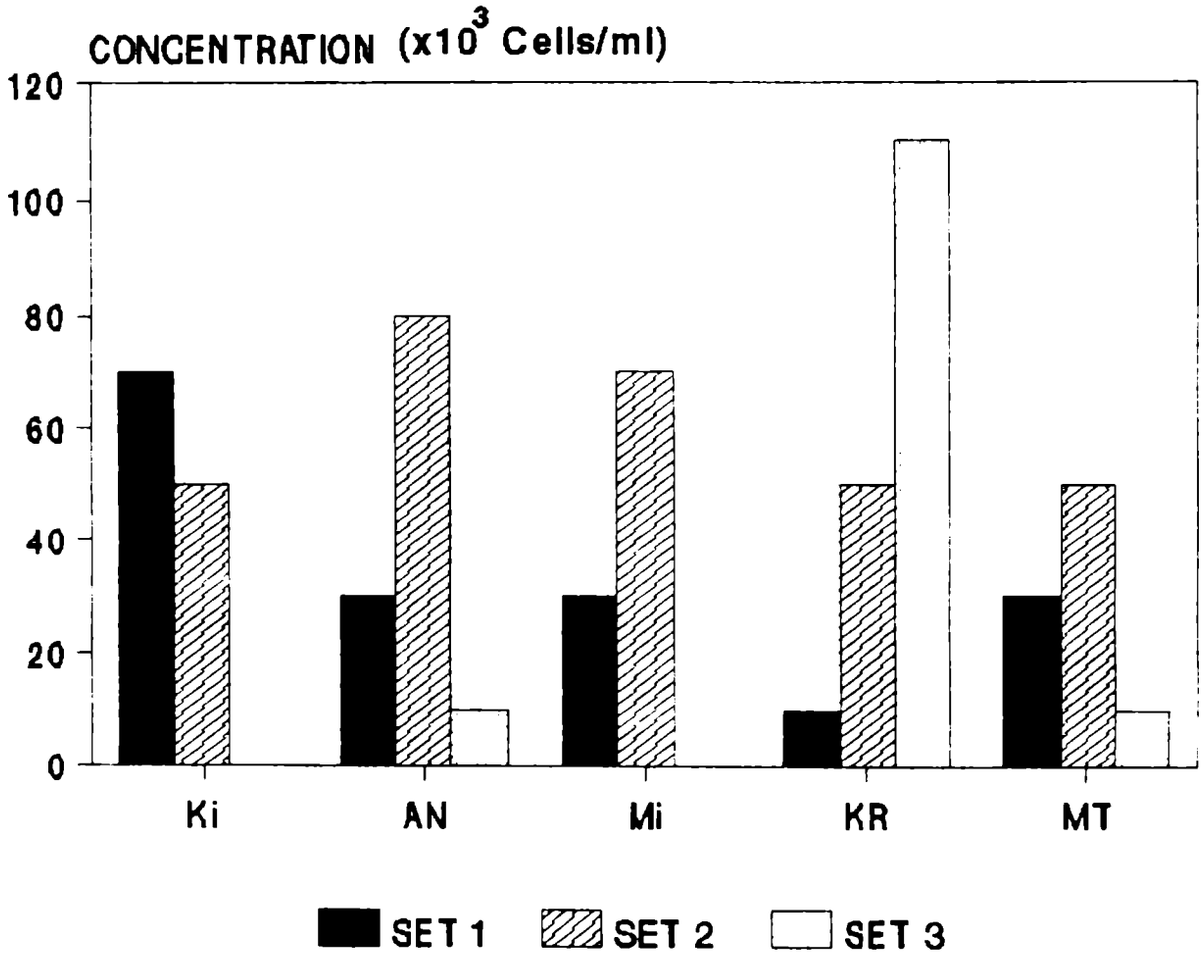


Fig. 5. Growth of S. salina in five different media under three different sets of conditions - after 5 days.

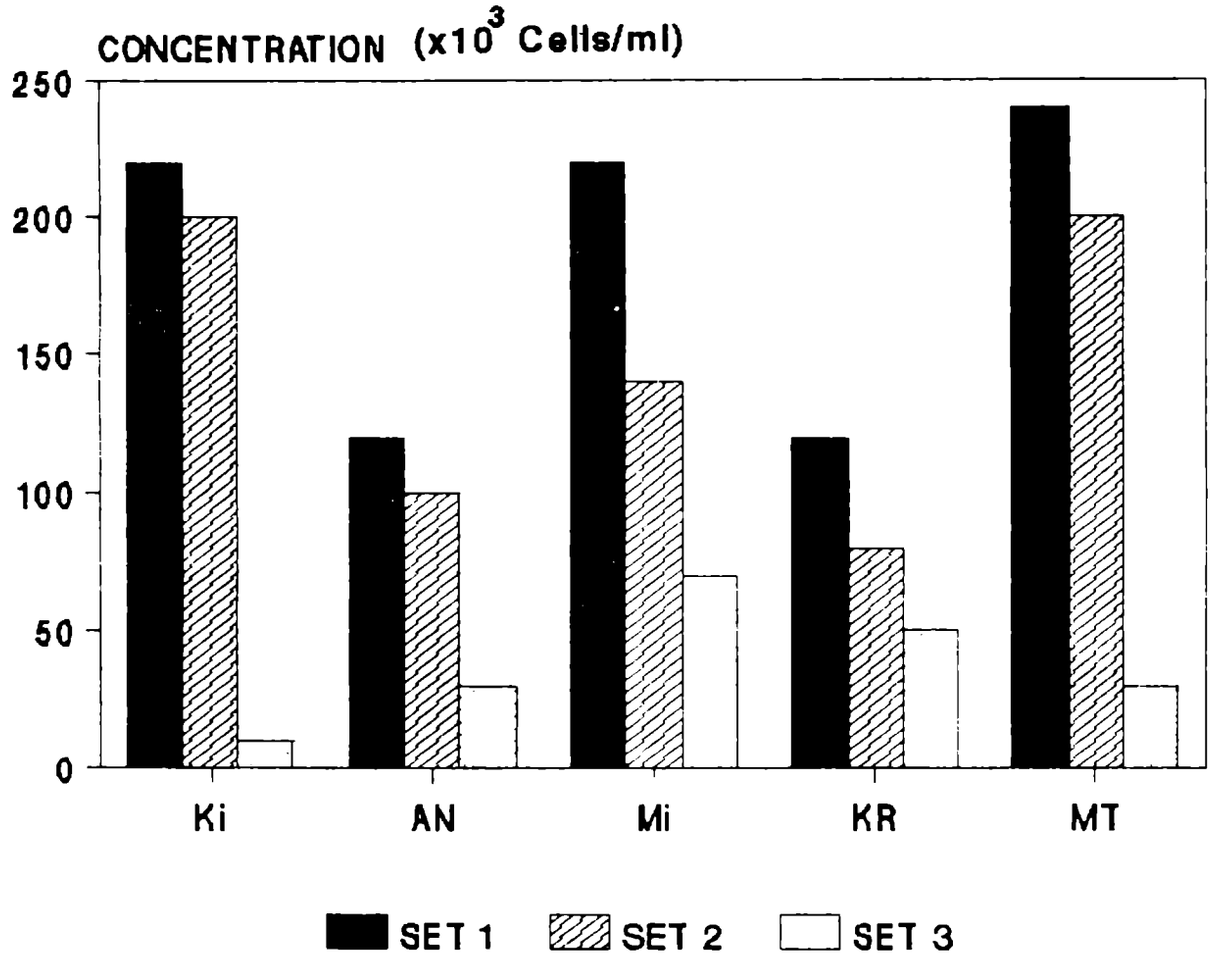


Fig. 6. Growth of S. salina in five different media under three different sets of conditions - after 10 days.

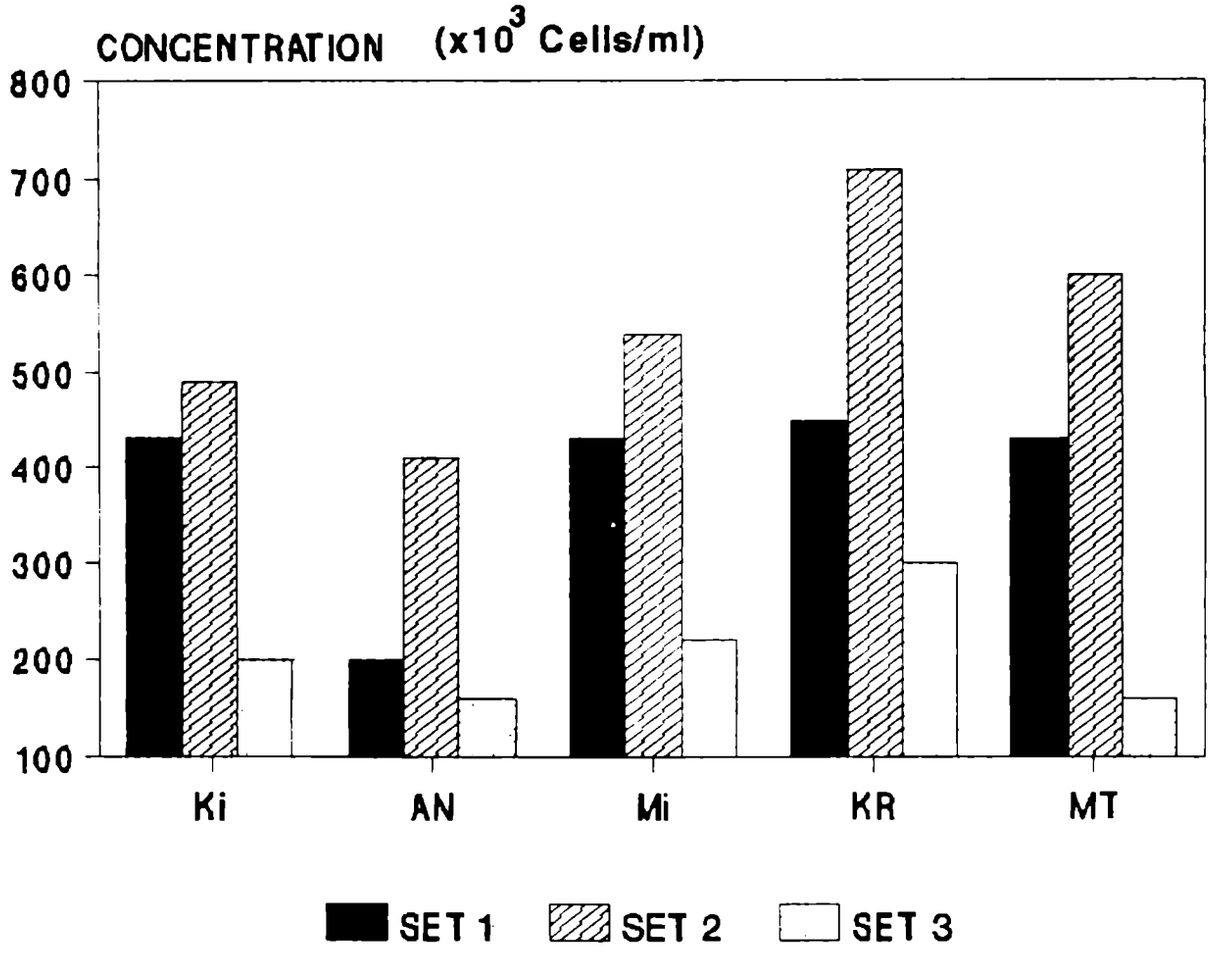


Fig. 7. Growth of S. salina in five different media under three different sets of conditions - after 15 days.

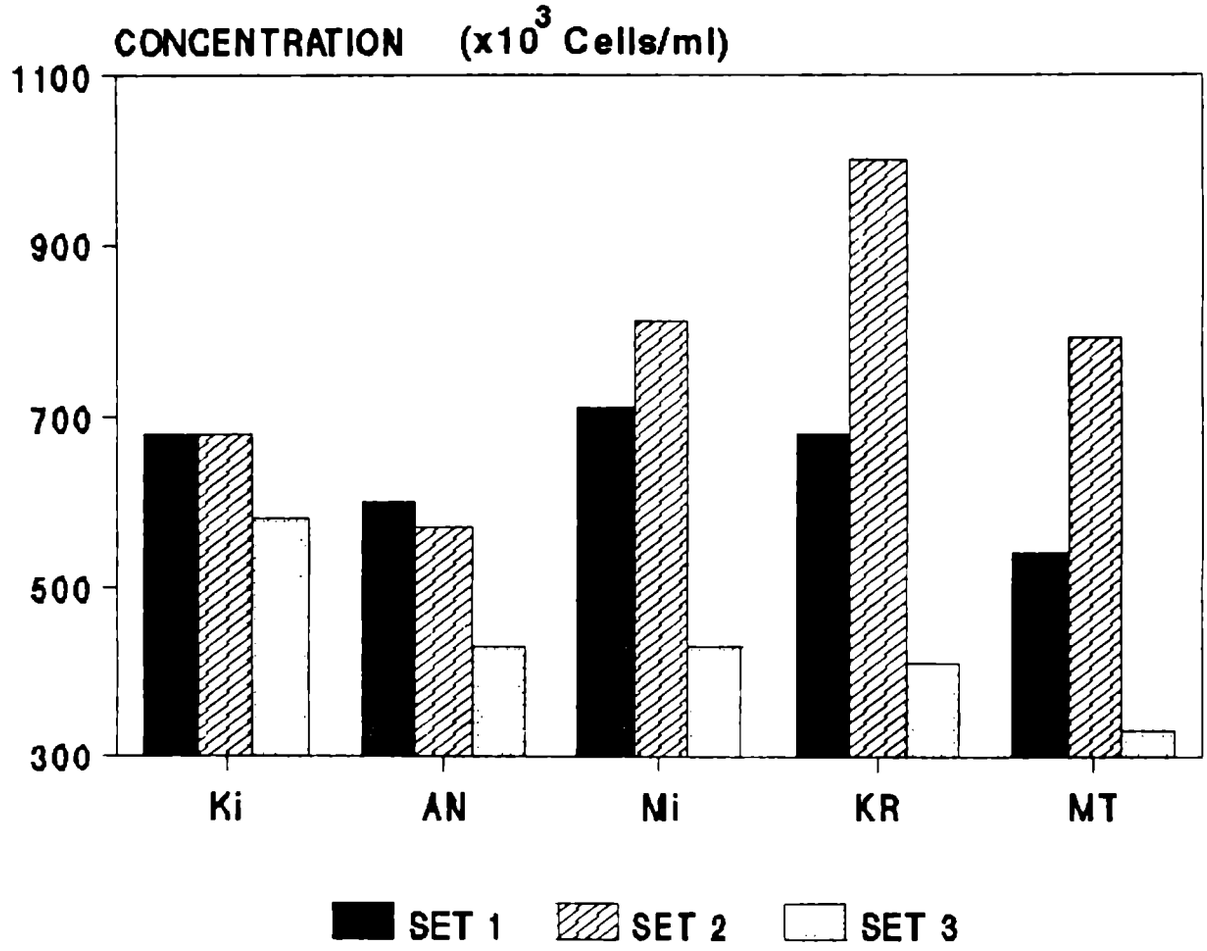


Fig. 8. Growth of S. salina in five different media under three different sets of conditions - after 20 days.

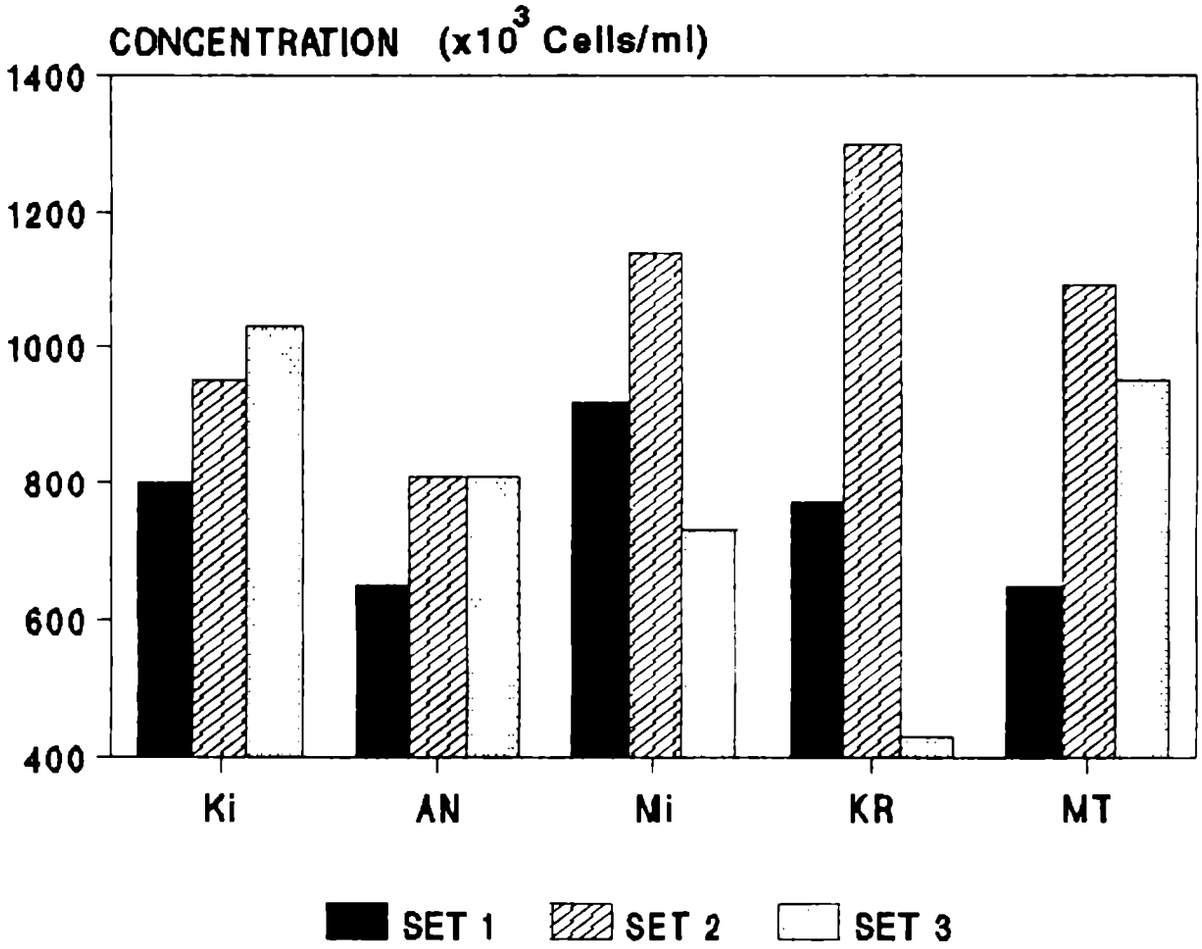


Fig. 9. Growth of S. salina in five different media under three different sets of conditions - after 25 days.

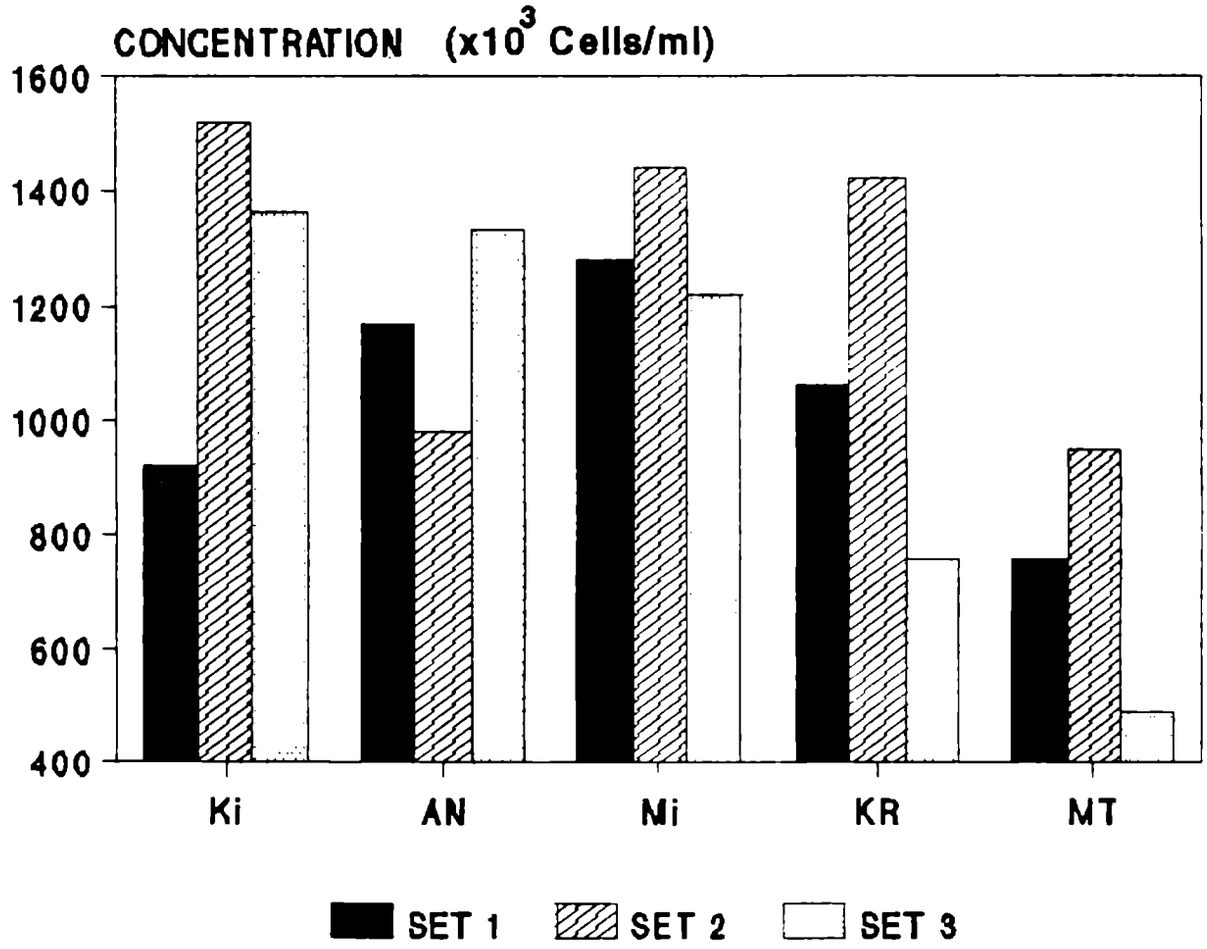


Fig. 10. Growth of S. salina in five different media under three different sets of conditions - after 34 days.

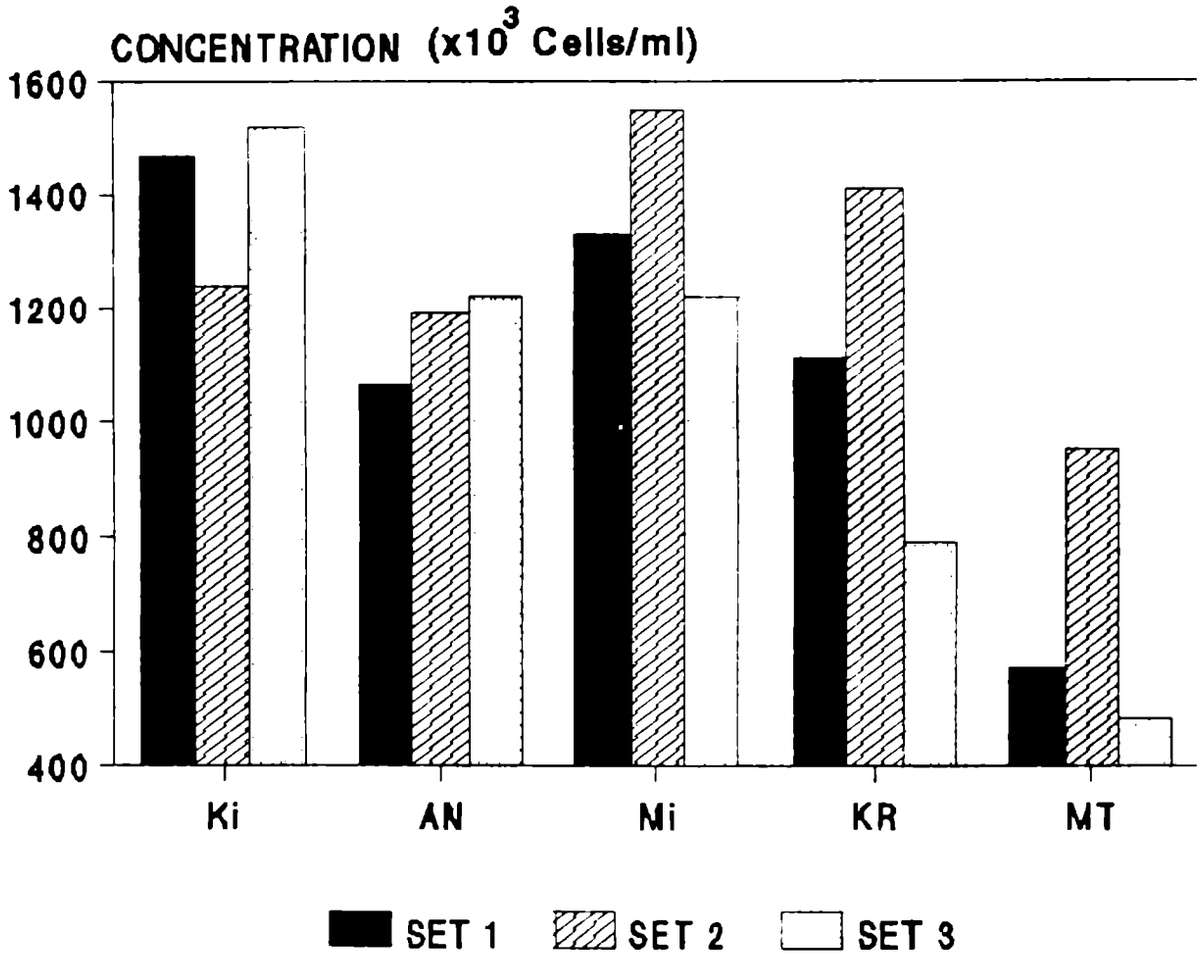


Fig. 11. Growth of S. salina in five different media under three different sets of conditions - after 41 days.

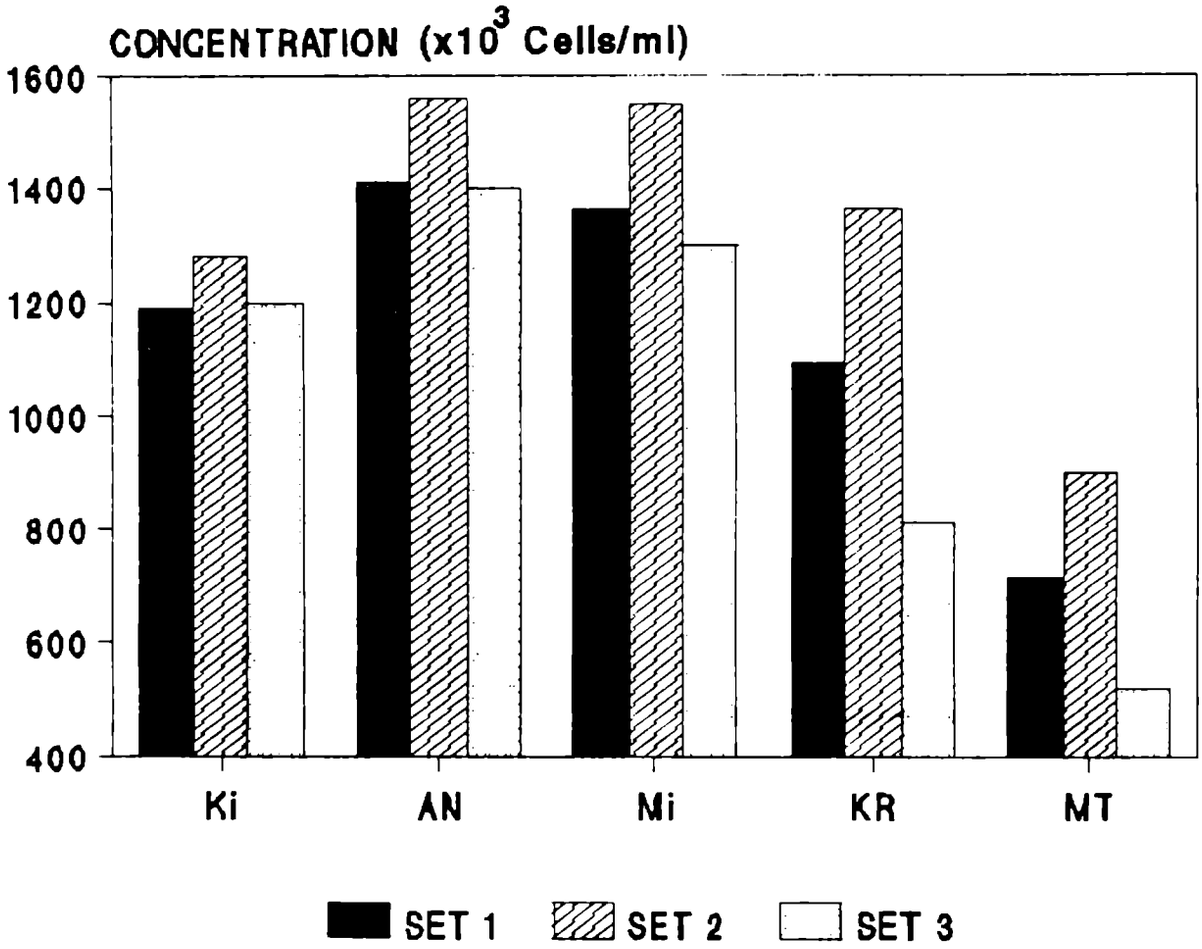


Fig. 12. Growth of S. salina in five different media under three different sets of conditions - after 46 days.

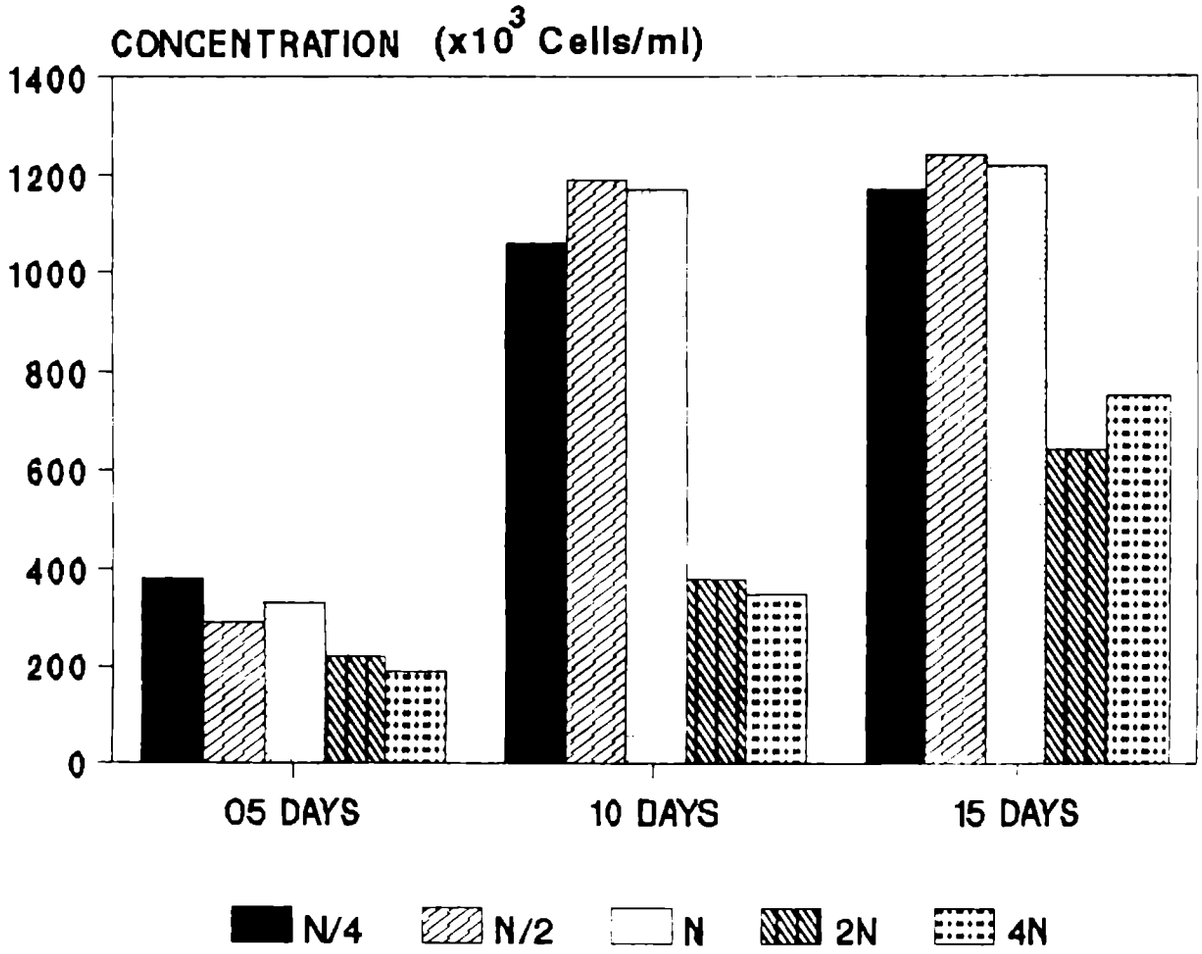


Fig. 13. Effect of NO_3^- concentration on the growth of S. salina

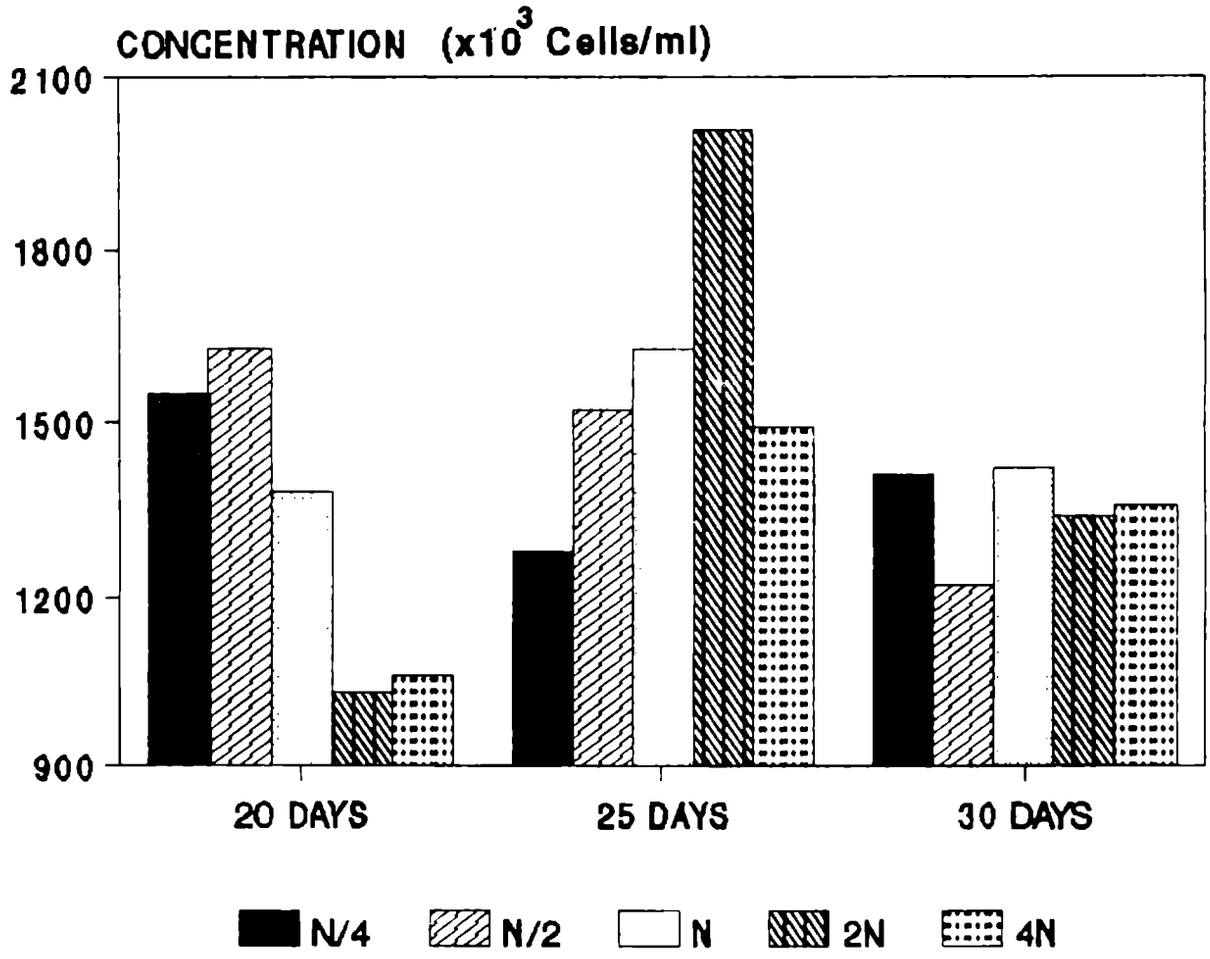


Fig. 14. Effect of NO_3^- concentration on the growth of S. salina

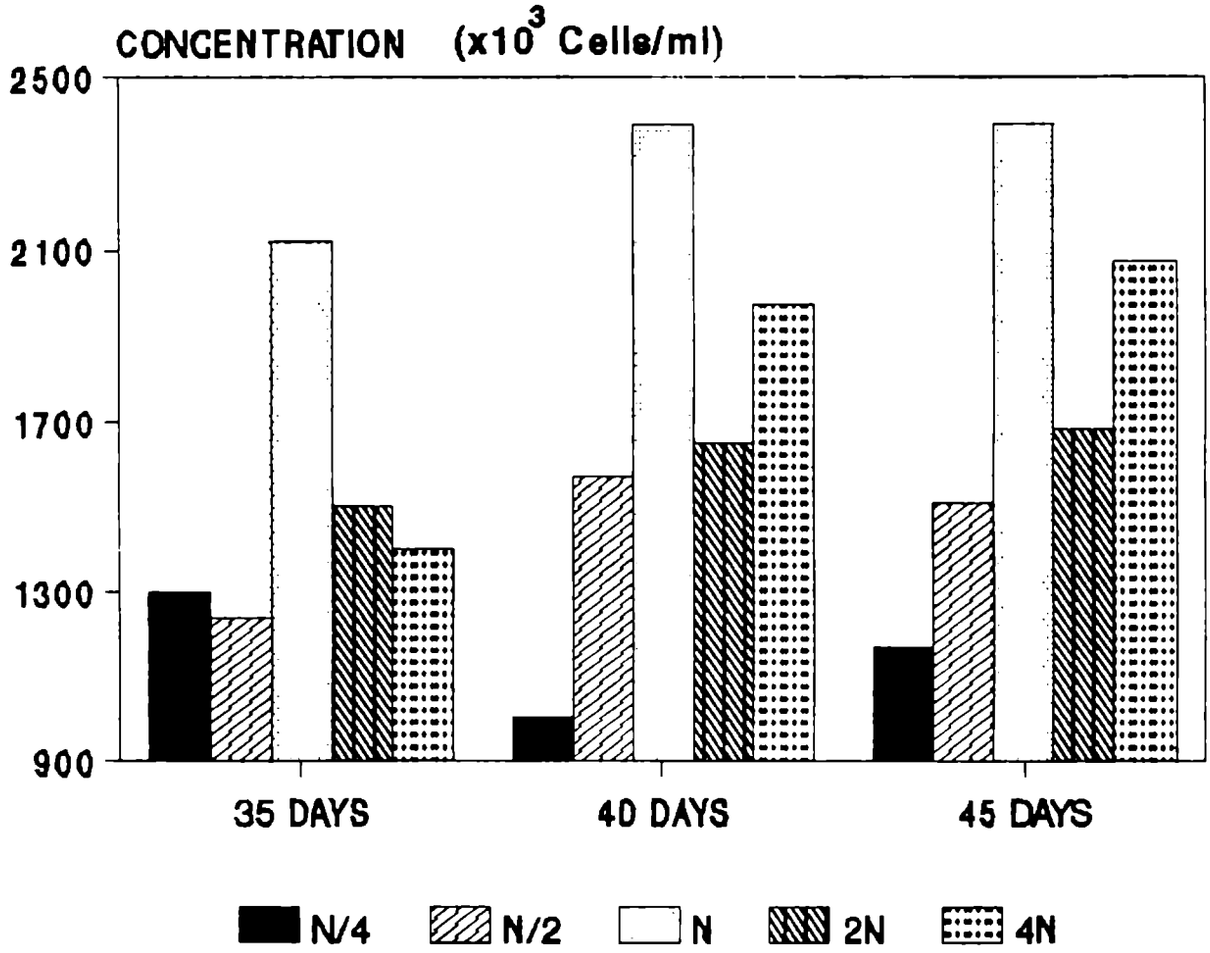


Fig. 15. Effect of NO_3^- concentration on the growth of S. salina

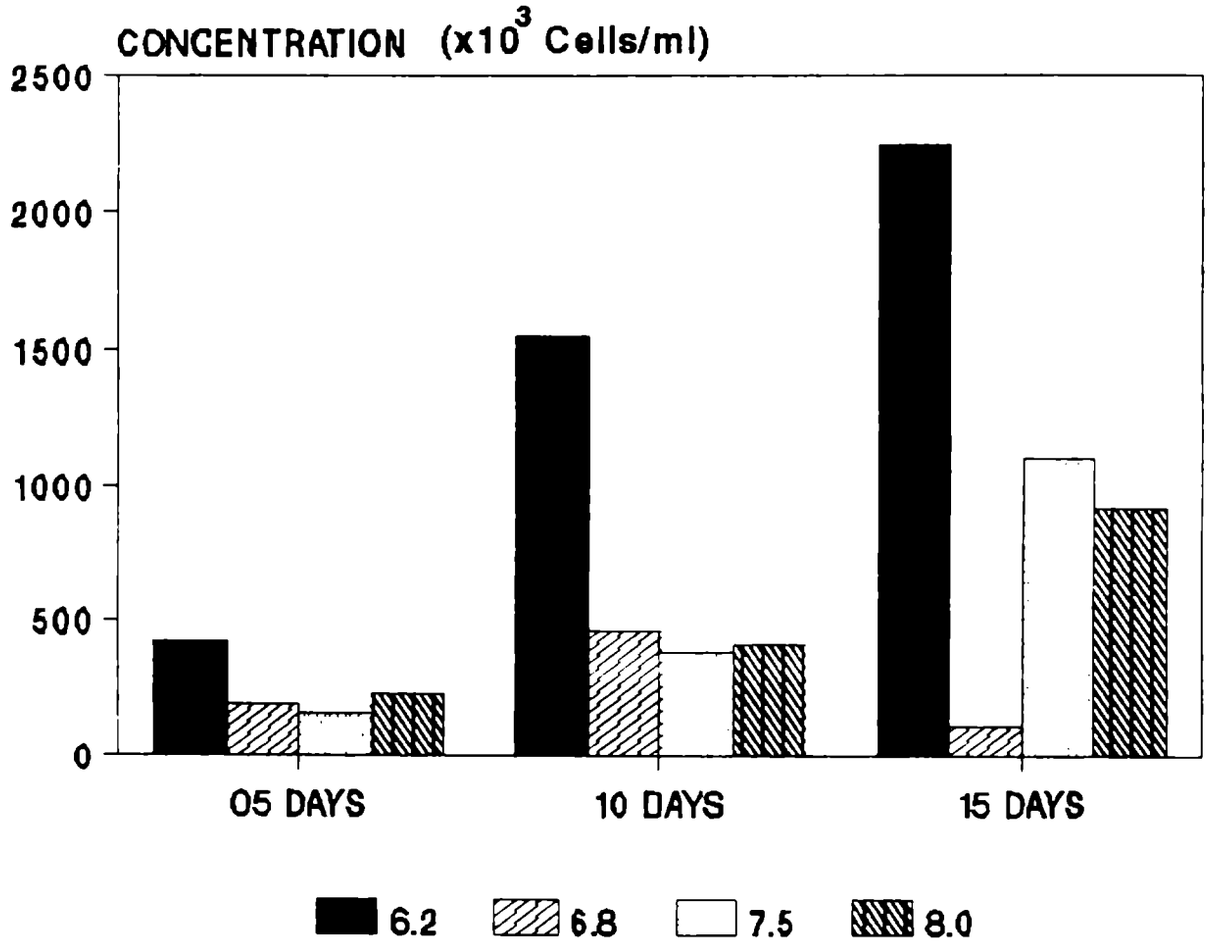


Fig. 16. Effect of initial pH on the growth of S. salina

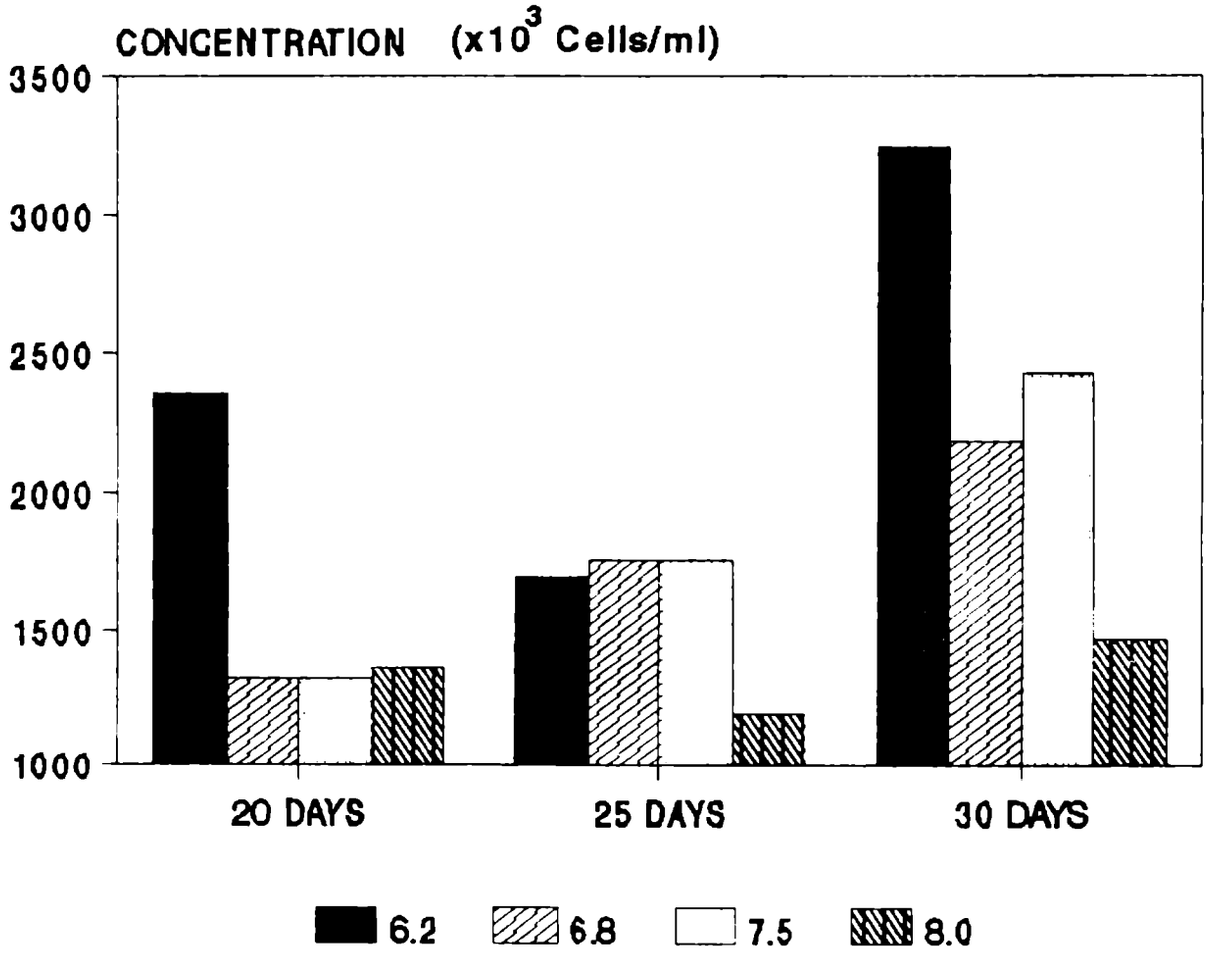


Fig. 17. Effect of initial pH on the growth of S. salina

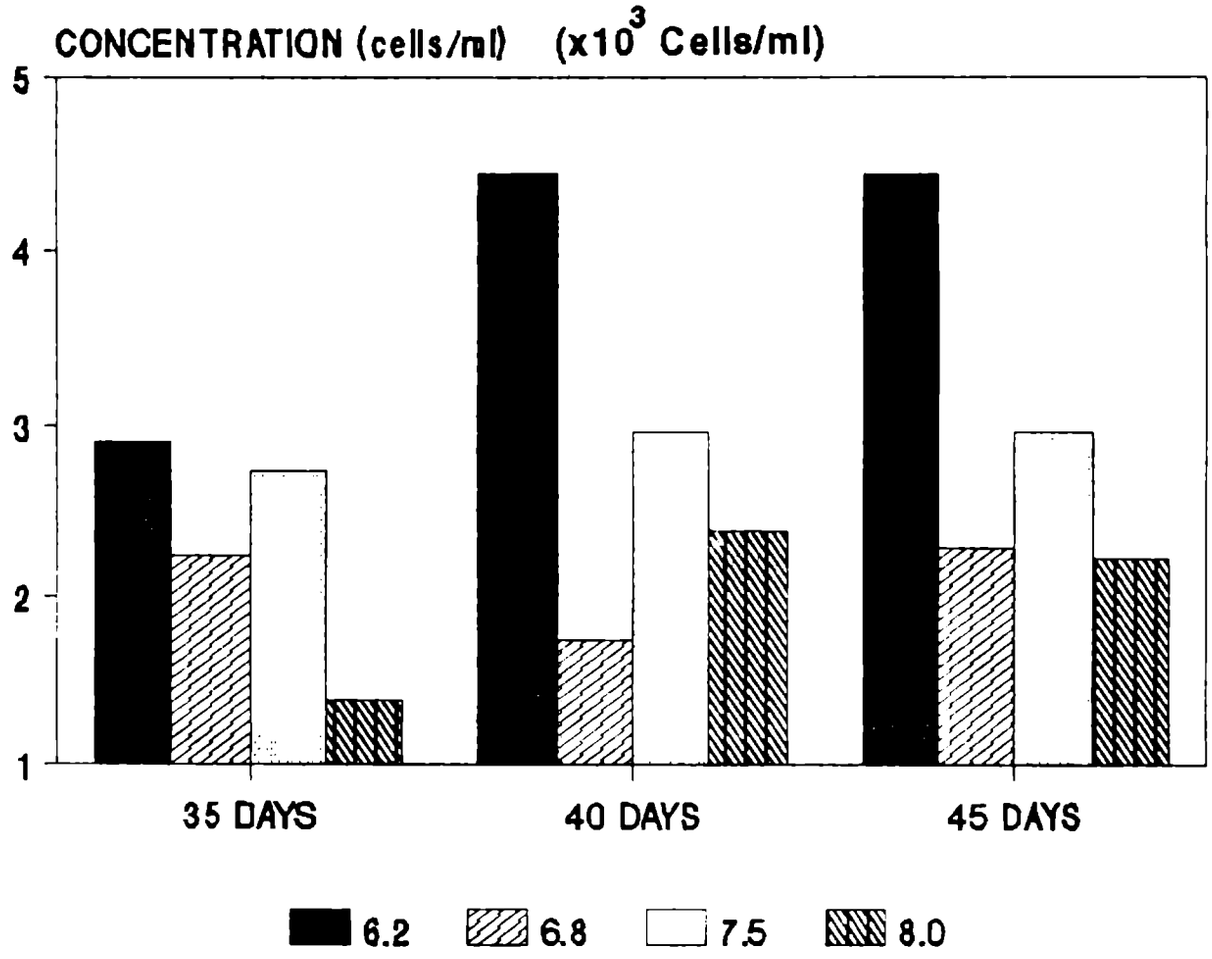


Fig. 18. Effect of initial pH on the growth of S. salina

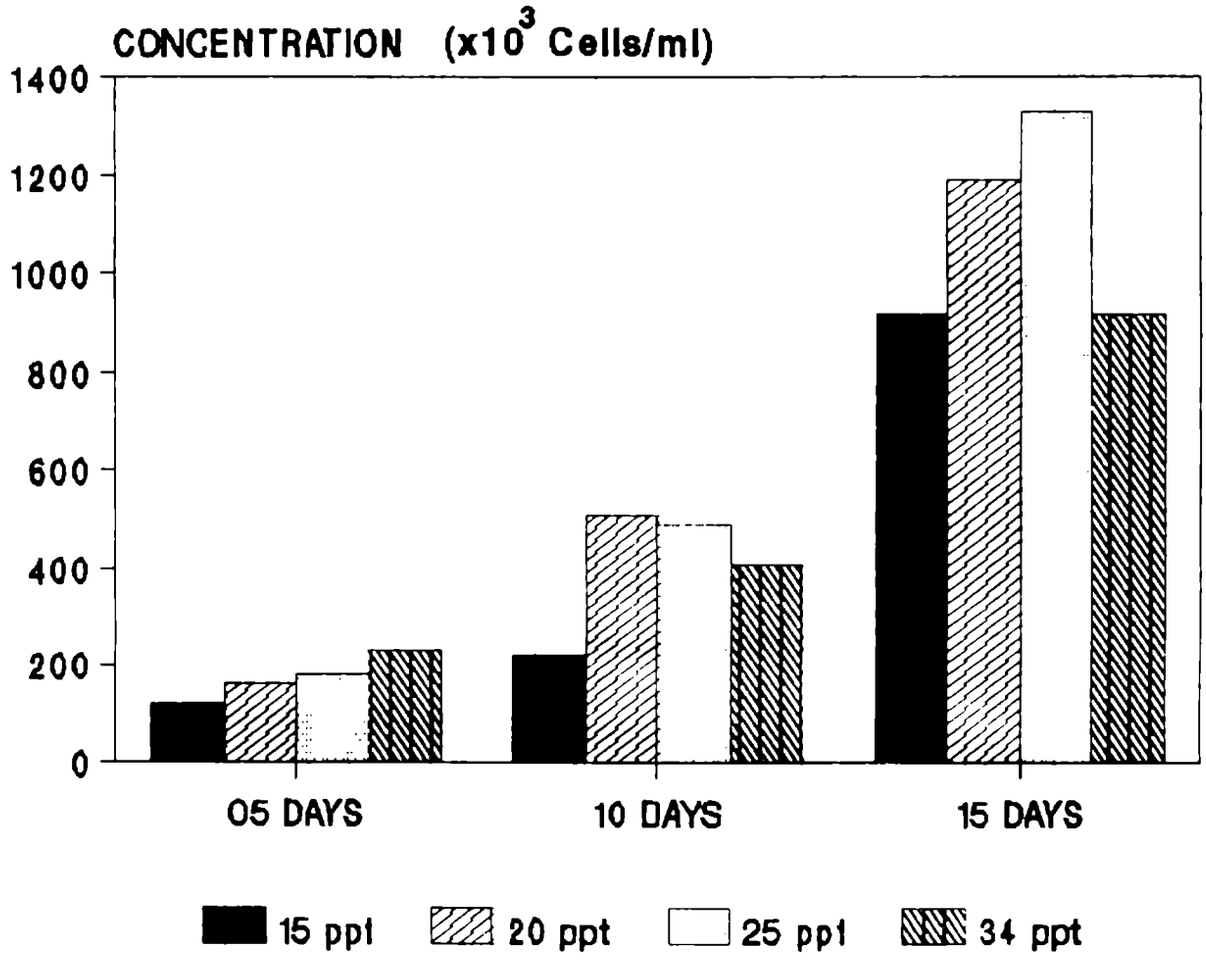


Fig. 19. Effect of salinity on the growth of S. salina

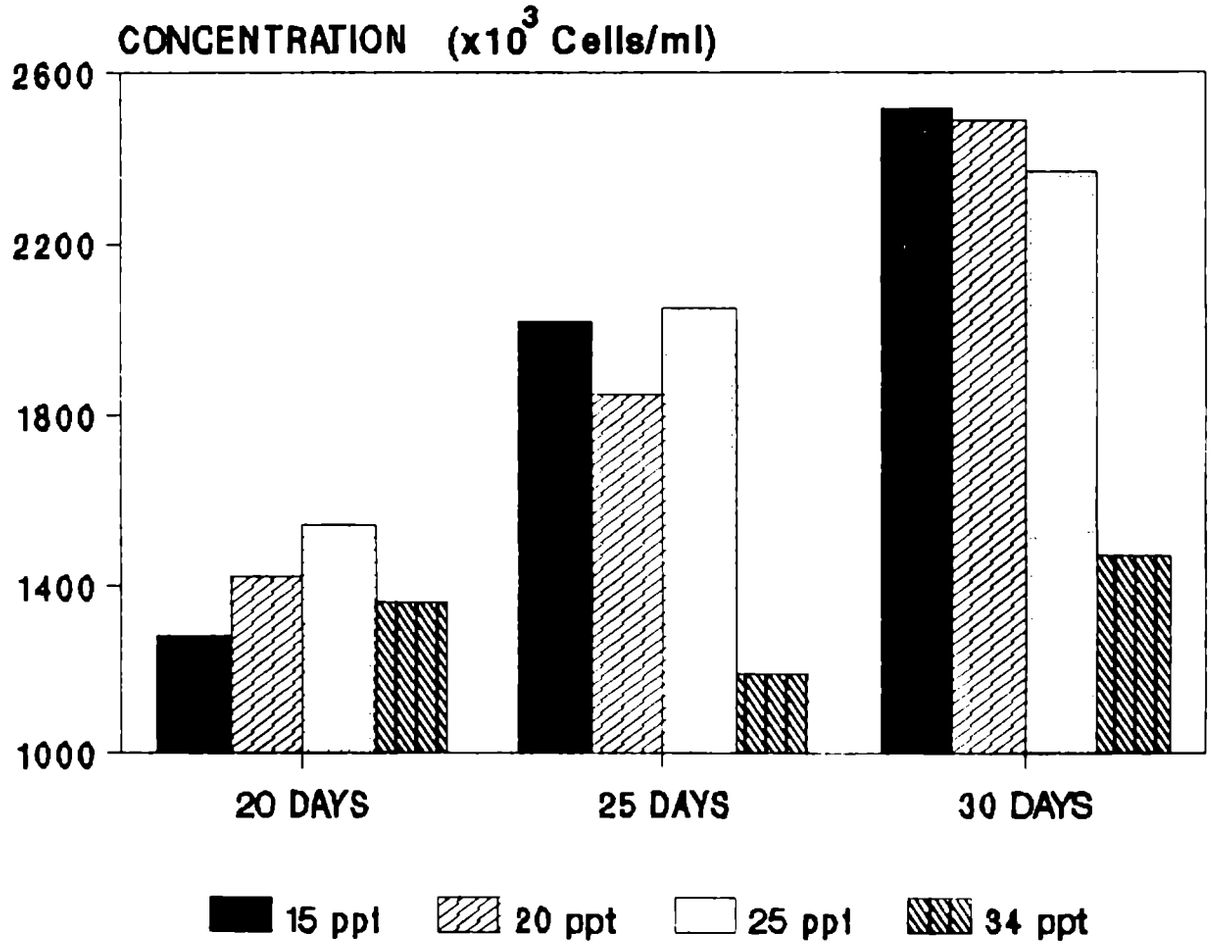


Fig. 20. Effect of salinity on the growth of S. salina

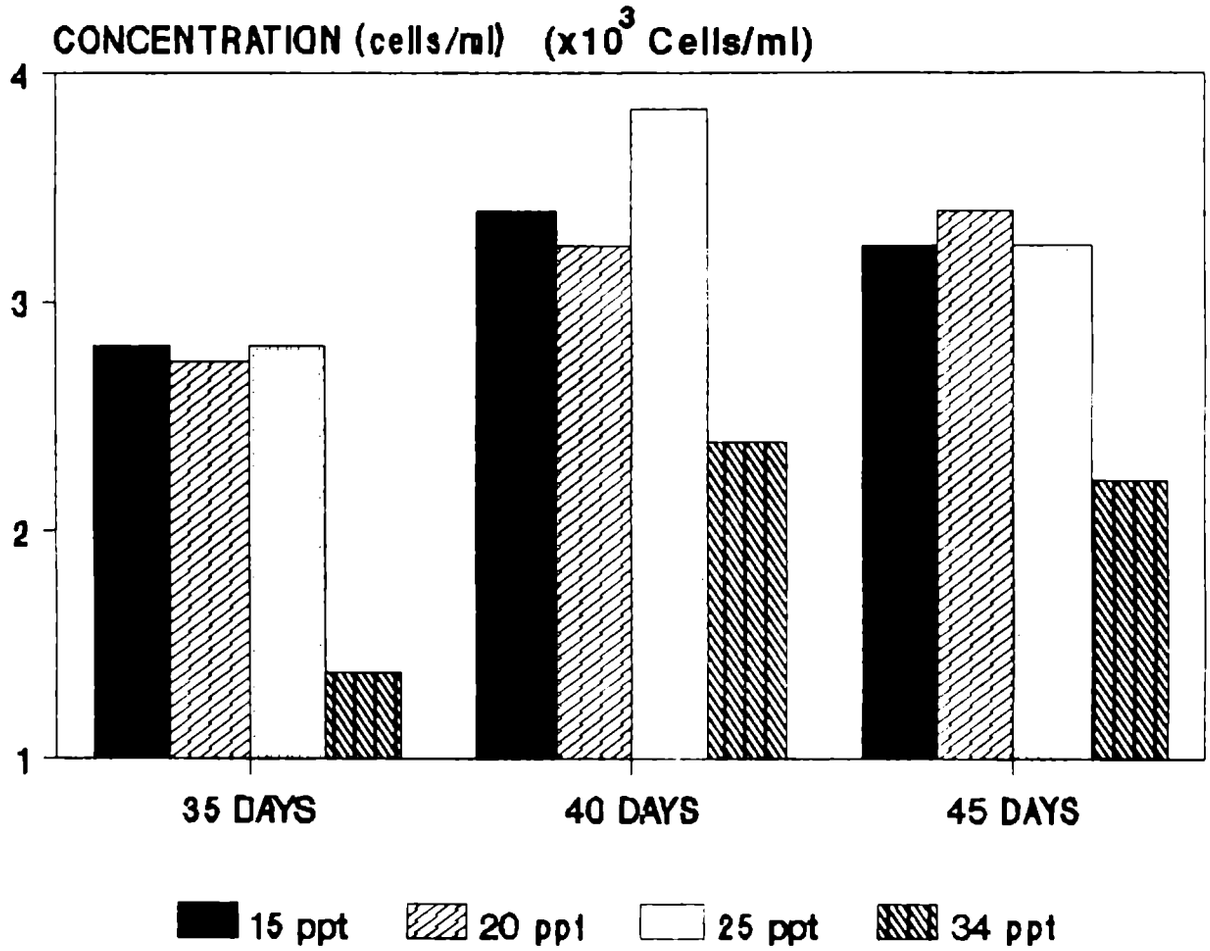


Fig. 21. Effect of salinity on the growth of S. salina

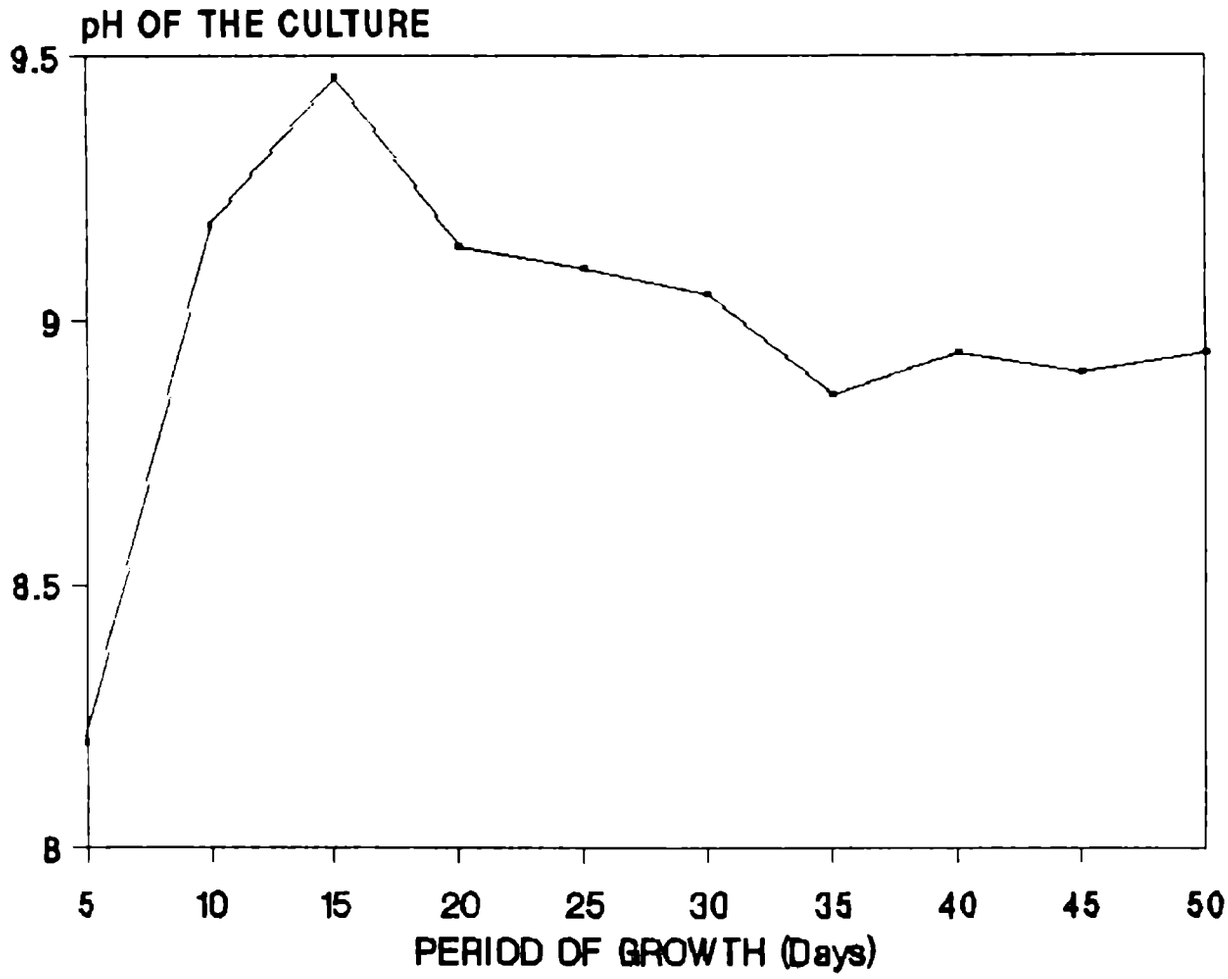


Fig. 22. Variation in the pH of the culture medium at different growth intervals.

CHAPTER 3

3. EFFECT OF HEAVY METALS ON THE GROWTH OF SYNECHOCYSTIS SALINA WISLOUCH

3.1 INTRODUCTION

The term heavy metal has generally been used to describe those metals having an atomic number greater than Iron or having a density greater than 5g/ml (Passov et al., 1961). Some are essential as trace nutrients for plant life while others are superfluous or even toxic. Most of them are capable of causing the disruption and/or death of algae (Sorentino, 1979). Heavy metals are found in the aquatic environment as inorganic cations, or as complexed species. They mainly originate as a result of weathering and leaching processes or from anthropogenic sources. They may be concentrated by the primary trophic levels and thus incorporated in to the food chain (Sorentino, 1979). This has given rise to the upsurge in research into the longer term sub lethal aspects of metal toxicity towards marine plankton and the way in which metals are accumulated at the first and second trophic levels.

The effect of various heavy metals on human beings and plants is reviewed by Mhatre (1991). Biochemical effects of trace metals at subcellular level is reviewed by Viarengo (1985). Molybdenum, Manganese, Copper, and Iron have been demonstrated to be essential nutrients for all algae (Round, 1973). Vanadium, Zinc and Cobalt are necessary for healthy growth and reproduction of some species (Noda and Horiguchi, 1971). Cationic uptake mechanisms have been developed by algae to absorb and concentrate nutrients from the surrounding

medium, even when metal concentrations are very low. This mechanism may also be used to uptake non essential or toxic elements. Most algae have the capacity to uptake most heavy metals to some extent. The major mechanisms used for this purpose are divided into passive and active. Sorentino (1979) and Venkataraman et al. (1992) summarised the literature available under these heads. The toxicity of heavy metals to algae is dependent on factors such as degree of chelation, concentration of cells and nutrients, physiological state of cells, salinity and temperature (Mowat and Reid, 1977). The effect of various heavy metals on the structure and physiology of algae at different concentration is given below.

Mercury

It is an extremely toxic pollutant which enters the environment from both natural and anthropogenic sources. Mercury affects membrane permeability in a number of species by changes in cell volume and density (Venkataraman et al., 1992). It causes potassium loss and change in cation exchange capabilities (Fujita et al., 1978). Nuzzi (1972) reported 50% reduction of photosynthesis in Microcystis pyrifex with 50 µg/l of mercury as $HgCl_2$. Venkataraman (1992) reported that marine algae are able to concentrate mercurials up to more than 100 times the concentration in the surrounding water. Mercury toxicity appears to be a consequence of the metal capacity to form stable mercaptides with proteinic thiol groups (Benesch and Benesch, 1952). The interaction of mercury with the enzyme generally results in enzymatic inactivation and metabolic

inhibition (Eichhorn, 1974). Sorentino (1979) reviewed the various biochemical and physiological mechanisms associated with mercury intake.

Copper

It is an essential micronutrient for algae. Copper is a constituent of plastocyanin, which affects the electron transport from cytochrome to the photocatalyst P_{700} in the photosystem I (Markley et al., 1975; Gregory, 1977). The use of copper sulphate as an algicide was described as early as 1904. It is a common practice to control the algal blooms in reservoirs by the addition of cupric sulphate in concentration between 110 and 660 ng/ml. (Courchene and Chapman, 1975). Sorentino (1979) reviewed in detail the biochemistry and physiology of copper toxicity and tolerance. Gupta et al. (1985) reviewed the response of blue green algae to copper.

Lead

Photosynthesis and cell division of various algae are inhibited by lead (Rivkin, 1979). Various reports show that lead is toxic to phytoplankton only at very high concentrations. It has been unequivocally demonstrated that lead ions first are quickly and reversibly bound to the cell surfaces and only later penetrate to deeper sites, where they may be expected to exert their main biological effects (Blades and Lewin, 1976). Higher concentrations of lead can also inhibit respiration (Woolery and Lewin, 1976).

Cadmium

Toxicity and accumulation of cadmium with respect to algae and cyanobacteria is extensively reviewed by Vymazal (1987). There are reports saying that cadmium disturbs cell division and stimulates the formation of microcolonies and spores, symptoms of environmental stress (Anikeeva et al., 1975). Kogan et al. (1975) reported that the presence of cadmium increased the ultraviolet induced mutation in Chlorella pyrenoidosa. Asterionella formosa accumulated most of the cadmium in the cell contents and Phaeodactylum tricornutum retained in the cell wall (Cain, 1980). In marine phytoplankton assemblages, cadmium decreased photosynthetic rates at concentrations as low as 10 ng/ml. (Monahan, 1967 a; Zingmark, 1972)

Zinc

At high concentration, zinc is toxic. But it is essential for the healthy growth of algae. Fucus and Ascophyllum grown in sea water accumulated 138 and 236 ppm of zinc and 398 and 278 ppm when grown in river water respectively (Foster, 1976). Chlorophyll content and carotenoid: chlorophyll ratio decreased after the addition of zinc to the medium (Fillips and Pallaghy, 1976). In Euglena 7.5 ng/ml of zinc inhibited enzyme synthesis and prevented growth (Mills, 1976). According to Venkataramn et al. (1992) the zinc concentrating ability of algae could be utilised to treat specific zinc related deficiencies in humans.

3.2 MATERIALS AND METHODS

Unialgal cultures of Synechocystis Salina were used for the experiment. Inocula for the experiment were taken after thorough shaking from 15 days old cultures, incubated at room temperature (8:16 light, dark at 3000 lux). The inocula were discharged @ 2.5 ml/100 ml of the medium. Allen and Nelson's medium was prepared with clean autoclaved sea water (32 ‰). Solutions of stable cadmium, zinc and mercury were prepared by dissolving their chlorides while the solutions of lead and copper were prepared by dissolving their nitrate and sulphate respectively. The experiments were conducted at concentrations of 0.1 ppb, 1 ppb and 10 ppb of above metals. Each concentration was made in hexaplicates (100ml) in conical flasks and was incubated at 8:16 light, dark cycle at 3000 lux. Controls were prepared by dissolving equivalent amounts of the corresponding sodium salts: NaCl, $NaNO_3$ and Na_2SO_4 respectively to compensate for the possible effects of the anions. Cl^-, NO^-_3 or SO_4^{2-}

Growth was measured at definite intervals by appropriating the optical density at 665 nm using a spectrophotometer as described in the previous chapter.

The aim of this experiment was to study the effect of the above heavy metals at very low concentrations.

3.3 RESULTS AND DISCUSSIONS

Mercury

At higher concentrations, mercury is extremely toxic. The primary effect of mercury on cells appears to be binding with sulf hydryl groups on surface membrane proteins. Mercury has an extremely high affinity for sulfhydryl groups (Leland and Kuwabara, 1985). As virtually all proteins have sulfhydryl groups and their conformations are dependent on these functional groups, at some concentration, mercury can inhibit the function of all enzymes. The important lesion for mercury toxicity in plants and animals appears to involve cell membrane and affect membrane permeability. One important effect is that sodium leaks in and potassium leaks out with subsequent volume shifts in cell (Leland & Kuwabara, 1985).

In the present study, using sublethel concentrations of mercury (0.1 ppb, 1 ppb and 10 ppb of Hg), it is showing an enhanced rate of growth over the control (Fig. 23-31 & Table 6)·The result is found to be statistically significant ($P < 0.01$). There are reports saying that at 1 ppb of Hg, growth and photosynthesis of several phytoplankton communities are inhibited (Harris et al., 1970; Hannan and Patouillet, 1972; Hannan et al., 1973; Holderness et al., 1975). But in Anabaena inaequalis, growth, photosynthesis and acetylene reduction were reduced only at 500 ppb. (Venkataraman et al., 1992). They also suggested that marine algae can concentrate mercurials up to more than 100 times the concentration found in surrounding water.

Copper

Copper is an essential component of many enzymes. However, not all of these enzymes activities are so decreased in copper deficiency that they are metabolically limiting (Leland & Kuwabara, 1985). The importance of copper as a trace nutrient in plant metabolism is well recognized. It is necessary for plastocyanin synthesis and functions in photosynthetic electron transport. It is also involved in enzymatic oxidation of ascorbate and polyphenolic compounds (Bidwell, 1974). The optimal concentration range for essential trace elements in aquatic environments may be very narrow (Leland & Kuwabara, 1985). Cyanobacteria are considered to be most sensitive to copper (Brand et al., 1986). Toxic effect of copper on different forms of cyanobacteria was studied by Gupta et al. (1985).

In the present studies Cu at very low concentrations (0.1 ppb, 1 ppb,10 ppb) enhances growth over control. However with prolonged incubation, it can retard the growth. This may be because of the reduced nitrate level in an aged medium. Gupta et al. (1985) reported reduced copper toxicity at high nitrate concentrations. Among heavy metals, trace amount of copper is required for various metabolic processes. From the studies it is clear that the concentration of Cu used is within these limits. (Fig. 23-31 & Table 6)

Cadmium

Organisms can acquire Cd through direct contact with contaminated air and/or water through the food chain. The growth of

Chlorella is stimulated by low concentration of Cd and inhibited by high concentrations (Venkataraman et al., 1992). As observed with copper, the cyanobacteria are most sensitive to cadmium toxicity (Brand et al., 1986)

In the sublethel concentrations (0.1 ppb, 1 ppb and 10 ppb), it is found that Cd enhances growth over the control. (Fig. 23-31 & Table 6)

Lead

Lead retarded the flow of electrons in electron transfer reactions of plant mitochondria (Koeppe & Miller, 1970) and chloroplasts (Bazzaz and Govindjee, 1974) and thus can be expected to have a detrimental effect on both respiration and photosynthesis. Sublethal concentrations of lead (2.5 - 10 mg/l) retard population growth by delaying cell division and daughter cell separation. Lethal concentrations cause inhibition of growth and cell death (Leland and Kuwabara, 1985). Whitton, (1970) reported increased tolerance of organisms for lead over copper and zinc. Lead is adsorbed strongly by biological membranes. There is a high passive affinity of lead for mitochondiral membranes (Bittel et al., 1974). Very high lead concentration can occur in benthic algae in streams receiving effluents from lead mines and mills (Gale et al., 1973).

But in the present study, all the concentrations used enhanced the growth of S. Salina over the control. (Fig. 23-31 & Table 6)

Zinc

Zinc is a ubiquitous trace metal essential for normal cell differentiation and growth both in plants and animals. It is essential because it is an integral part of a number of metalloenzymes and a cofactor for regulating the activity of specific zinc dependent enzymes. The concentration of zinc in cells can govern many metabolic processes specifically carbohydrate, fat and protein metabolism and nucleic acid synthesis or degradation through initiation and/or regulation of the activity of these enzymes (Leland and Kuwabara, 1985). Some zinc dependent enzymes contain metal binding sites that are essential for structural stability. Zinc is also an essential constituent of DNA dependent DNA polymerase and RNA polymerases. These enzymes have a key position in nucleic acid metabolism and hence also in protein biosynthesis (Leland and Kuwabara, 1985).

Zinc is usually toxic only at higher concentrations. In the present investigation, at lower concentrations (0.1 ppb, 1 ppb and 10 ppb) zinc enhances the growth of Synechocystis over the control. Price and Quigley (1966) reported that specific growth rates of Euglena gracilis are linear functions of internal zinc concentrations. Since Zinc is an essential constituent of a number of enzymes required for the growth and development of cells, it is quite convincing that at lower concentrations zinc enhances growth. (Fig. 23-31 & Table 6)

Factors affecting Toxicity

It is not clearly understood why heavy metals like mercury, lead and cadmium enhance growth of Synechocystis salina at lower concentrations. The present result is comparable to the observations of Ibragim and Patin (1976). They found that at a concentration of 1 µg/l of copper, cadmium or lead, primary production rates were increased over that in the control, even on the first day after the metals had been added. According to them at 1 µg/l, mercury was either nontoxic or stimulatory. Eichhorn et al. (1969) suggested that the binding of metallic cations to enzymes could alter their activity not only by inhibiting but also by stimulating the catalytic function of the enzymes. It could be anticipated that the effective concentration of metals available to the cells may be far less, since toxicity depends on factors such as degree of chelation, concentration of cells, nutrients, physiological state of cells, salinity and temperature (Mowat and Reid, 1977). Mayers et al. (1975) observed decrease in net charge of cell surfaces caused by increasing salinity. This is as a result of interaction of cations in sea water with the negative groups on the surfaces. So increased salinity will increase the nonavailability of space to be occupied by heavy metals which in turn reduce the toxicity. According to Davies (1978) the toxicity of heavy metals is dependent upon the following factors:

a. Phytoplankton species

b. Composition of the sea water, supporting the plankton

c. The cell population

d. Changes in the metal tolerance of the cells or in the chemical state of the metal during the period of growth.

e. Concentration of the metal

f. Temperature, and

g. Level of illumination

Davies (1978) also suggested the better tolerance of phytoplankton in the presence of metal chelators. High concentration of nutrient ions such as nitrate and phosphate may also reduce the toxic effect. According to Gupta et al. (1985), the nutritional status of an organism may be an important factor while determining the heavy metal toxicity.

Peterson et al. (1984) pointed out that metal toxicity to algae is a highly pH dependent phenomenon. They have suggested that both inorganic and organic complexation of metals generally increase with increase in pH, and metal solubility decreases causing reductions in the free ion pool. Copper, lead and zinc speciation is much dependent upon the pH while cadmium and mercury are the least affected (Freedman et al., 1980; Zirino and Yamamoto, 1972; Peterson et al., 1984).

Monitoring the pH of the growth medium at 5 day intervals revealed that it increases regularly until it reaches a constancy around 9. This could be explained as a result of increased nitrate utilization, which leaves K^+ in the medium in the form of KOH. This is similar to the observations made by Egorov (1985). This increased pH might have reduced the toxicity further.

Struempler (1973) studied the adsorption of different heavy metals on various containers and found that various heavy metals are adbsorbed to the borosilicate glass at a basic pH. Since the pH of sea water is basic, adsorption of heavy metals to the glass might have reduced the stress of heavy metals on Synechocystis salina. It is also suggested that in growth experiments algae liberate products capable of forming complexes with heavy metals (Leland and Kuwabara, 1985).

Ibragim and Patin (1976) ascribed the reduction in toxicity in the longer term experiments to "biological dilution". i.e., the decrease in the metal burdens of the plant cells as they increased in number in the presence of fixed amount of metal. A decrease in the total metal concentrations due to losses on the container would have reinforced this effect. Davies (1978) suggested that, a further possible explanation of the apparent moderation in the effects of the metals could be that the phytoplankton which grew during the experiment were more metal resistant than those initially present. The present observations are also in concordance with the findings of Sreesudha (1989). According to her heavy metals like Cu, Mn & Zn which are toxic at higher concentrations may be growth promoting at lower concentrations. Since the effective concentration of free metals available in the medium depends on factors such as pH, speciation, complex formation, salinity, nature of containers used etc. and each factor is specific for each metal, the results available cannot be compared in a meaningful way.

Table 6

Con. of metal	Metal species	Age of Culture (Days)								
		6	10	14	20	25	31	35	41	49
		x 10^4 cells/ml								
0.1 ppb	Cu	30 ± 8	53 ± 8	94 ± 15	123 ± 28	146 ± 32	159 ± 30	183 ± 22	221 ± 24	202 ± 23
	Hg	24 ± 7	69 ± 27	98 ± 5	139 ± 16	184 ± 14	210 ± 11	234 ± 10	270 ± 32	295 ± 26
	Zn	22 ± 8	52 ± 17	93 ± 10	138 ± 11	166 ± 20	187 ± 9	201 ± 04	256 ± 18	262 ± 16
	Pb	24 ± 6	49 ± 10	105 ± 23	139 ± 10	165 ± 20	184 ± 14	219 ± 18	252 ± 12	242 ± 14
	Cd	36 ± 11	34 ± 11	97 ± 29	119 ± 12	142 ± 17	155 ± 17	178 ± 31	205 ± 42	199 ± 43
1 ppb	Cu	22 ± 6	53 ± 12	92 ± 5	137 ± 18	169 ± 27	169 ± 22	163 ± 16	195 ± 15	180 ± 11
	Hg	22 ± 4	49 ± 4	92 ± 18	143 ± 11	178 ± 25	200 ± 16	237 ± 27	257 ± 9	264 ± 15
	Zn	24 ± 4	86 ± 18	114 ± 12	139 ± 14	174 ± 23	196 ± 17	213 ± 23	258 ± 16	263 ± 7
	Pb	28 ± 6	63 ± 11	97 ± 18	129 ± 19	146 ± 13	161 ± 24	190 ± 24	206 ± 30	183 ± 28
	Cd	23 ± 14	28 ± 16	125 ± 30	122 ± 32	158 ± 23	186 ± 36	199 ± 24	232 ± 18	229 ± 22
10 ppb	Cu	22 ± 2	41 ± 6	73 ± 21	125 ± 9	172 ± 14	169 ± 9	156 ± 21	200 ± 14	191 ± 16
	Hg	30 ± 7	53 ± 12	98 ± 14	140 ± 17	188 ± 25	195 ± 24	210 ± 20	257 ± 33	242 ± 50
	Zn	30 ± 8	81 ± 18	119 ± 14	138 ± 20	176 ± 14	187 ± 8	219 ± 22	268 ± 15	255 ± 15
	Pb	35 ± 3	65 ± 9	106 ± 11	142 ± 15	153 ± 23	168 ± 24	199 ± 25	234 ± 41	235 ± 32
	Cd	27 ± 9	42 ± 21	119 ± 9	141 ± 11	185 ± 27	131 ± 23	218 ± 39	255 ± 24	263 ± 62
Control		23	41	92	136	119	147	138	248	222

Effect of sublethal concentrations of 5 different heavy metals on the growth of Synechocystis salina

* ANOVA significant ($P<0.01$)

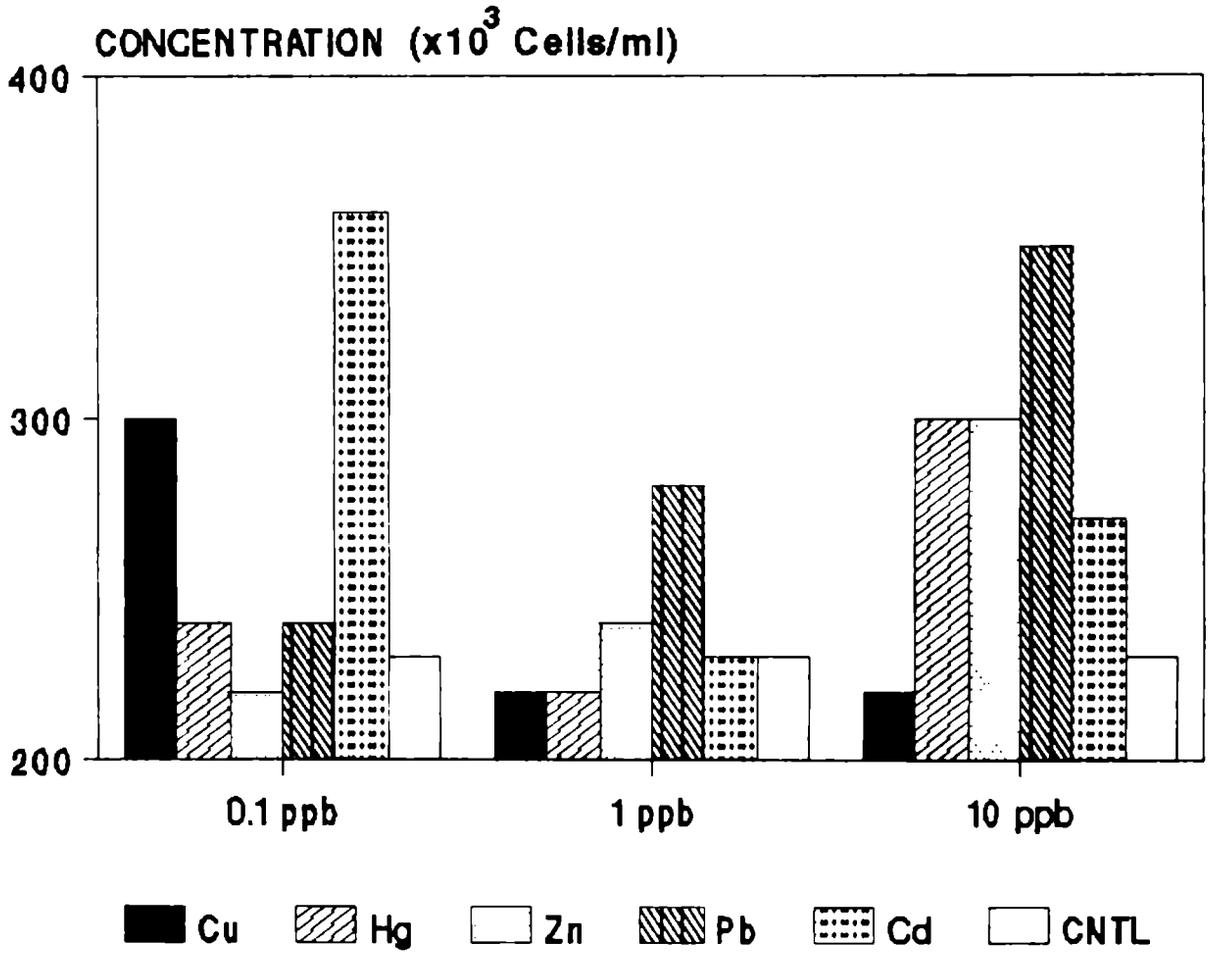


Fig. 23. Effect of different heavy metals on the growth of S. salina at different concentrations-after 6 days of inoculation.

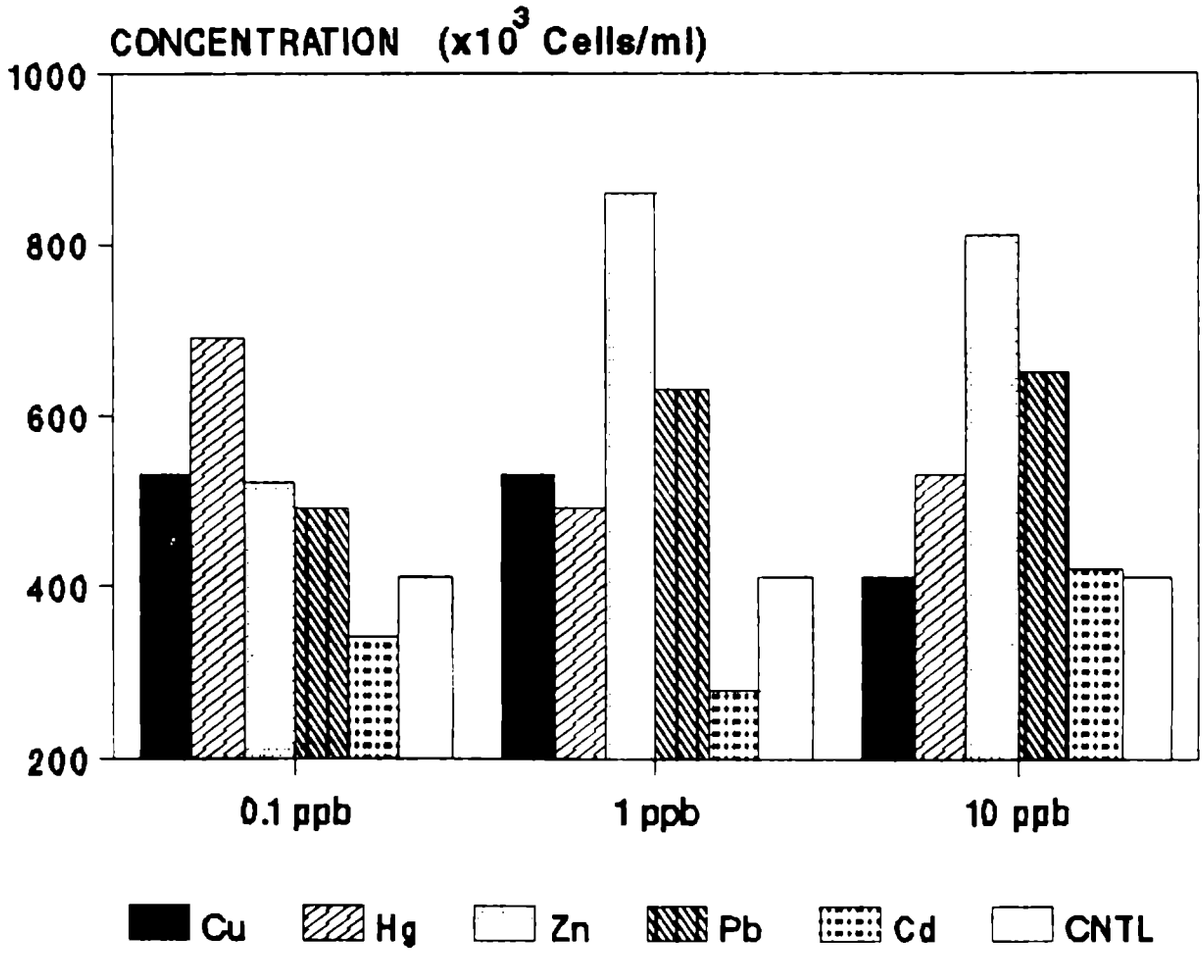


Fig. 24. Effect of different heavy metals on the growth of S. salina at different concentrations-after 10 days of inoculation.

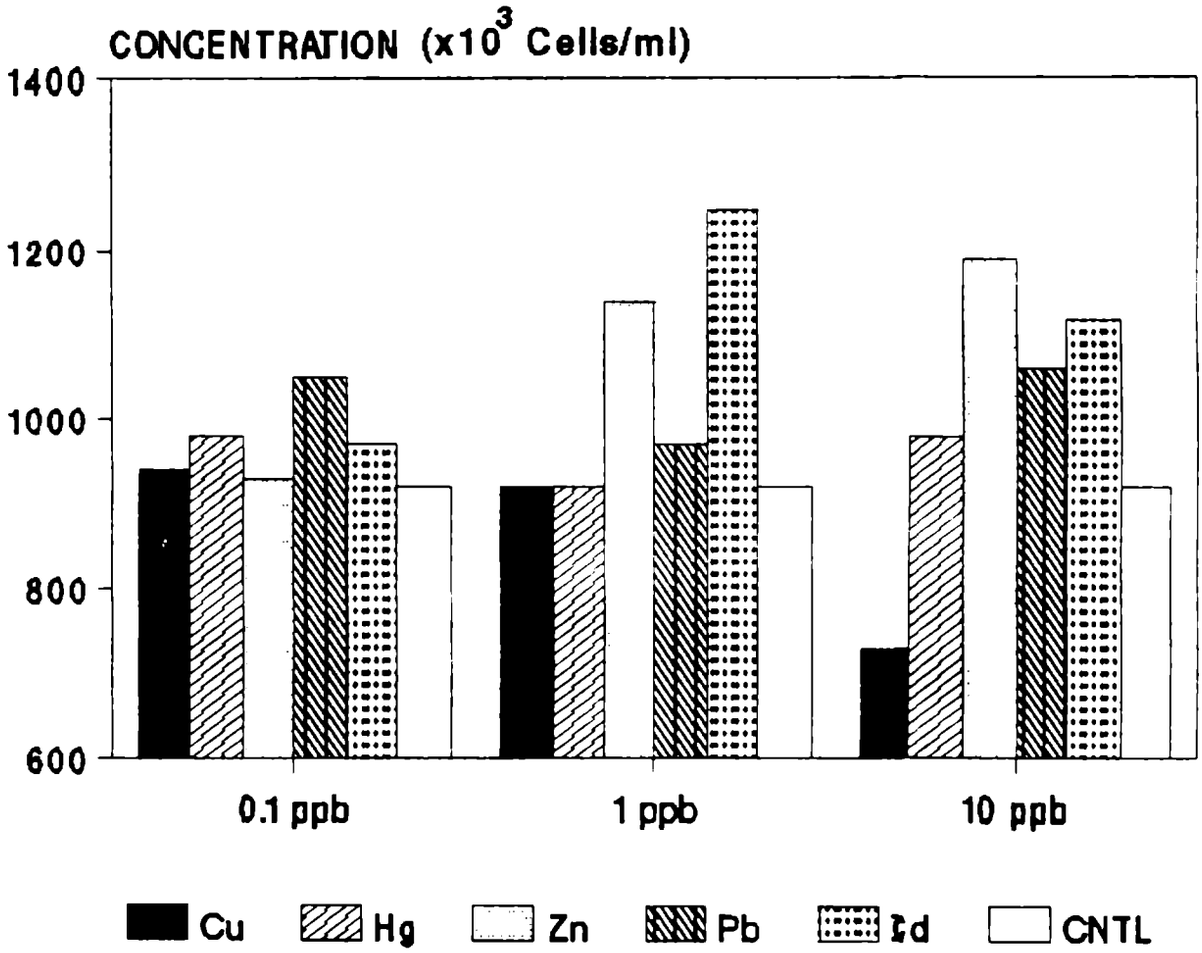


Fig. 25. Effect of different heavy metals on the growth of S. salina at different concentrations-after 14 days of inoculation.

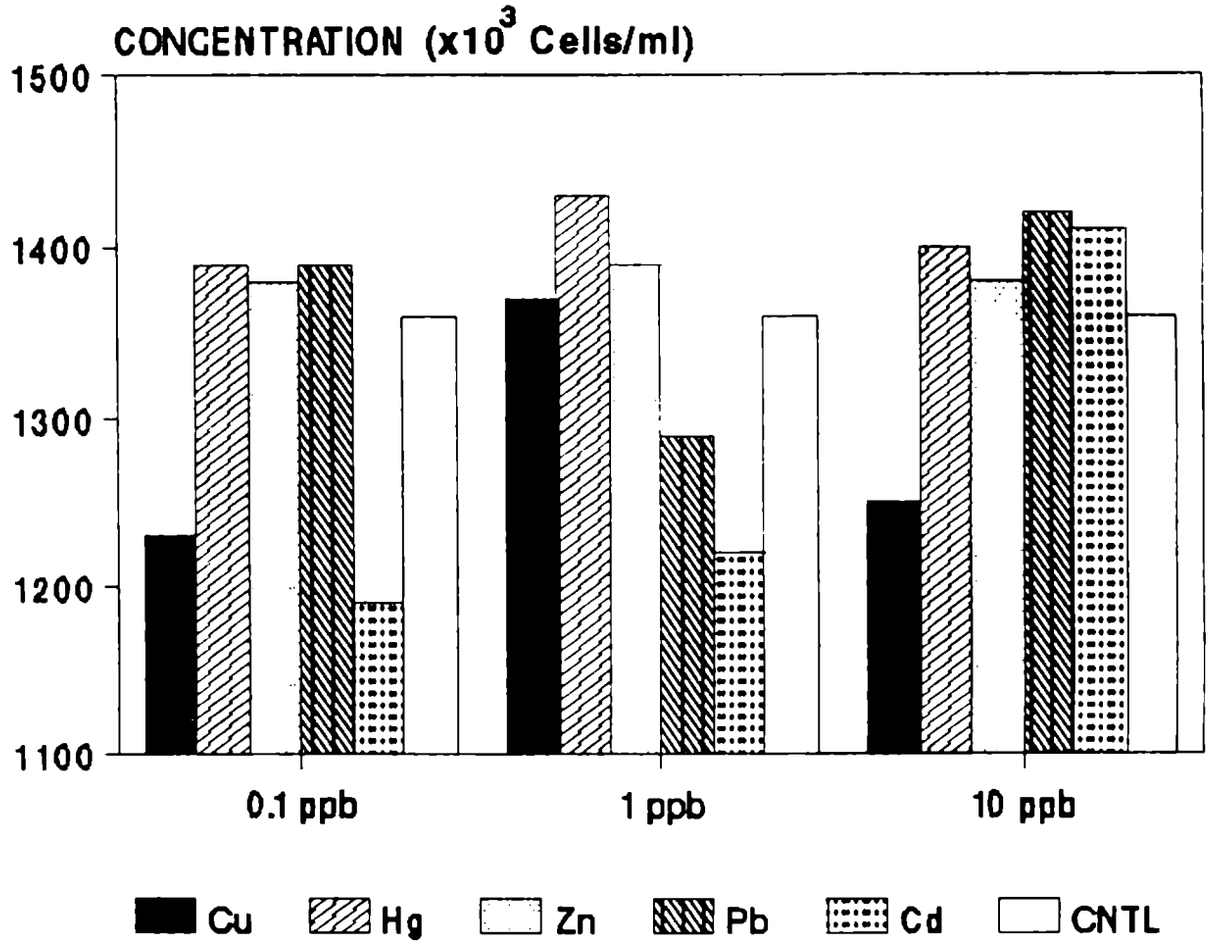


Fig. 26. Effect of different heavy metals on the growth of S. salina at different concentrations-after 20 days of inoculation.

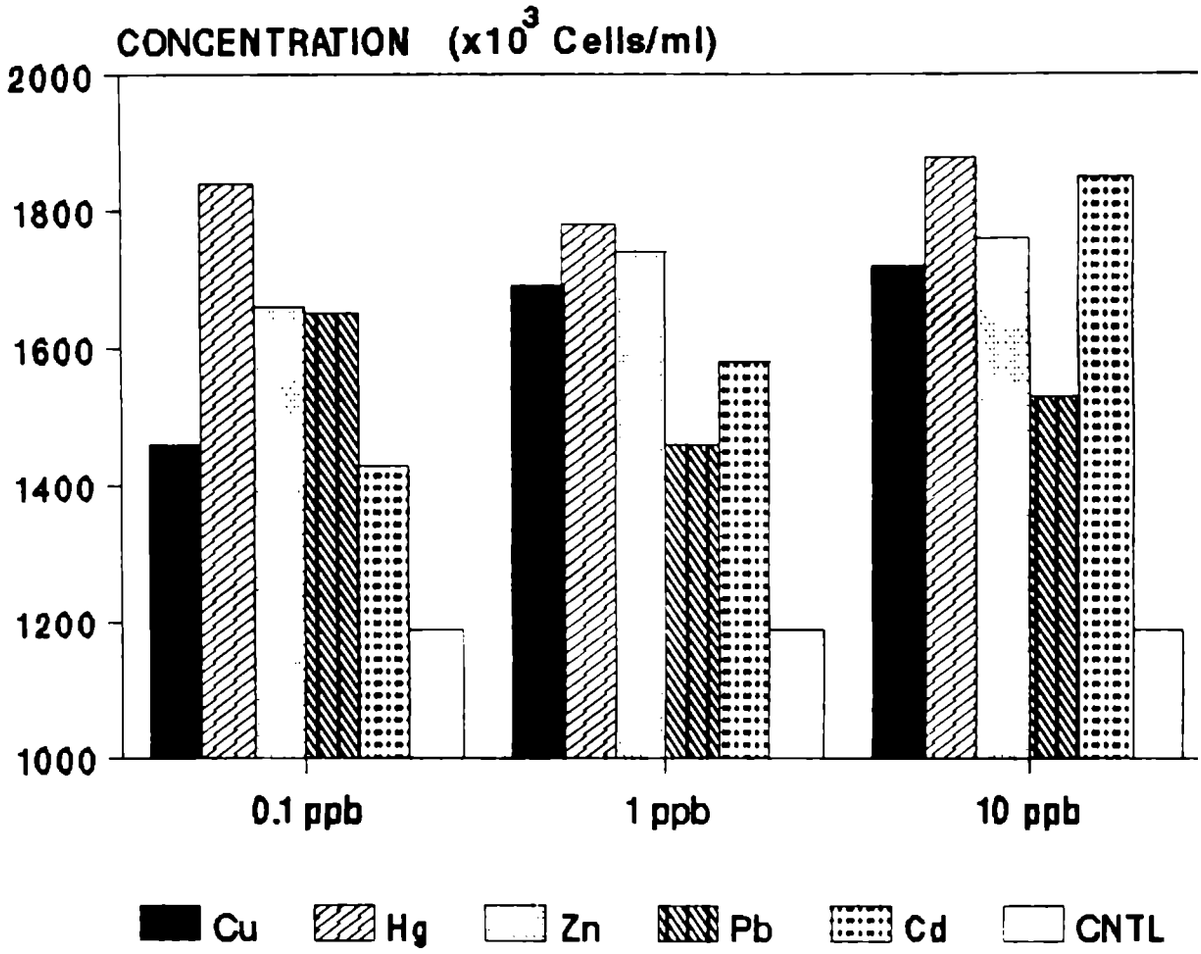


Fig. 27. Effect of different heavy metals on the growth of S. salina at different concentrations-after 25 days of inoculation.

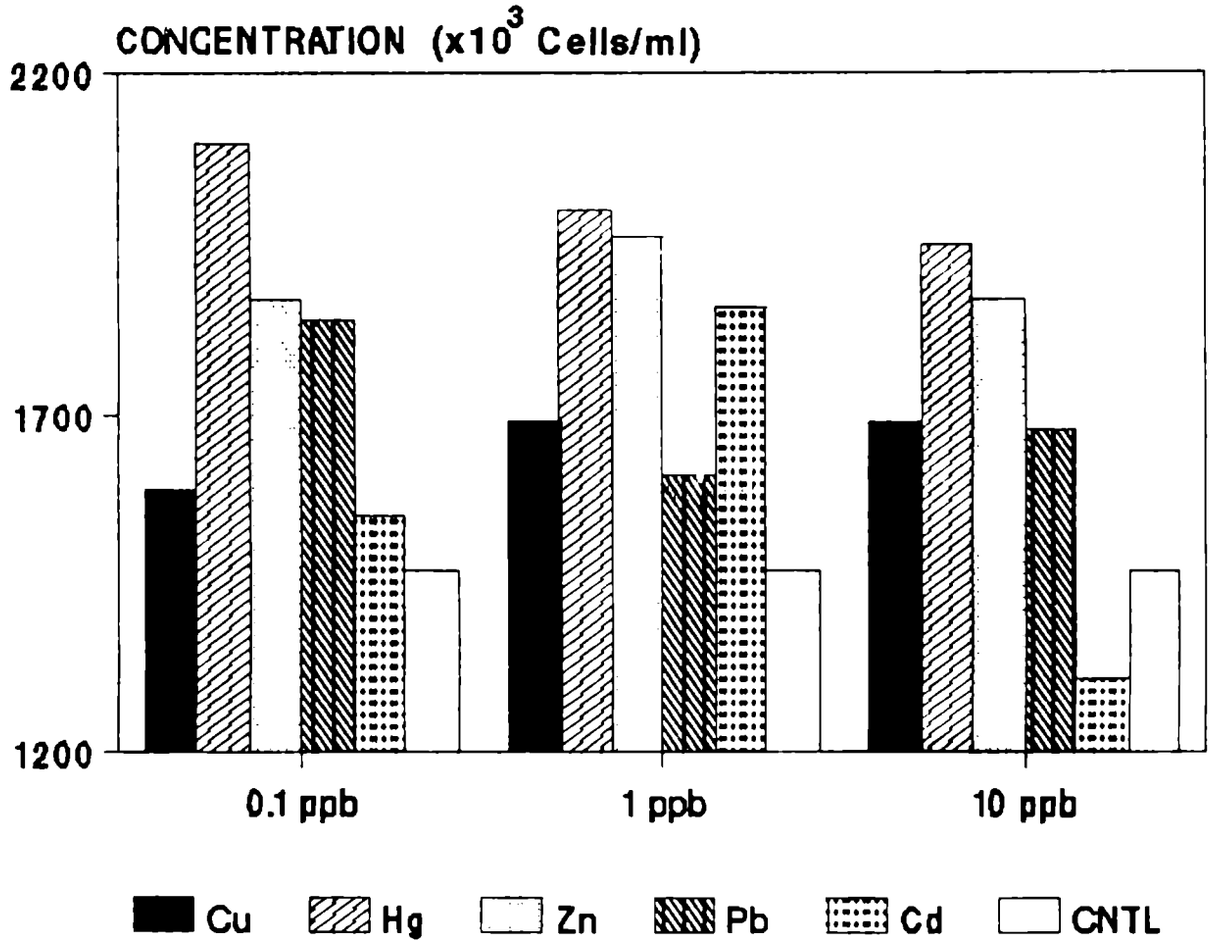


Fig. 28. Effect of different heavy metals on the growth of S. salina at different concentrations-after 31 days of inoculation.

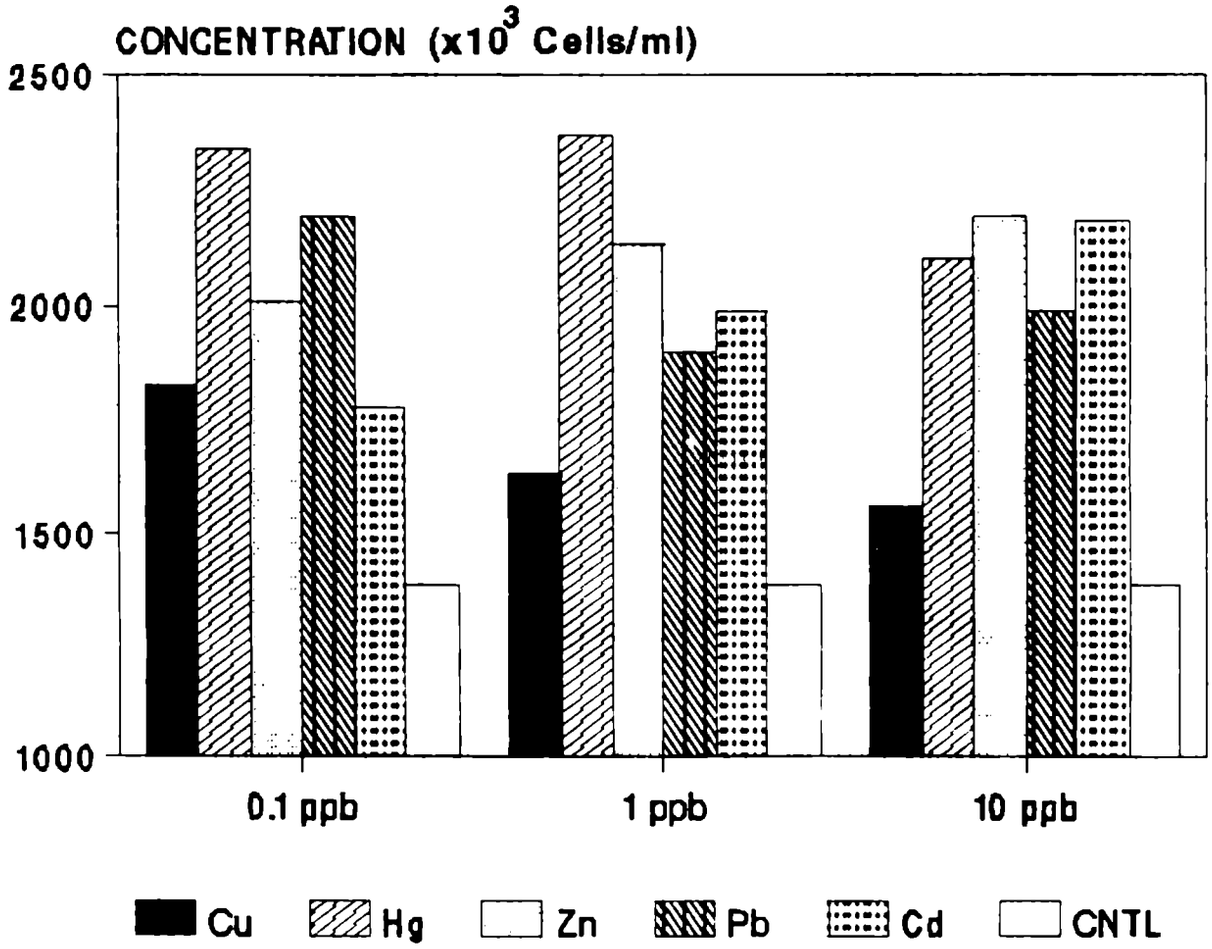


Fig. 29. Effect of different heavy metals on the growth of S. salina at different concentrations-after 35 days of inoculation.

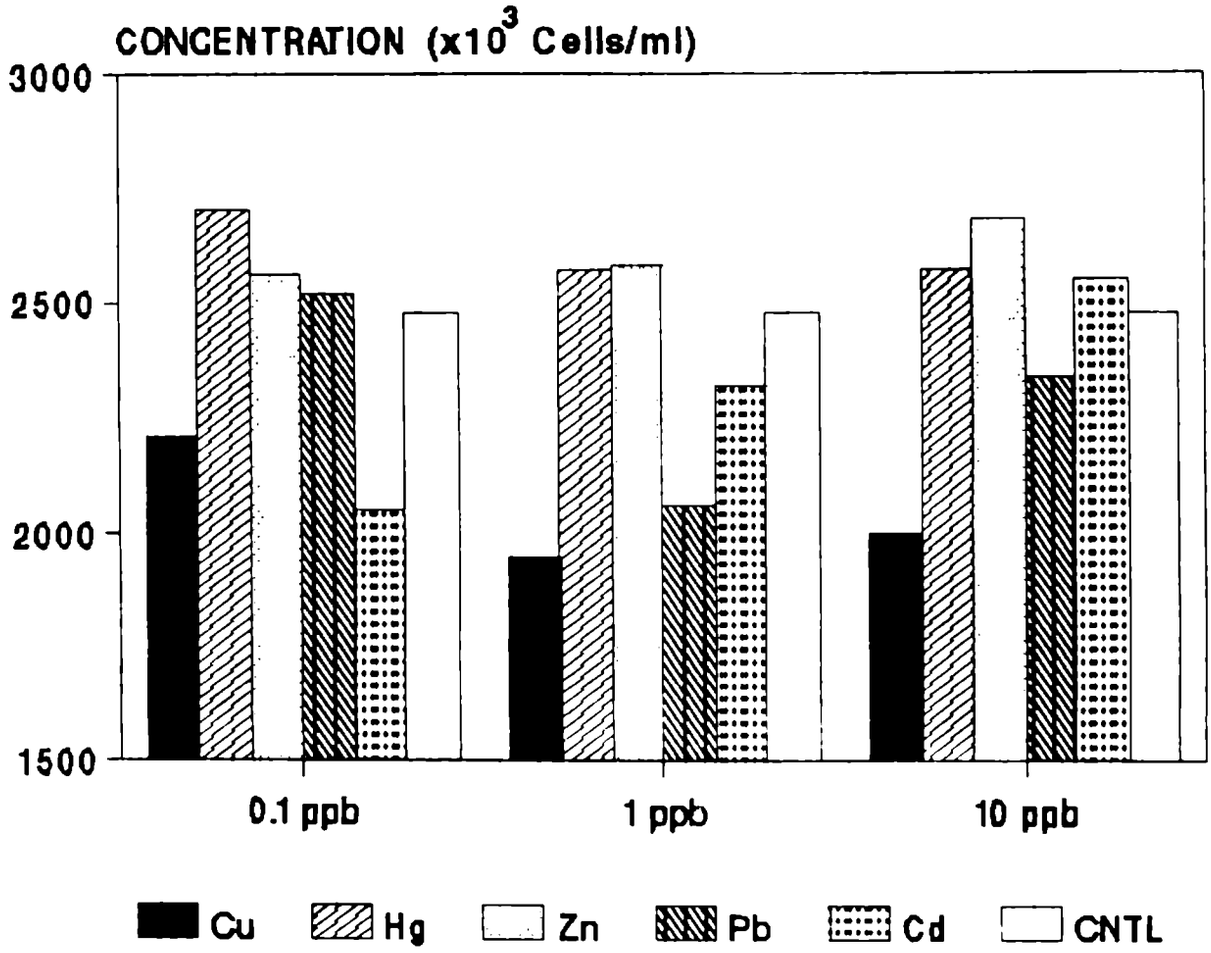


Fig. 30. Effect of different heavy metals on the growth of S. salina at different concentrations-after 41 days of inoculation.

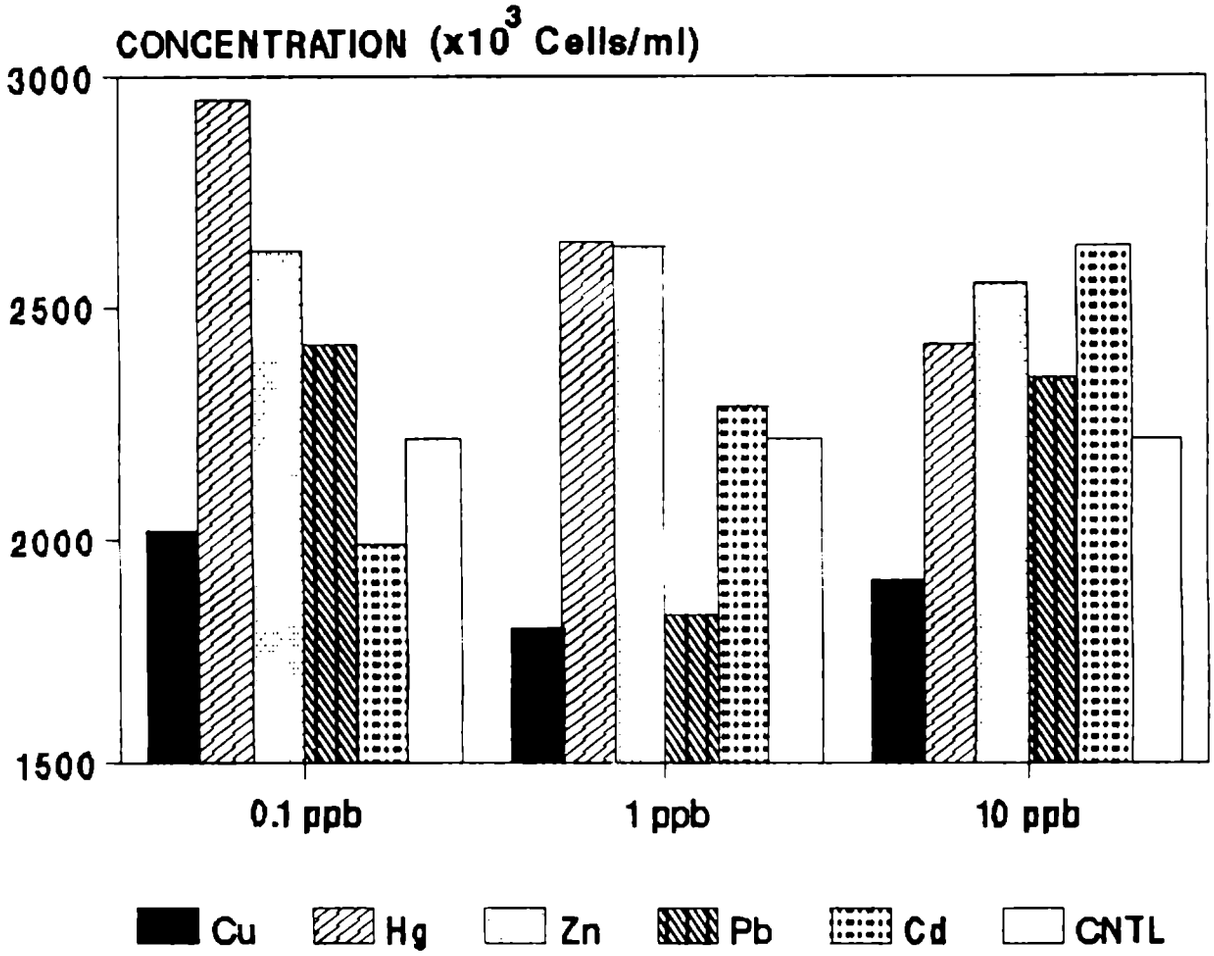


Fig. 31. Effect of different heavy metals on the growth of S. salina at different concentrations-after 49 days of inoculation.

CHAPTER 4

4. HEAVY METAL UPTAKE BY SYNECHOCYSTIS SALINA WISLOUCH

4.1 BATCH CULTURE

4.1.1 INTRODUCTION

Selective uptake and accumulation of heavy metals by algae has gained significance in the present context of legislations of effluent treatment, pollution control, hazards to organisms at higher trophic levels and to produce safe, good grade algae for their multifaceted use (Venkataraman et al., 1992). According to them the uptake and tolerance of different heavy metals have an interplay with

a. Each algal form
b. Its sensitivity
c. Concentration in the environment
d. Composition of medium
e. Toxicity of individual heavy metals and
f. Cation exchange capabilities

The last decade has seen greater interest and thrust on heavy metal uptake by algae from the point of view of possible removal of problematic toxicants from effluents, pollution control and safety of cultivated algae which may remove them from the medium containing heavy metals inadvertantly (Venkataraman and Becker, 1986). Thus uptake, accumulation and consequent toxicity in algae assumes a practical significance.

Heavy metals have important industrial applications and those using them often cause environmental contamination. Several heavy

metals have no known biochemical role and are toxic even at low concentrations. The introduction of heavy metals in to aquatic habitats creates potential threat to aquatic food webs. Heavy metals enter the food chain through uptake by plants and ingestion by animals. The fraction which is assimilated either accumulates in tissues or is metabolised by the organism. Algae can also accumulate and concentrate heavy metals to levels substantially higher than those found in the surrounding environment. Such concentration by primary producers could be hazardous to organisms at higher trophic levels (Venkataraman et al., 1992). According to Fisher (1985) picoplankton based food webs would represent a major route for the movement of particle reactive metals in marine ecosystem.

Though accumulation of heavy metals in cultivated good grade algae is generally considered undesirable, it is possible to use it to advantage in some cases. Desirable concentration of specific metal in some cultivated algae can be achieved by introducing this metal in the medium and this high metal (such as Zn) containing biomass can be produced to correct specific metal related deficiencies in humans (Venkataraman et al., 1992). Phytoplankton populations present surprisingly large surface areas to the sea water or culture medium in which they are growing. It is therefore not surprising that adsorption on to the outside of cells represents an important aspect of metal uptake by phytoplankton (Davies, 1978).

In general all heavy metals are inhibitory to growth, pigments, macromolecule content, nutrient uptake (NH_4^+, NO_3^-, PO_4^{-3}), ATP content, photosynthesis (carbon fixation and oxygen evolution), and

the enzymes of nitrogen metabolism such as nitrogenase, nitrate reductase, glutamine synthetase, urease and alkaline phosphatase (Venkataraman et al., 1992).

There are differences in the degree to which various species of algae react to the environmental concentration of heavy metals. The essential elements such as Cu, Zn, Fe and Co have important biochemical functions in the micro algae. The concentration of trace elements is generally higher in the organism than in the aquatic system and if it is otherwise, the metal content in the organism is regulated by homeostatic control mechanism (Bryan and Hummerstone, 1977). At higher concentrations of heavy metals the homeostatic mechanism ceases to function and the heavy metals act as toxicants. Thus during bioaccumulation of heavy metals the organism may be damaged (Venkataraman et al., 1992).

For several years it has been repeatedly suggested by many authors that heavy metals from polluted aquous systems may be removed by phytoplanktonic algae. It is concluded that this method, including the separation of the metal saturated algae from the medium is an economic method for removing heavy metals from waste waters resulting in high quality reusable effluent water and valuable algal biomass, which could be used for different purposes (Becker, 1994). It is well established that several marine and fresh water algae are able to take up various heavy metals selectively from aquous media and to accumulate these metals within their cells (Lorenz and Krumbein, 1985; Garnham et al., 1993; Fisher, 1985; Fisher et al., 1984). However the efficiency of the

concept of using algae for heavy metal removal will be determined principally by the following parameters.

1. Growth rate of the algae
2. Metal concentration factor of the algae
3. Concentration of heavy metals in the medium
4. Desired percentage of metal removal from the medium, and
5. Metal recovery in relation to capital and operation costs (Becker, 1994)

4.1.2 MATERIALS & METHODS

Inorder to study the heavy metal uptake the best growth medium and the best set of conditions were provided (Allen & Nelsons medium, 3000 lux). The experiments were performed in duplicate. Salts of the 5 heavy metals (Hg, Cu, Pb, Cd & Zn) at 1 ppm metal level were used for the experiments. A local control was always maintained. 8 h light 16 h dark cycle was always maintained. samples were drawn at 5 days intervel upto 60 days and growth was measured using a UV-visible spectrophotometer (Hitachi) by noting optical density at 665 nm. Samples were withdrawn from the medium at definite growth intervals. Algal cells present in 25ml of the medium were harvested by centrifugation. The pellet obtained thus was resuspended in 10ml of 0.I N HCl, shaken well and centrifuged further. The supernatant was discarded and the pellet was carefully transferred into the digestion tubes by rinsing with 0.I N HNO_3. The cells were digested for 3 hrs in 5ml Con H_2SO_4. When the tubes became colourless, the sample was carefully transferred to a standard flask and was made upto 25ml using 0.I N HNO_3. Concentration of the heavy metals in the samples was detected using

a atomic absorption spectrophotometer (Perkin - Elmer, model No. 2380)

4.1.3 RESULTS & DISCUSSION

From the results it is clear that Synechocystis salina wislouch can concentrate lead and cadmium. Phytoplankton populations present surprisingly large surface areas to the sea water or culture medium in which they are growing. The adsorption of heavy metals on to the outsides of cells represents an important aspect of metal uptake by phytoplankton (Davies, 1978). He explained the physico chemical nature of the surface of phytoplankton as consisting of a mosaic of interspersed cationic and anionic exchange sites, provided by carboxylic, sulphydryl, phosphatidic, amino and other groups. The net charge on the surface being related to the degree to which the sites are occupied by protons and other ions present in sea water. The initial uptake of a positively charged heavy metal ion can then be envisaged as occurring by the displacement of the cations already occupying the binding sites. Studies by Xue et al. (1988) on the binding of heavy metals to algal surfaces revealed that the functional groups of biological cells bind metal ions in a similar way as solubel ligands. The amount of metal finally bound on to the surface at equilibrium being determined by the relative affinities of the sites for the metal and the sea water cations and also the concentrations of each remaining in solution in accord with the principles of ion exchange (Davies, 1978). According to him the metal once bound on to the surface would be suitably placed for being transported by the cell membrane in to the cytoplasm. Fisher et al. (1984) reported that dead cells accumulated metals comparably

to living celles, indicating that initial association of metal with the cell is governed by adsorption. According to them the degree of metal association with the cells was in direct proportion to the external metal concentration and is explained by the Freundlich isotherm. The early stages of uptake of heavy metals by initially metal free phytoplankton could be explained as being due to rapid adsorption of the metals on to externally exposed binding sites on the cell surfaces followed by passive diffusion - controlled transport in to the cytoplasm at rates proportional to the concentration of surface bound metal (Davies, 1978). Experiment by Fisher et al. (1984) confirmed that accumulation of Cd, Zn, Hg and Ag was directly related to the total metal concentration in solution. Once associated with the plasmalemma the metals may be translocated in to the cell via carrier protein molecules or they may remain bound to cell surfaces (Fisher et al., 1984).

The cellular metal values reported in the present study are for metals firmly bound either on the cell surface or to the interior of the cells, since the cells are washed and centrifuged in 0.1 N HCl. Crist et al. (1981) suggested that the nature of binding of metals to algal surfaces is still largely uncharacterized and probably varies among metals and algal species including covalent bonding to proteins for the highly reactive metals and ionic charge bonding for the less reactive metals. According to Davies (1978) the biochemical basis of heavy metal tolerance is in general, very poorly understood.

The ability of Syncehocystis salina wislouch to accumulate lead and cadmium is highly significant. Toxicity studies on different

phytoplankters by Brand et al. (1986) using copper and cadmium revealed that in general cyanobacteria are most sensitive to both copper and cadmium, compared to other phytoplankters. According to them it is possible that copper and cadmium bind more strongly to the nucopolysacharide cell wall of cyanobacteria than to the fundamentally different types of cell walls of eukaryotic algae. This could result in stronger interference with cell surface properties and functions of cyanbacteria. According to them the high sensitivity of cyanobacteria to the heavy metals is innate because of their primitive cellular features. But the present study is not in agreement with these findings. S. salina could take up both lead and cadmium in appreciable quantities. But it is not strictly obeying the principles of Langmuir adsorption isotherm as suggested by Fisher (1985).

Davies (1978) pointed out the significance of large surface areas in heavy metal uptake. Studies by Fisher (1985) reiterated the significance of surface areas and surface area: volume ratios as a function of cell number and heavy metal uptake.

Being a nanoplankton of 3 μ in size, S. salina can be cultured to the extend of 8 million cells/ml with a division time of 15 hrs and having a growth constant 0.047. One lakh cells will expose a surface area of 2.83 m^2

However all the heavy metals retard the growth of S. salina (Fig.32). Unlike other plant cells, cyanobacterial cell wall is made up of murein, which is a peptidoglycan (Venkataraman & Becker, 1985). Cyanobacteria are having a three membrane system - the

thylakoid membrane, cytoplasmic membrane and an outer membrane. Studies on Synechocystis Sp. PCC 6714 by Jurgens and Weckesser (1985) revealed that the outer membrane is composed of LPS, proteins, lipids and carotenoids. 50% of total outer membrane dry weight is represented by lipopolysaccharides and 30% dry weight by outer membrane proteins. Amino sugar Glc N is a major component in the polysaccharide of lipopolysaccharide fraction. This three membrane system with COOH, SH, PO4 and NH_2 groups provide ample surface area for attachment with heavy metals.

The present study also revealed that physiology of the cell is playing a major role in sequestering the heavy metals. At 1ppm of Hg, growth of S. salina was totally inhibited before two days. With regarding Cu, compared to control, growth is retarded from the beginning itself (Fig. 32). However on 40th day maximum intake is noticed (Fig. 33). Maximum intake of zinc is noticed on 25th day (Fig. 34), for lead 20th day (Fig. 35) and for cadmium 45th day onwards. (Fig. 36). At 1ppm of metal concentration, the toxicity index can be expressed as Hg > Cu > Cd > pb > Zn (Fig. 37). Nevertheless it is important to note that in the initial stages Zn enhances the growth over control. This may be the increased need of this trace metal for rapid cell division better affinity of cell wall for zinc and enhanced metabolic rates in the initial stage.

The differential behaviour of the cell to various metals at different phases of growth may be because of the biochemical differences exhibited by the ingredients of the cell or cell membranes at different growth periods.

Table - 7

Age of Culture (Days)	No. of cells (x 10^3)/ml				
	Control	Cu	Zn	Pb	Cd
05	1470	1090	1410	1520	1140
12	2430	1030	3200	2390	1580
19	3640	760	3170	2460	1770
27	3850	230	2720	2840	1650
30	3260	410	4140	3140	1580
38	6810	1470	4740	4560	1380
43	4140	650	4280	3760	1580
51	7250	1090	5650	5770	2090
55	5330	330	5560	5620	2700
60	8290	270	6010	5560	3550

Effect of different heavy metals (1 ppm) on the growth of Synechocystis salina

Table - 8

Age of Culture (Days)	pH				
	Control	Zn	Cu	Pb	Cd
05	8.55	8.15	8.36	8.22	8.09
12	8.83	8.62	8.22	8.53	8.11
19	8.87	8.80	8.18	8.64	8.24
27	8.95	8.43	8.13	8.70	8.26
30	8.93	8.73	8.20	8.72	8.29
38	9.01	8.84	8.23	8.79	8.28
43	9.1	8.87	8.18	8.79	8.16
51	9.01	8.89	8.13	8.79	8.19
55	9.03	8.91	8.12	8.81	8.29
60	8.60	8.88	8.09	8.74	8.52

Variation in the pH of heavy metal exposed cultures of S. salina at different growth intervels.

Table - 9

Age of Culture (Days)	Cu		Zn		Pb		Cd	
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
05	0.027	0.097	0.064	0.08	0	0.087	0	0.026
12	0.008	0.061	0.04	0.066	0	0.064	0.018	0.006
19	0.003	0.083	0.028	·0 67	0.018	0.135	0	0.005
27	0.002	0.029	0.02	0.1	0.015	0.115	0	0.023
30	0.003	0.155	0.027	0.032	0.008	0.115	0	0.021
38	0.001	0.057	0.013	0.045	0	0.073	0	0.010
43	0.002	0.064	0.023	0.079	0	0.087	0.001	0.032
51	0.027	Cells die out	0.015	0.069	0	0.069	0	0.134
55	0.004	Cells die out	0.021	0.050	0.013	0.071	0	0.110
60	0.002	Cells die out	0.012	0.059	0	0.093	0.001	0.154

Quantity of metal ions (in ppm) per 10^5 cells in control and heavy metal exposed cultures of Synechocystis salina at different growth intervels.

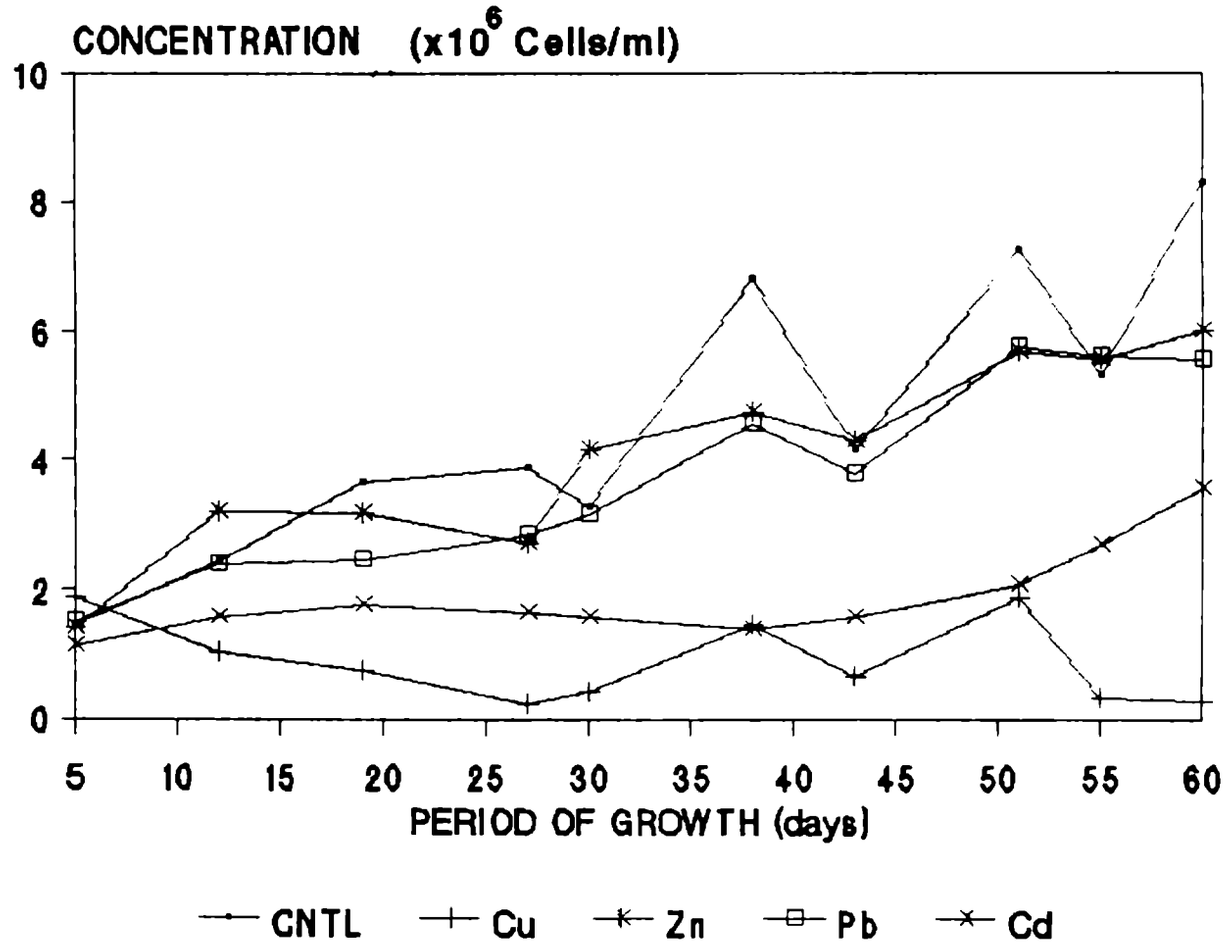


Fig. 32. Effect of 5 different heavy metals at 1 ppm on the growth of S. salina

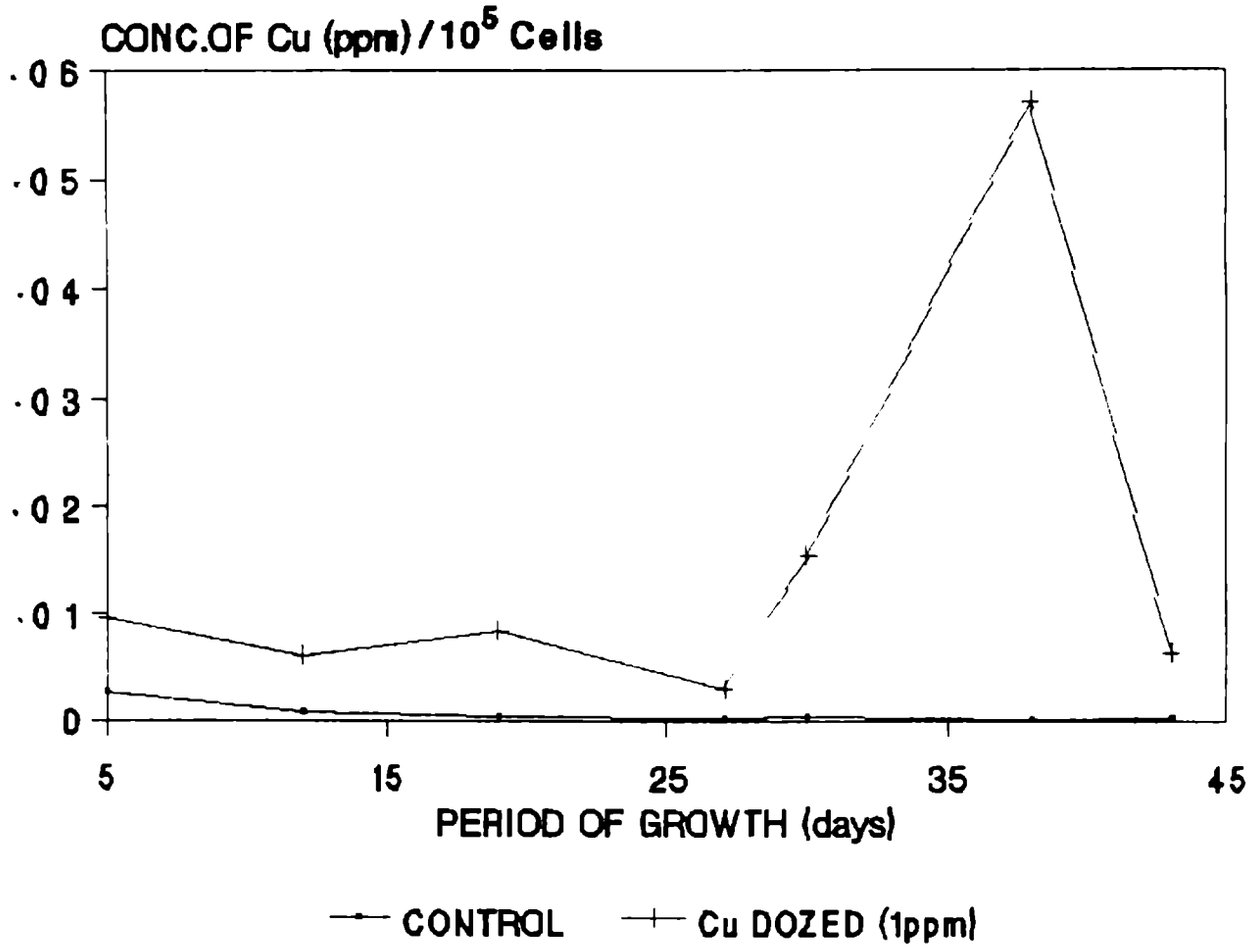


Fig. 33. Quantity of copper in control and copper exposed cultures at different growth intervals.

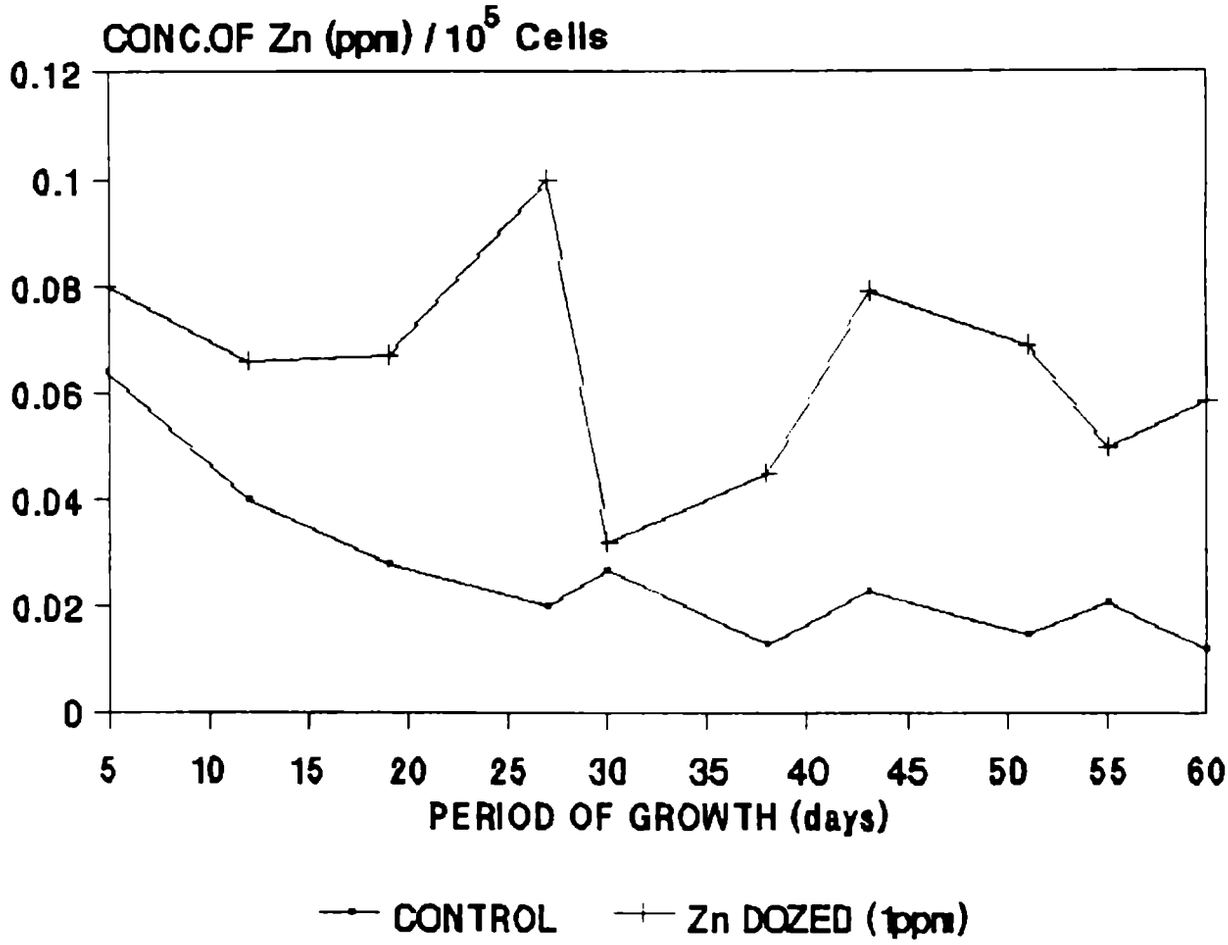


Fig. 34. Quantity of zinc in control and zinc exposed cultures at different growth intervals.

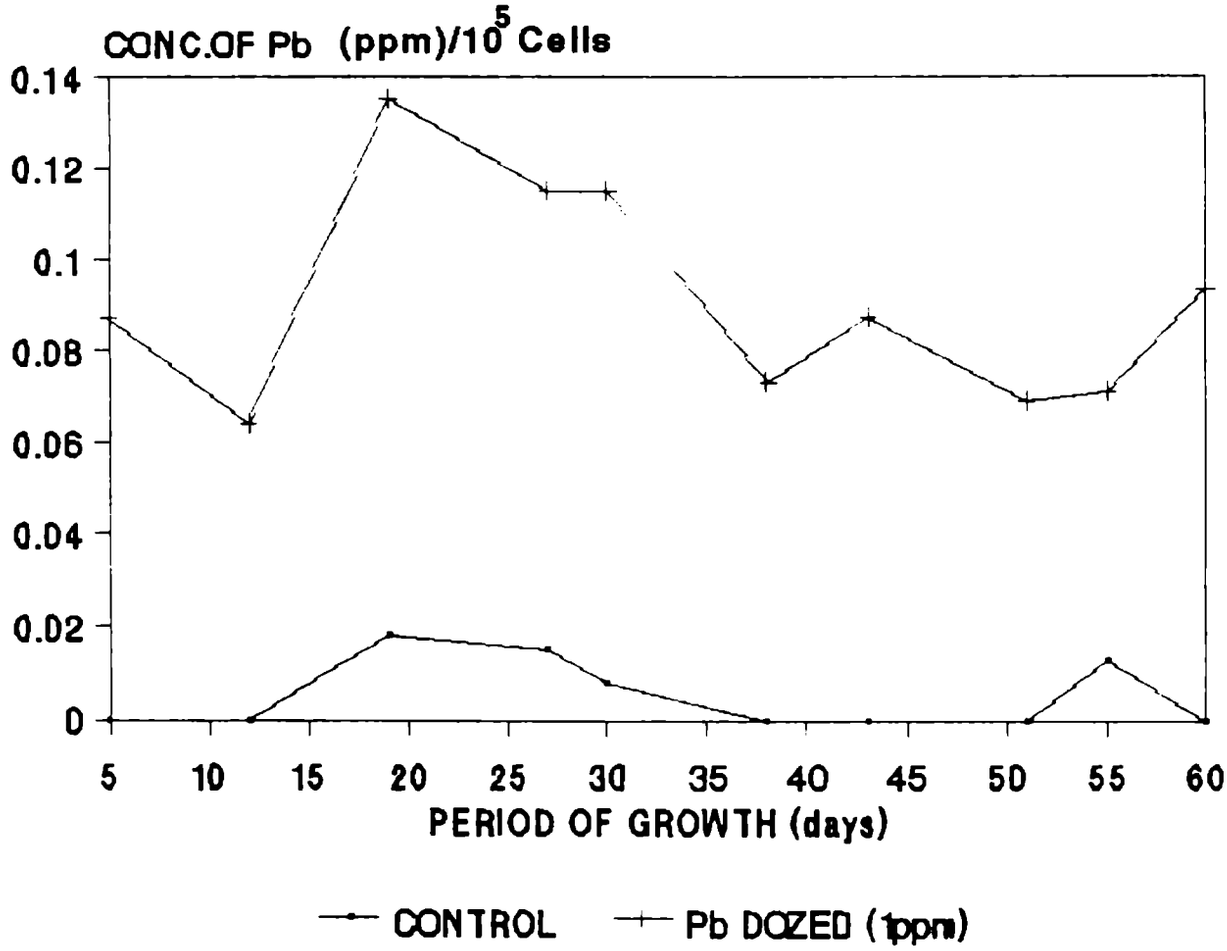


Fig. 35. Quantity of Lead in control and Lead exposed cultures at different growth intervals.

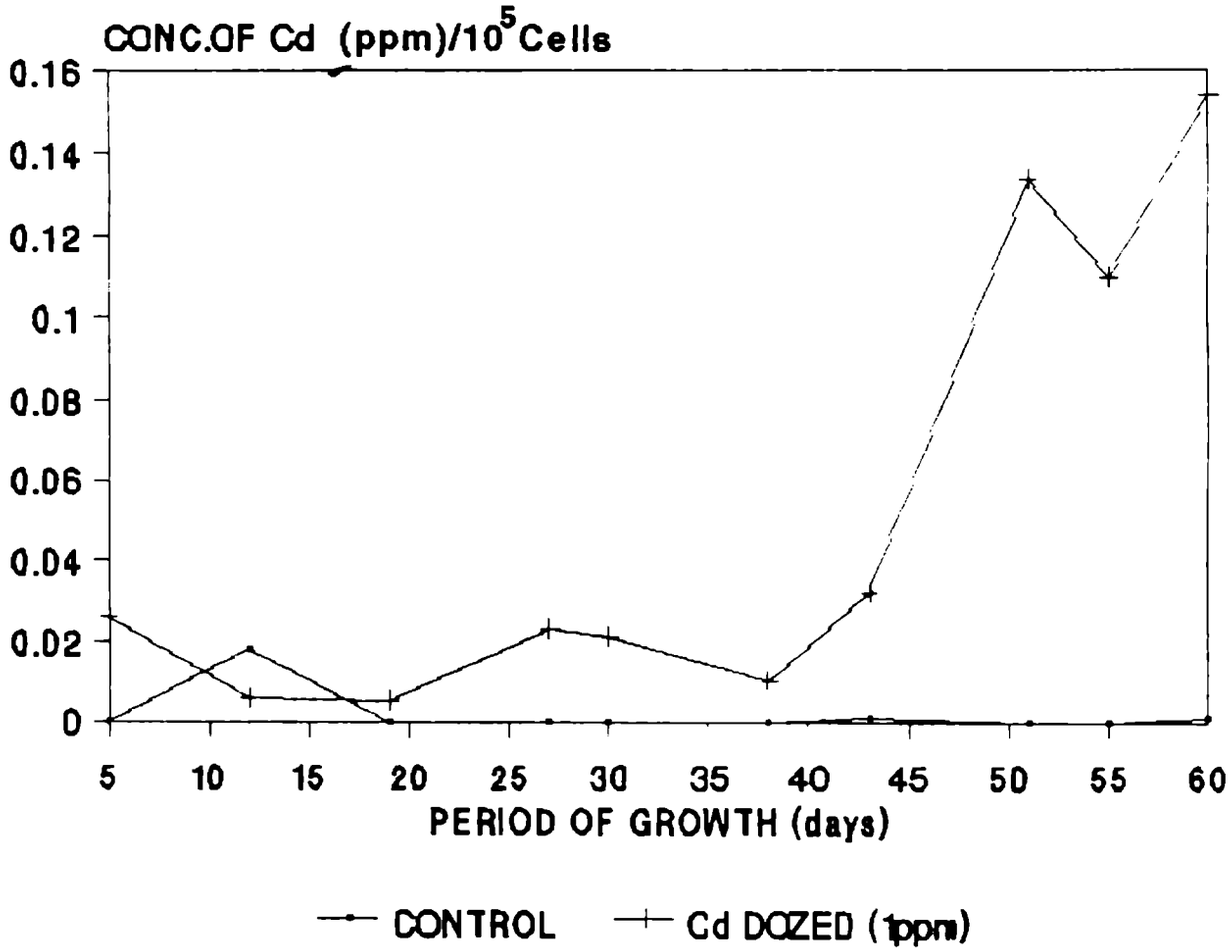


Fig. 36. Quantity of cadmium in control and cadmium exposed cultures at different growth intervals.

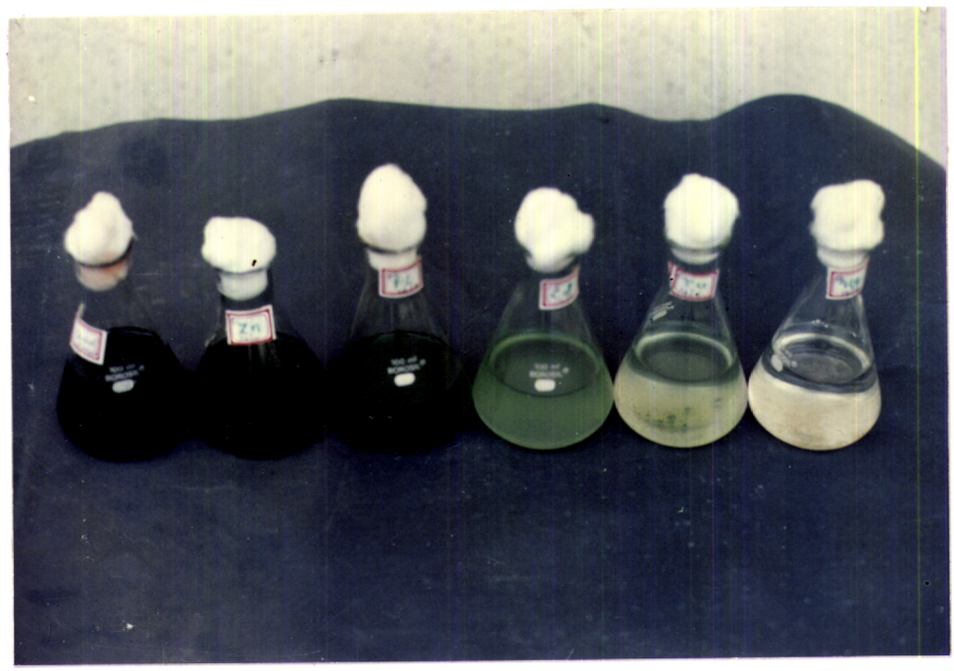


Fig. 37. Effect of heavy metals on growth of S. salina showing toxicity index. (Hg > Cu > Cd > Pb > Zn)

4.2 IMMOBILIZATION OF *SYNECHOCYSTIS SALINA* WISLOUCH

4.2.1 INTRODUCTION

In the past 20 years the use of immobilized enzymes or cell components for the production of a series of metabolites has become a branch of biotechnology of rapidly growing importance (Becker, 1994). Although in the initial stage most of the research work on immobilization deal with systems designated for the release of products, synthesised by enzymes or multi enzyme complexes, a more recent development focuses on the immobilization of complete cells or cell agglomerates (Becker, 1994). To a certain extent, these systems resemble natural environmental conditions as many micro organisms in a biotope where they are also immobilized by encapsulation in slimes or as a partner of symbiotic systems. Although the pioneering work with immobilized cells mostly employed heterotrophic organisms, a number of scientific reports today deal with studies on plant cells, algae, cyanobacteria and photosynthetic bacteria. Various applications of immobilized micro algae are reveiwed by Robinson et al. (1986).

Algae excrete a wide range of secondary metabolites including organic acids, amino acids, peptides and polysaccharides. Algal cells thus retain the essential virtue of microbial cells to excrete many of their metabolites while exhibiting characterisitics of plant cells. A more promising approach for recovering excreted algal products would be the autotrophic or phototrophic cultivation of axenic algal cultures in enclosed bioreactors such as transparent

plastic tubes or illuminated fermentors. But immobilized algae have the advantage that a good ratio of algal biomass to volume can be achieved, thus reducing the toal volume of medium from which the desired bioproduct has to be extracted (Becker, 1994).

It has been known for many years that algae are involved in the purification processes occurring in percolating filters of waste water treatment plants, where they can be found as thin films in the form of encrustrations or sheets in the upper zone of filter beads. These intrinsic tendencies of algae to adhere to the surface or to flocculate has been taken out for the development of various techniques of preparing immobolized algal cells (Becker, 1994). Various immobilization techniques are developed. They are

1. Adsorption
2. Entrapment in polymers or gels
3. Covalent coupling and
4. Cross linking

Different natural compounds and synthetic polymers are used for this purpose. They include alginate, agar, agarose, carraageenan, serum albumin, glutaraldehyde, polyurethane, polyvinyl foams, acrylamide, ceramic, glass beads etc. The qualities required for a mitrix to be used for immobilization and various procedures, factors affecting the strength, their pros and cons are discussed in detail by Becker (1994).

In general two types of algal entrapment can be differentiated

1. Thin gels less than 1mm thick

2. Round matrix droplets or beads with diameters up to 5mm.

Different types of bioreactors are used for hosting the immobilized cells. Their merits and defects are discussed by Becker (1994). The major fields envisaged for the utilization of immobilized algae are as follows:

a. Accumulation and removal of waste products in aqueous systems
b. Biosynthesis and biotransformation of different natural products such as polysaccharides, enzymes etc.
c. Production of ammonia
d. Production of photosynthetic oxygen in combined bacteria - algae system
e. Production of hydrogen

The various optical and electron microscopy studies revealed that the entrapment alters the morphology of algal cell function very little, but allows better separation of biocatalyst and product. According to Robinson et al. (1986) the immobilized algae might even be utilised for bioaccumulation in effluent streams containing levels of toxins usually lethal to the algae. He also discussed in detail about the future prospects of immobilized algae. According to him genetic manipulation studies in cyanobacteria using vectors like plasmids and cyanophages are important because of the inability of cyanobacteria for sexual fusion. Robinson et al. (1986) stremlined the future prospects as follows:

a. Increased understanding of the effects of immobilization on algal physiology and biochemistry.

b. Advancement in immobilization technology itself.

c. Screening for new algal products, and

d. Advances in our understanding of algal genetics and genetic manipulation.

4.2.2 MATERIALS AND METHODS

IMMOBILIZATION

Axenically grown cultures of 15-20 days' age were used for immobilization. The principle of ionotropic gelation of polyelectrolytes was used for the entrapment of algal cells. 6% sodium alginate was prepared by dissolving 6 gms sodium alginate in 100 ml of sea water and the content were stirred vigorously for 10 minutes to obtain a thick unifrom slurry without any undissolved lumps. The slurry was passed through a 40 μ mesh. The cells harvested were washed repeatedly with sterile sea water and 100 ml of algal suspension were mixed in 1:1 ratio and stirred for 10 minutes to have a uniform mix. The uniform slurry is extruded through a nozzle with a help of a peristaltic pump into $CaCl_2$ solution of 0.05M strength. The resultant droplets were cured for 3 hours and preserved in 0.025 M $CaCl_2$ solution at $4^{o}C$ for use. By adjutsting the speed of the pump and changing the nozzle pore size, desired sizes of the cell-immobilized beads were prepared.

Estimation of Bead diameter

The diameter of randomly selected fifty beads was measured using a screw gauge. The mean value obtained was taken as the diameter of

a bead. The mean diameter was also found out by measuring the volume of water displaced by 50, 100 and 150 beads. The mean diameter obtained by this method was in agreement with that obtained by the former method.

50 beads were put in different petri dishes containing medium with 1 ppm of Pb and cadmium. A control was also kept.

Heavy metal uptake

Heavy metal uptake by algal beads was measured by periodically withdrawing aliquotes of medium and appropriating the concentration using an atomic absorption spectrophotomer (Perkin - Elmer, Model No. 2380).

4.2.3 RESULTS AND DISCUSSION

Synechocystis salina cells are with an average diameter of 3 μ. This sort of nanoplankters can be accumulated to the extent of several millions cells/ml. Davies (1978) highlighted the significance of this vast surface area. Fisher (1985) highlighted the significance of greater surface: volume ratios in metal concentration factors. A comparison between algae with high surface: volume ratio and low surface: volume ratio revelaed that the former can concentrate the metal 1 to 10 times compared to the latter.

In culture conditions Synechocystis salina Wislouch can be concentrated to the extent of 8 million cells per ml. This will be exposing a surface area of 22 6 m^2. This area may act as an ion

exchange carrier. Studies using atomic absorption spectrophotometry revealed that the metals are very firmly bound to the membrane or it is sequestered inside. It is also revealed that the physiology of the cells is playing a major role in the intake of heavy metals.

In order to concentrate the cells further, immobilization technique is employed. According to Becker (1994) immobilized algae can remove heavy metals as well as nitrogen and phosphorous. A study conducted using Scenedesmus immobilized in Carrageenan revelaed that the system can remove appreciable quantities of phosphate and ammonia from typical urban secondary effluents at a similar rate to free living cells. This indicates the possible application of such systems in the treatment of waste waters (Becker, 1994). He suggested a further improvement of this system and a possibility of reducing the costs by using hyperconcentrated algae inorder to get a higher cell load in the beads by using less costly less purified Carrageenan. According to Becker (1994) similar results were obtained on the removal of heavy metals in industrial effluents, where the immobilizing matrix seems to protect the algae to a certain degree against the toxic effects of heavy metal ions.

As a result of immobilization, due to higher cell load there may be poor light penetration. Due to mutual blocking, the effective surface area exposed to the metal ions may be also less. But studies by various authors revealed that the heavy metal intake is more related to the cell surface characteristics. Studies conducted by Fisher et al. (1984) revealed that heat - killed cells accumulate

metals comparably to living cells. Rabsch and Elbrachter (1980) noted that heat killed (50°C) diatoms (Coscinodiscus granii) accumulated 3 times more Cd and 4 times more Zn than did living cells and Glooschenko (1969) found that formalin killed cells (Chaetoceros costatum) accumulated more Hg than did living cells; the differences presumably resulted from significant changes in the surface chemistry caused by the heat or formalin treatment. Conway and Williams (1979) found equivalent Cd accumulation by live and cold killed cells of one fresh water diatom (Fragilaria crotonensis) but not another (Asterionella formosa).

The present study revealed that heavy metal uptake ability is proportional to the total surface area available. Even if there is increased shading loss of surface area due to "mutual blocking", by immobilization method a heavy cell load could be achieved which is very much essential for getting large surface areas.

25 ml algal suspension immobilised in to beds, when incubated in 25 ml of 1 ppm solutions of Pb and Cd reduced the quantity of dissolved metals in each medium to a value as low as 0.1 ppm within 24 hrs. (Fig. 38).

The beads are getting deeper green colour as they become older (Fig.39a-e). This may be because of the increased proliferation of the cells within the beads or increased formation of photosynthetic thylakoid membranes. According to Robinson et al. (1986) The chlorophyll content of immobilized cells generally has been found to be higher than that of free cells, probably because of self shading

and subsequent reduction in incident light in the immobilized state results in a promotion of photosynthetic pigment synthesis. Robinson et al. (1986) also opined that "so far studies using chlorophyll as an index of biomass have not taken this phenomenon in to account- they would therefore probably over estimate the numbers of immobilized cells and thus underestimate mean cellular activity."

For a living organism a lot of energy is spent for the molecular mechanisms associated with cell division and sexual reproduction. But when an autotrophic unicellular organism is immobilised the energy associated with the above processes is saved. This may result in increased longivity of the cells. As suggested by Robinson et al. (1986) there may be increased synthesis of thylakoids which may increase the surface area further and there by increased intake of heavy metals. Cell leakage is a usual problem associated with immobilization. But microscopic obsrevation of the medium indicates the absence of cell leakage in the present study.

Increased longivity of the beads (green colour) noticed (6 months) for cadmium (1 ppm) treated beads compared to lead (4 months) and control (3 months). This phenomenon points towards the ability of cadmium and lead to retard the cellular activity and there by to increase the longivity of the cells as seen in batch cultures where these metals induced a longer lag phase.

However the commercial use of this information is much dependent upon designing a viable bioreactor, finding out the optimum conditions including the cell load/bead and rechargability.

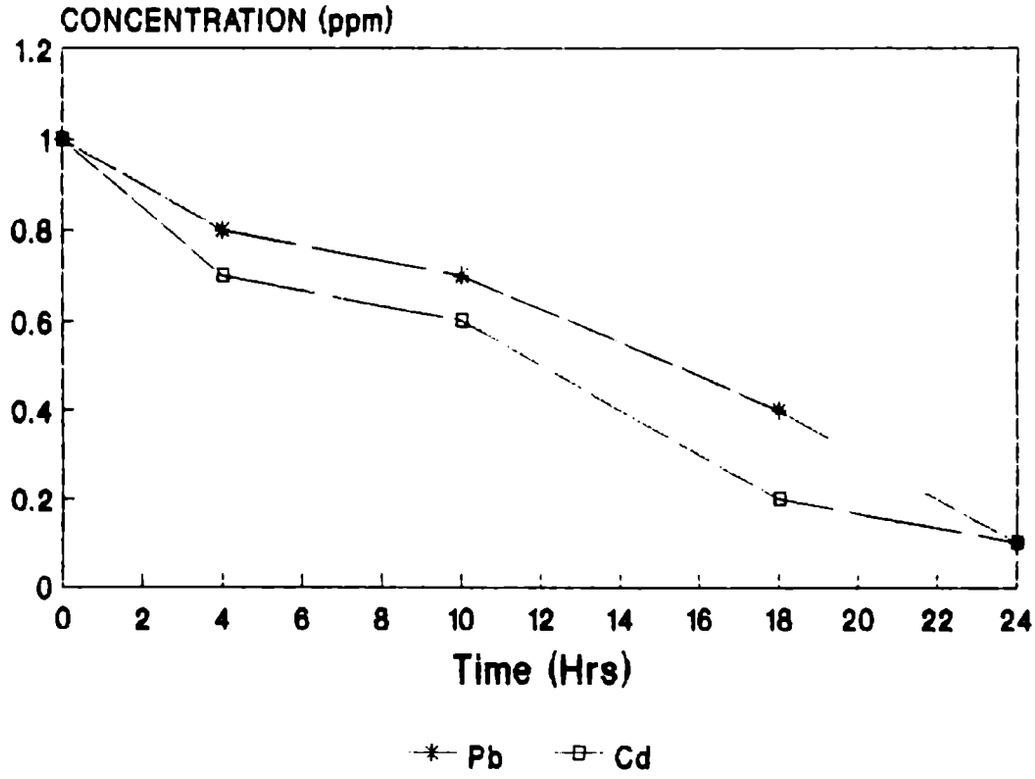


Fig. 38. Heavy metal uptake by immobilised beads containing S. salina

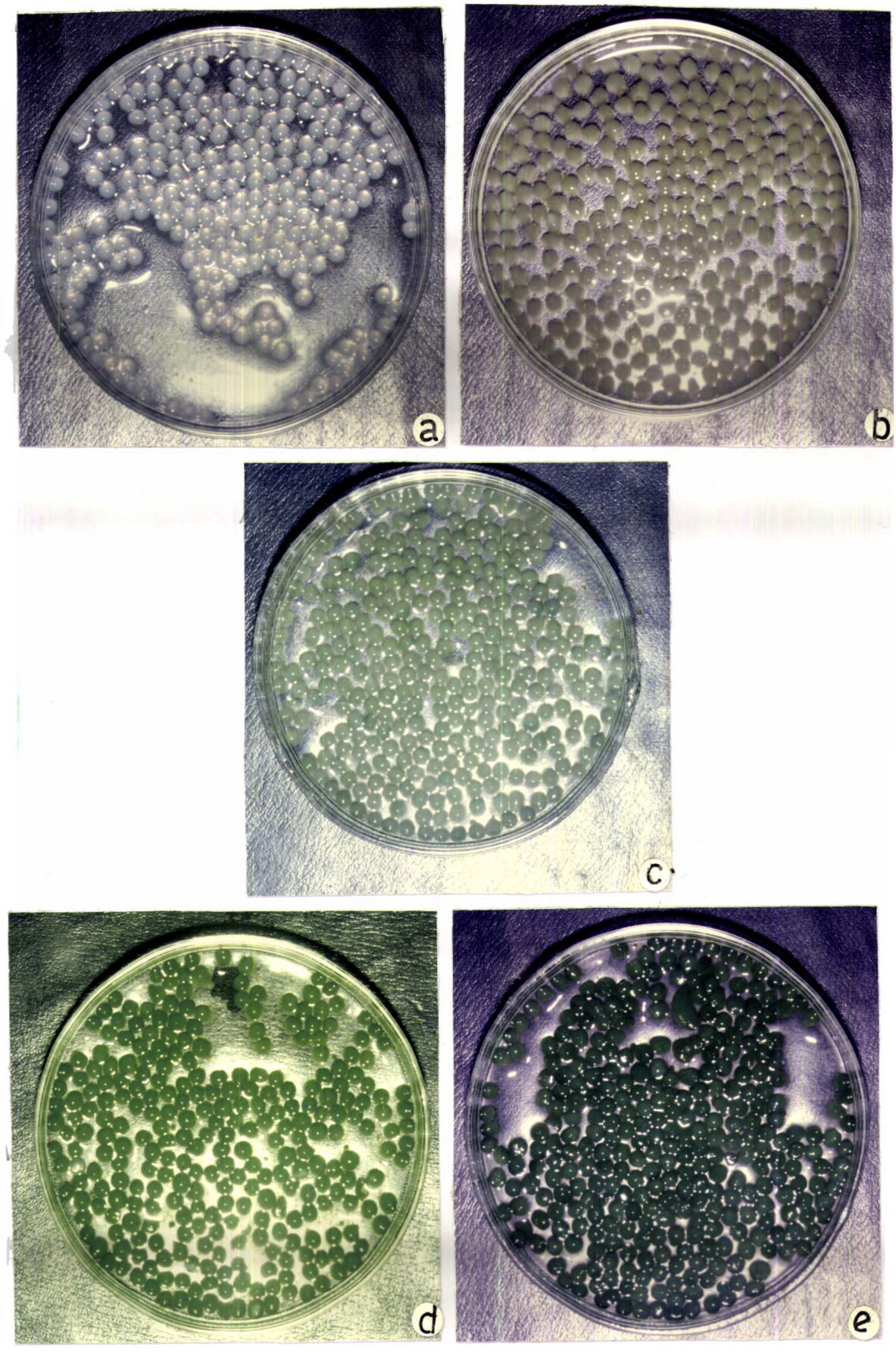


Fig. 39 a-e. Progressive greening of S. salina immobilised beads at 15 days intervals.

CHAPTER 5

5. METALLOTHIONEIN IN *SYNECHOCYSTIS SALINA* WISLOUCH

5.1 INTRODUCTION

The name metallothionein was first given to the Cd, Zn, and Cu containing S-rich protein from equine renal cortex (Kagi & Vallee, 1960). Metallothioneins are low molecular weight cysteine - rich, polypeptides that complex soft metal ions in thiol clusters. The characteristics and probable functions of animal metallothioneins were discussed in detail by Hamer (1986). Plant metallothioneins are structurally diverse. Some are gene products while others are secondary metabolites (Robinson, 1989). They are also known as poly (gamma glutamyl cysteinyl) glycine (Robinson & Jackson, 1986; Robinson et al., 1988), Cadystin (Murasugi et al., 1981; Kondo et al., 1985), gamma glutamyl metal binding peptide (Reese et al., 1988), phytochelatin (Grill et al., 1985), phytometallothionein (Rauser, 1987a) etc.

Metallothioneins have attracted interest from researchers involved in a wide range of disciplines including bio organic chemistry, biochemistry, molecular biology, physiology, toxicology, environmental science and medicine. Although the precise physiological roles of these polypeptides remain undefined, a large number of functions have been speculated. These molecules chelate toxic trace metals such as Cd, there by reducing the concentration of cytotoxic free metal ions. They are also believed to be involved in zinc and copper homeostasis (Robinson, 1989).

Three classes of metallothioneins have been defined (Fowler et al., 1987). Class I metallothioneins are proteins with locations of cysteine closely resembling those of equine renal metallothionein and they are not identified in algae (Robinson, 1989).

Class II metallothioneins are proteins with locations of cysteine only distantly related to those of the equine product (identified in the cyanobacterium Synechococcus Tx-20; Olafson et al., 1988). Metallothionein Class III represent non translationally synthesised metal thiolate polypeptides (Gekeler et al., 1988; Shaw et al., 1989) common in algae. A common feature of all metallothioneins is an abundance of Cys-xaa-Cys sequences where Xaa is an aminoacid other than cysteine (Kagi & Kojima, 1987). These sequences are involved in binding metal ions in metal thiolate clusters (Kagi & Kojima, 1987) and their synthesis increases in organisms exposed to elevated concentrations of certain trace metal ions (Robinson, 1989). Class II metallothionein of Synechococcus TX-20 is the most hydrophobic metallothionein to be described and at neutral pH was resolved in to seven isoforms (Olafson et al., 1988).

Cd binding polypeptides which form aggregates in the presence of Cd were first identified in extracts from the fission yeast Schizosaccharomyces pombe and called Cadystins (Murasugi et al., 1981; Kondo et al., 1985). These polypeptides are composed of the repeating dipeptide unit gamma glutamyl cysteine with a single carboxy terminal glycine residue, usually represented as (Gamma EC)$_n$ G (Robinson, 1989). Similar polypeptides were subsequently isolated

from higher plants (Bernhard & Kagi, 1985; Grill et al., 1985; Robinson et al., 1985) and then from eukaryotic algae (Gekeler et al., 1988; Shaw et al., 1989). The cadmium binding peptides of Chlorella fusca and Euglena gracilis possess inorganic sulphide, S^{2-} (Weber et al., 1987; Gekeler et al., 1988; Shaw et al., 1989), a characteristic of some Cd (gamma EC)$_n$ G complexes (Murasugi et al., 1983; Reese et al., 1988).

The pH at which 50% of the metal is displaced provides an estimate of the affinity of Cd (gamma EC)$_n$G for metals (Robinson, 1989). Metal ions are co-ordinated to a cluster containing several (gamma EC)$_n$G molecules in a metal (gamma EC)$_n$G aggregate (Reese et al., 1988). Two different forms of Cd (gamma EC)$_n$G aggregate are produced. One form contains acid labile S in the cluster. The S is present as reduced sulfide, S^{2-} (Murasugi et al., 1983). Aggregates containing S^{2-} have both higher affinity and capacity for Cd (Reese et al., 1988; Shaw et al., 1989). S^{2-} has not been identified as a component of isolated Cu aggregates. Furthermore, the pH of half dissociation of Cu ions indicates a much higher affinity for Cu than for Cadmium (Robinson, 1989).

Although synthesis of class III metallothionein, (gamma EC)$_n$G has been shown to increase following exposure of cells to metals other than Cd, Cu and Ag (Grill et al., 1985; 1987), there is no evidence that other metals form complexes with (gamma EC)$_n$G in vivo. Prolonged exposure to metal may elicit increased transcription of genes encoding enzymes involved in (gamma EC)$_n$G synthesis.

(Robinson, 1989). A number of Cd induced sequences have been identified in cells producing (gamma EC)$_n$G, but it has not yet been established that any of these correspond to enzymes involved in (gamma EC)$_n$G synthesis (Jackson et al., 1989).

Class II metallothioneins from Synechococcus Tx-20 is the only prokaryotic metallothionein to have been isolated and characterized (Robinson, 1989). Despite the high frequency of cysteine residues, Synechococcus TX-20 metallothionein is distinct from eukaryotic class I metallothionein, class II metallothioneins of Echinoidea Sp, Saccharomyces cerevisiae (Robinson, 1989) and metallothionein like Ec protein from wheat germ (Olafson et al., 1988; Hofmann et al., 1984).

Most eukaryotic metallothioneins have two metal binding domains designated alpha and beta (Hamer, 1986). However the Cu containing class I metallothioneins of Neurospora crassa and Agaricus bisporous are similar to mammalian class I metallothioneins, in the fact that both possess a single similar domain (beta domain). Spectroscopic data obtained for Zn-metallothionein and metallothionein substituted with Cu from Synechococcus TX-20 suggest that the prokaryotic protein may have a metal thiolate cluster similar to that of eukaryotic metallothionein, but in a single domain (Olafson et al., 1988).

Synechococcus TX-20 metallothionein possesses six long chain aliphatic residues and two aromatic aminoacid residues, making this the most hydrophobic metallothionein to have been characterized.

The presence of aromatic aminoacids is also a distinguishing feature, since these are absent from other metallothioneins causing characteristic low absorbance at 280 nm (Kagi & Kojima, 1987; Olafson et al., 1988).

Mammalian metallothionein genes are regulated by a wide range of factors and possess a complex array of elements determining basal metallothionein expression and response to a number of materials, hormones and other factors associated with acute stresses (Palmiter, 1987). By contrast regulation of fungal metallothionein genes is less complex with the metal regulatory elements of S. cerevisiae and Neurospora crassa responding only to elevated concentrations of Cu (Hamer, 1986). These fungal metal regulatory elements have been isolated and extensively characterized. However increased synthesis of class II metallothionein in Synechococcus 2X-20, following exposure to Cd and Zn is regulated at transcriptional level (Olafson, 1984; Olafson, 1986). The regulatory elements are not responsive to copper. Cyanobacterial metallothionein genes have been identified. This provides an opportunity to produce oliogodeoxy nucleotides which can be used to isolate the corresponding gene from genomic DNA libraries (Robinson, 1989). Transformation protocols are also available for nonfilamentous cyanobacteria (DzKelzkalns et al., 1988).

The proposal that Zn homeostasis may be the primary function of Class I metallothionein in mammals has been discussed (Karin, 1985). This has also been proposed as a possible function of class II

metallothionein in Synechococcus Tx-20 (Olafson et al., 1988). However, Euglena gracilis when exposed to cadmium, the majority (>80%) of the cellular Zn occurs in a low molecular weight peak distinct from Cd- (gamma EC)$_n$G (Gingrich et al., 1984). This implies that (gamma EC)$_n$G may not be involved in Zn homeostasis. This may also be true for other species and it has been proposed that Zn may only weakly bind to (gamma EC)$_n$G in Vivo (Reese & Wagner, 1987a). Therefore, the presence of (gamma EC)$_n$G in a particular species does not mean that class I or class II metallothionein genes would be absent a priori (Robinson, 1989). Study of metallothionein has become an important aspect in biotechnology such as:

1. The synthesis of class I metallothionein in animals and class II metallothioneins in fungi has provided useful model systems for the study of environmentally modulated gene expression (Hamer, 1986; Palmiter, 1987).

2. Studies on the cyanobacterial metal regulatory element could provide a useful model system for the environmentally modulated gene expression in a prokaryote (Robinson, 1989). (These sequences may be useful for the manipulation of prokaryotic genes, as other metallothionein genes and promoters have been used in eukaryotes.)

3. The structure of (gamma EC)$_n$G has been used as a model to produce synthetic genes that encode protein analogues of these

small metal binding polypeptides which differ from (Gamma EC)$_n$G in containing only α-carboxamide bonds (Robinson et al., 1988). Associated with appropriate expression vectors these genes may confer metal tolarance (Micro organisms and algae containing such constructs may be used either to accumulate metals in effluent treatment or applied to other bioprocessing applications which require growth in the presence of supra optimal concentrations of trace metals).

4. The possibility that (gamma EC)$_n$G could be involved in the detoxification of free radicals has encouraged researchers to investigate the possible role of these polypeptides in resistance to ionizing radiations (Robinson, 1989).

5. (Gamma EC)$_n$G has been immobilized in a functional form to produce columns that remove metals from solution (Robinson, 1989).

6. The structure of (gamma EC)$_n$G has also been used as a model for the chemical synthesis of biomimetic ligands to perform equivalent functions (Furlong et al., 1988). (unlike proteins gamma EC)$_n$G is resistant to degradation by peptidases and is therefore more amenable to such applications).

7. It is possible that metal specific ligands could be used in the production of metal specific biosensors for detecting elevated concentrations of metals in both critical chemical processes and for monitoring aquatic environments. (Robinson, 1989).

8. In genetic engineering - Inorder to construct organisms that overproduce desirable gene products or that display useful physiological traits, metallothionein genes could be used. They are well suited for such experiments because metallothionein genes are inducible, selectable and expressed at higher levels (Hamer, 1986).

9. Metallothionein genes can be used as either selectable markers in screening protocols or as reporter genes to monitor promotor function in transformed organisms (Robinson, 1989).

5.2 MATERIALS AND METHODS

100ml of exponentially growing cultures of 15 days age were dosed with 1ppm Zn, Cu, Cd, Pb and Hg as their salts in different conical flasks of 250ml capacity. The cultures were incubated at room temperature under fluorescent lamp (3000 lux) in 8:16 light and dark period. After 24 hours, 40 ml samples were pipetted out from various flasks. Separation of metal binding protein was done following the procedure of Brown (1985). The samples were centrifuged and equal quantities (1gm) of precipitated cyanobacteria were resuspended in 2 ml of 0.9% NaCl. After repeated freezing and thawing the bacteria were ground in a mortar with the help of a pestle and centrifuged at 10,000 g for 20 min.

About 2g of Sephadex G 75 (Sigma) was suspended in excess of 0.01M ammonium bicarbonate buffer (pH 7.8), and kept for few hours for the complete swelling of gel particles. The swollen gel was then

packed carefully in a column (0.9 x 60cm), and was washed with the buffer until it is equilibrated to the pH of the buffer. 1 ml of the prepared sample was applied to the top of the column with least disturbance and was allowed to percolate through the column bed. Once the applied sample has completely entered into the column, elution was started with ammonium bicarbonate buffer. The absorbance of the eluting drops was monitored at 254nm using uvicord (LKB) and 50 fractions of 1 ml each were collected at defined flow rate in a fraction collector (LKB, Redirac). The fractions were then monitored in a UV-Visible spectrophotometer (Hitachi) at 254 and 280nm. The absorbance obtained at 254 nm is converted in to equivalent amounts of protein by interpolating from the standard graph of bovine serum albumin in ammonium bicarbonate buffer, read at the same wave length.

The metal content of each fraction from the control sample was analysed for copper and zinc using atomic absorption spectrophotometer (Perkin - Elmer, Model No. 2380) and the relative concentration of metal bound to the separated protein is calculated. Eluant fractions from the dosed samples were analysed for copper, zinc and corresponding dosed metal. Concentration of mercury was determined using Mercury Analyser (MA 5800 D)

Cultures exposed to 1ppm of lead and cadmium for one month which showed tolerance were transfered to another medium containing the same quantity of metal inorder to check the resistance of the cells in these cultures.

5.3 RESULTS AND DISCUSSION

The relative distribution of protein and metals in the eluant fractions of proteins extracted from control is given in Fig. 41. UV monitoring showed that the fractions constituting the second peak has got a higher absorbance at 254nm and a comparatively low absorbance at 280nm (Fig. 40), a chacteristic of metallothionein. A screening of eluant fractions for the metals revealed that the metallothionein or metallothionein like protein of S. salina is predominantly Cu bound, Zn being present in lesser amounts. This finding is in contrast to the suggestions of Robinson (1989) that unlike mammalian and fungal metallothioneins cyanobacterial class II metallothionein does not appear to play a role in either Cu homeostasis or Cu detoxification.

Concentration of zinc in the fractions corresponding to metallothionein or metallothionein like proteins of the sample from the cyanobacteria exposed to zinc is significantly increased, when compared to the concentration in the respective fractions of the control (Fig. 42). This is a clear indication of the role of metallothionein or metallothionein like protein in Zn homeostasis and detoxification. This observation is in agreement with the ability of majority of metallothioneins or metallothionein like proteins to sequester Zn^{++} (Robinson, 1989; Gekeler et al; 1988). It is also noticed that addition of Zn^{++} induces the production of metallothionein or metallothionein like proteins (Fig. 47). Similar observations were made earlier (Olafson, 1984; Olafson, 1986) in

which increased synthesis of class II metallothionein in Synechococcus TX-20 was observed following exposure to Cd and Zn. According to the author it is regulated at transcriptional level.

When dosed with copper, there occurs a reduction in the concentration of copper present in the fractions of metallothionein or metallothionein like proteins (Fig. 43). However the concentration of zinc in these fractions increased (Fig. 48) while that of copper is decreased. Similar increase in concentration of copper and zinc is also noticed in the high molecular weight protein fractions (Fig. 43). These observations reveal that even if copper is the principal metal present in metallothionein or metallothionein like protein of Synechocystis salina, a higher concentration is toxic and protein with still higher molecular weight is synthesised inorder to accomodate the excess copper. This is in confirmation with the suggestion by Robinson (1989) that the regulatory elements of class II metallothionein in Synechococcus TX-20 are not responsive to copper.

When dosed with cadmium there is a sharp increase in the production of metallothionein or metallothionein like protein (Fig. 44). The concentration of copper and zinc bound to the metallothionein or metallothionein like protein also increases (Fig. 48). In the cyanobacterium Anacytis nidulans protection against cadmium toxicity was correlated with production of a metallothionein like protein (Maclean et al., 1972). However in the present study cadmium is not found to be appreciably bound with the

metallothionein or metallothionein like protein. It is also noticed that when Synechocystis salina cells grown in a medium containing cadmium were subcultured into another medium containing cadmium, instead of the predicted growth lag, they grew immediately. This is in conformation with the observation by Olafson (1986) on synechococcus TX-20. According to him the cells were truely cadmium resistant. However, synthesis of metallothionein is noticed in both tolerant and sensitive cells of the angiosperm Datura innoxia (Jackson et al., 1989). The enzymes responsible for the synthesis of metallothioneins are present constitutively in both Cd tolerant and sensitive cells, even in the absence of toxic metal ions (Robinson et al., 1988). This suggests that either the metal binding polypeptides or the enzymes perform some other metabolic function in the absence of metal ions. (Jackson et al., 1989). According to them both cadmium tolerant and sensitive cells can synthesise these compounds upon exposure to cadmium. However only the tolerant cells survive. So they proposed that some other biochemical or physiological mechanism must also contribute to tolerance. In some mammalian cells, the tolerance to different concentrations of cadmium is tightly correlated with the ability to produce specific amounts of metallothionein immediately following exposure to cadmium (Hilderbrand,et al., 1980).

When dosed with mercury there occurs a reduction in the concentration of copper in the fractions of metallothionein or metallothionein like proteins (Fig. 48). However zinc concentration in the corresponding fractions were higher. Mercury is also not

taken by the metallothionein or metallothionein like protein. (Fig. 45). Even though there are reports saying that mercury induces synthesis of metallothioneins (Gekeler et al., 1988), it is not demonstrated to be bound to such compounds in extracts from algal cells (Robinson, 1989).

Concentration of zinc in the fractions corresponding to metallothionein or metallothionein like proteins of the sample from the Synechocystis exposed to lead is significantly higher while copper is less, when compared to their concentrations in the respective fractions of the control (Fig. 48 & 46). As in the case of zinc and cadmium exposed cells, metallothionein or metallothionein like protein production is also increased (Fig. 47) compared to the control. This is in contrast with the suggestion by Robinson (1989) that at present only cadmium and copper have been demonstrated to be bound to metallothioneins in extracts from algal cells.

Synechocystis salina cells grown in a medium containing 1 ppm of lead, when transfered to another medium containing the same concentration of lead behaved just like that in the medium with cadmium.

Algae are capable of accumulating heavy metals to concentrations several orders of magnitude higher than in the surrounding medium (Becker, 1992). Infact micro algae can be employed in the purification of heavy metal contaminated water. The high

accumulation capacity can even be used for the enrichment or recycling of valuable metals (Gekeler et al., 1988). According to Kagi and Nordberg (1979) algae detoxify metals like zinc, copper and cadmium via metallothioneins, evolutionarily strongly conserved proteins of low molecular weight and high cysteine and metal contents. Zinc and copper containing proteins in plants include enzymes performing key functions in diverse metabolic processes. However relatively little is known about the control of metabolism and distribution of these metal ions among tissues, cells, organelles or compounds in angiosperms (Robinson and Jackson, 1986). Same is the situation in algae and cyanobacteria. Walker and Webb (1981) stated that no compound has been ascribed the function of "copper storage", "copper membrane transport", or insertion of copper in to enzymes". This statement also applies to zinc metabolism. Metabollothionein and metallothionein like proteins may perform some of these functions for essential trace metal ions such as copper and zinc (Robinson and Jackson, 1986). According to them, these compounds may also be involved in the detoxification of non essential metal ions such as cadmium and excess amounts of essential metal ions. Karin (1985) speculated that metallothioneins may regulate the metabolic or proliferative status of a cell by altering the intracellular distribution and availability of zinc and consequently the activity of zinc requiring enzymes, which are involved in many biological processes such as DNA replication, RNA transcription, energy metabolism, protein synthesis and protein degradation.

In the present study Synechocystis which is resistant against cadmium, lead and zinc is capable of producing metallothionein or metallothionein like proteins to a greater extent than control organism (Fig. 47). Maximum induction is in presence of cadmium followed by lead and zinc. Copper and mercury dosed cells produce less metallothionein or metallothionein like protein compared to control and the cells die. In Scenedesmus actutiformis and Chlorella fusca, the class III metallothionein has been shown to increase following exposure to Cd, Pb, Zn, Ag, Cu and Hg (Gekeler et al., 1988). However only cadmium and copper have been demonstrated to be bound to such compounds in extracts from algal cells (Robinson, 1989). According to him there is no unequivocal evidence of modified expression of class II metallothionein genes conferring resistance to supra optimal concentrations of toxic trace metals in any algae. In contrast to these reports, the present study revealed that both zinc and lead induce metallothionein or metallothionein like protein as well as bind to it. However contradictory to many of the previous reports cadmium can induce the production of metallothionein or metallothionein like protein, but is unable to bind on it. As in the case of Synechococcus TX-20 cadmium appears to be a more potent inducer of metallotheionein than zinc (Olafson, 1986).

Since cadmium is not directly bound to the metallothionein , the molecular mechanism of cadmium tolerance is not fully clear. Studies on the heavy metal uptake (Chapter 4) revealed that the metal is taken inside or very strongly bound to the cell wall. Induction of metallothionein production points towards intake of cadmium and

amplification of metallothionein gene, probably an extrachromosomal one as suggested by Olafson (1986). The increased production of metallothionein and tolerance over the toxic metals reveals that metallothionein is playing a major role in metal detoxification. Heavy metal uptake studies (chapter 4) revealed that out of the 5 heavy metals studied zinc is least inhibitory at 1 ppm followed by lead, cadmium and copper. Mercury is found to be the most toxic. Invariably in all the heavy metal exposed cultures, the metallothionein fraction contains more than double the quantity of zinc compared to control while copper concentration fluctuates. Considering all these facts it could be speculated that in addition to the binding to the toxic heavy metals, metallothionein or metallothionein like protein may be also playing a major role in the detoxification mechanism by metallothionein mediated ion antagonism. It is possible that metallothionein induces some other molecule that could sequester the heavy metal at least in the case of cadmium tolerance of Synechocystis salina.

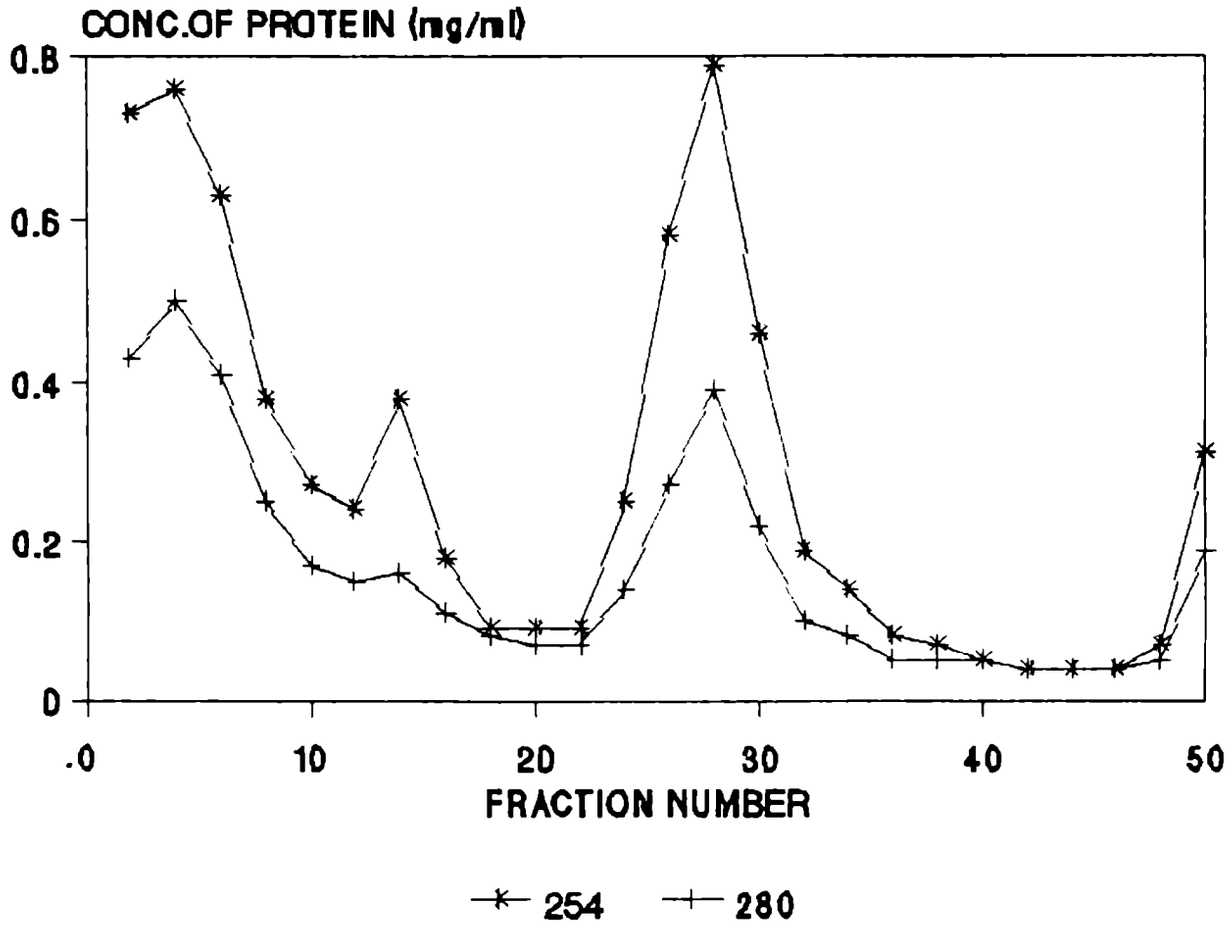


Fig. 40. Relative absorbance of different eluant fractions from the sephadex G75 column at 254 and 280 nm.

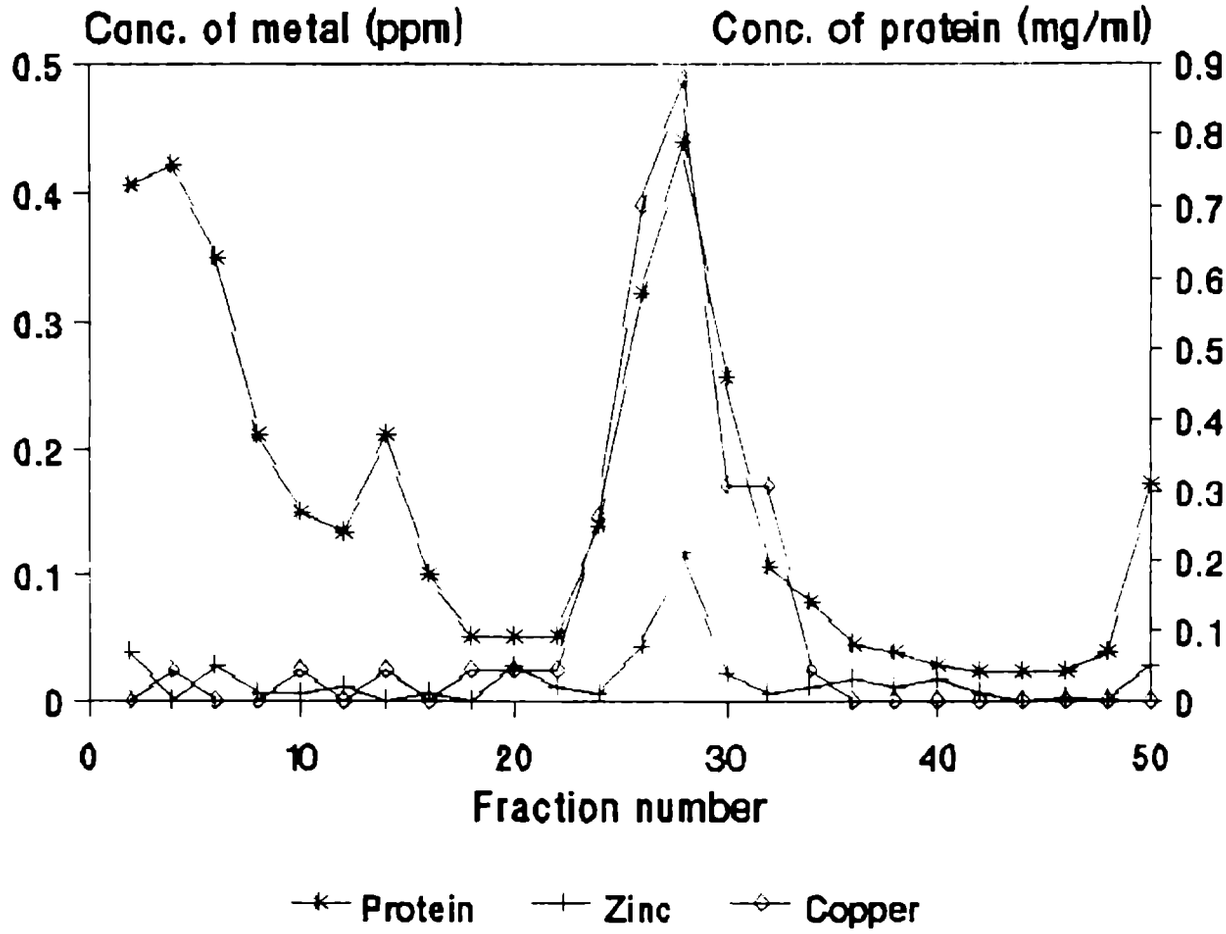


Fig. 41. Distribution of metals in relation to protein content of the eluant fractions from the sephadex G75 column in control.

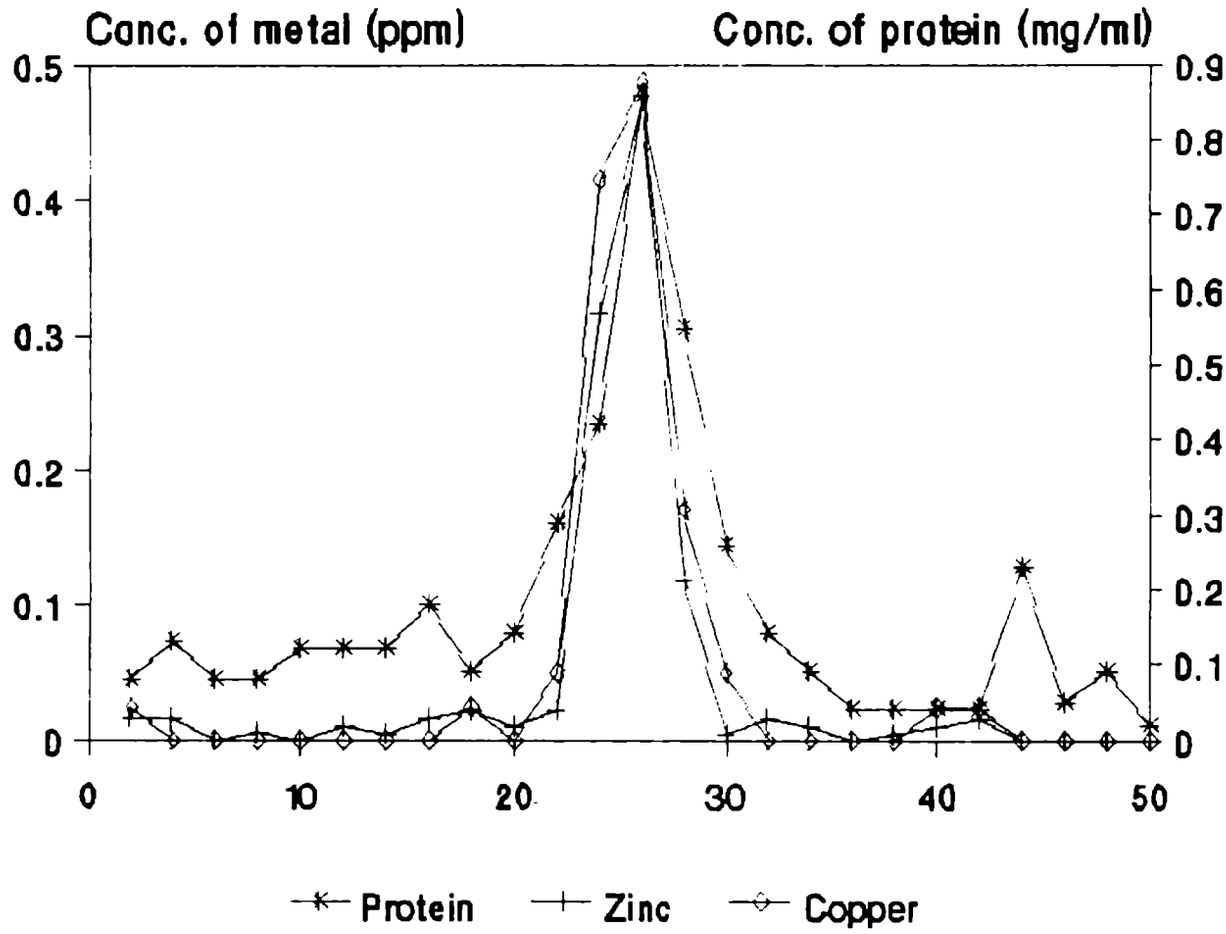


Fig. 42. Distribution of Cu and Zn in relation to protein content of the eluant fractions from the sephadex G75 column in the cyanobacteria exposed to zinc.

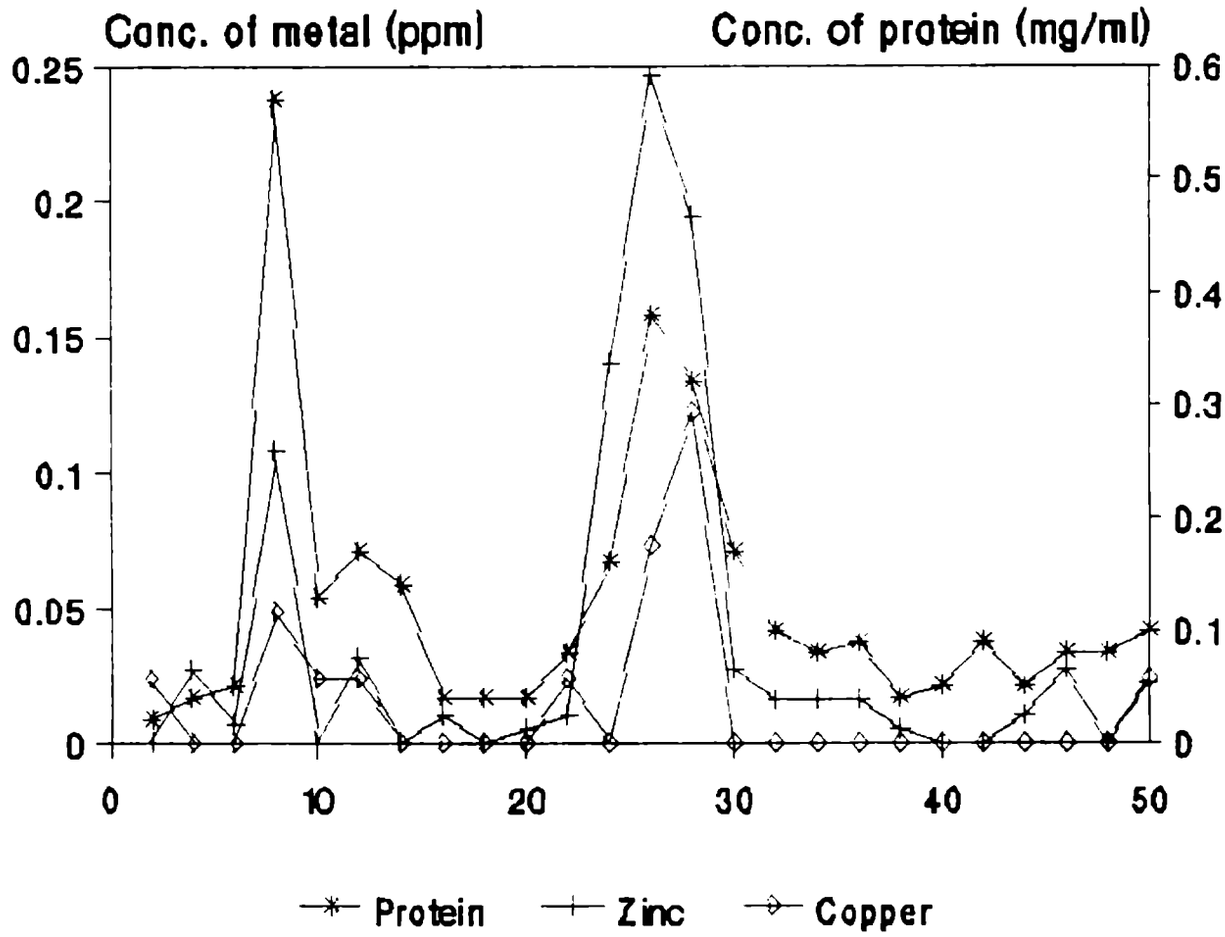


Fig. 43. Distribution of Cu and Zn in relation to protein content of the eluant fractions from the sephadex G75 column in the cyanobacteria exposed to copper.

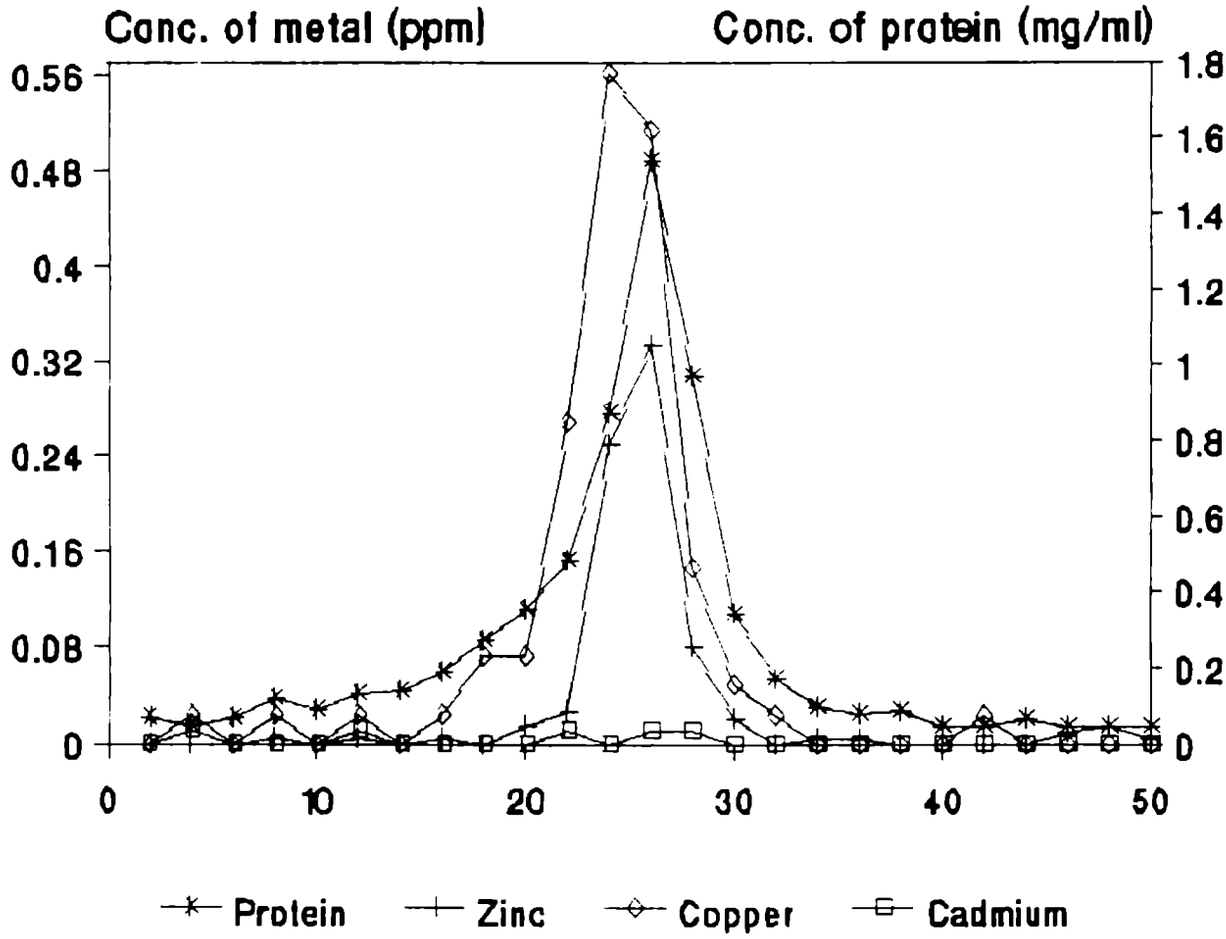


Fig. 44. Distribution of Cu, Zn and Cd in relation to protein content of the eluant fractions from the sephadex G75 column in the cyanobacteria exposed to cadmium

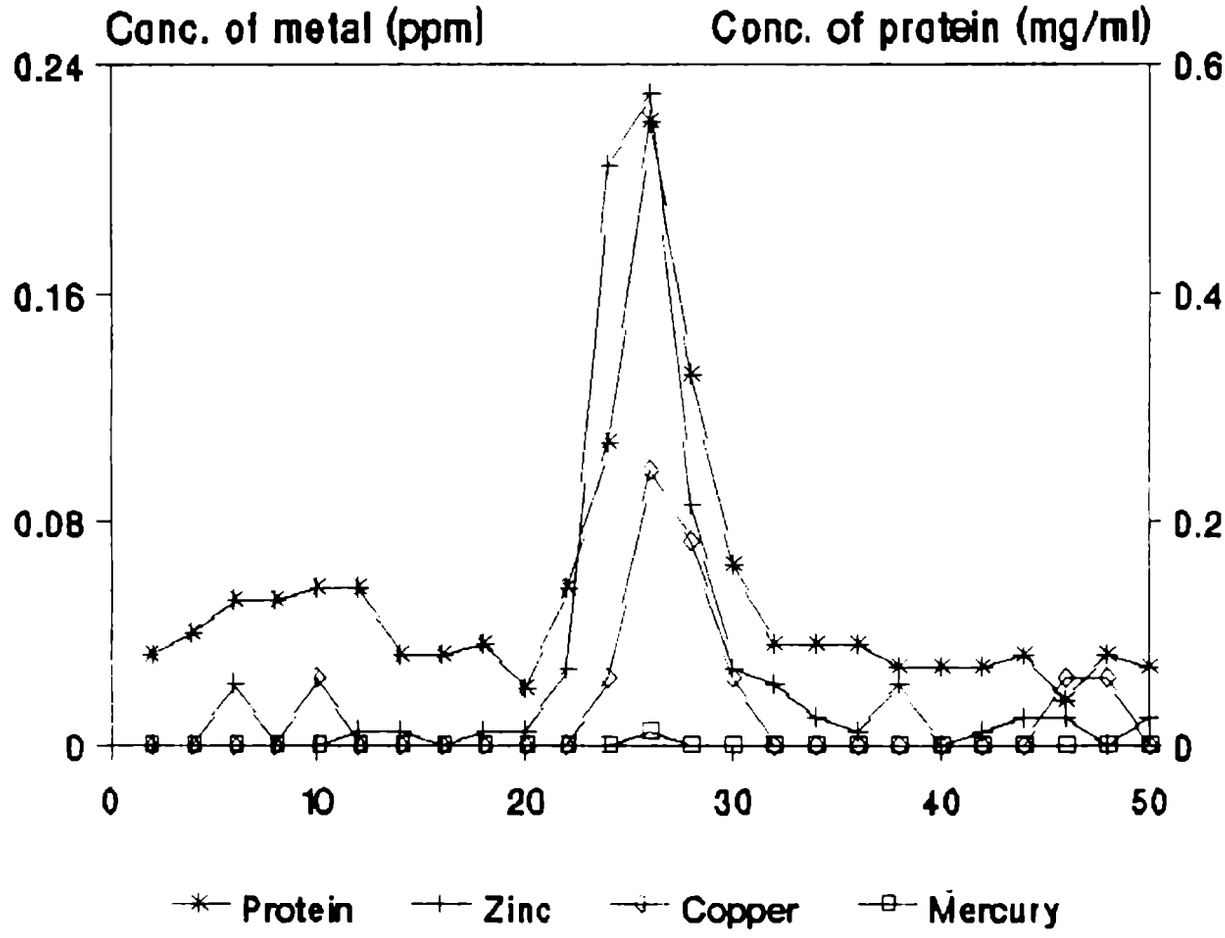


Fig. 45. Distribution of copper, zinc and mercury in relation to protein content of the eluant fractions from the sephadex G75 column in the cyanobacteria exposed to Hg

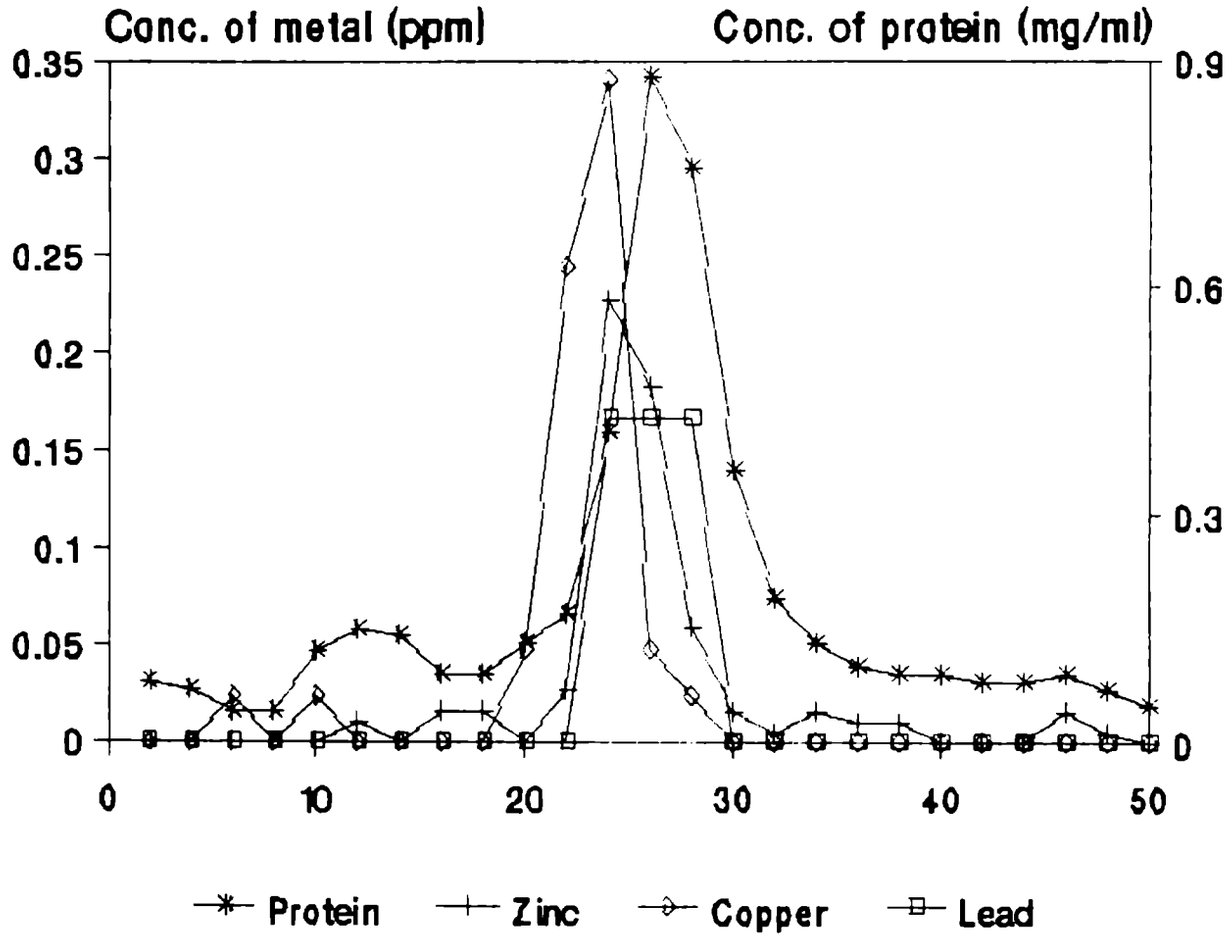


Fig. 46. Distribution of copper, zinc and lead in relation to protein content of the eluant fractions from the sephadex G75 column in the cyanobacteria exposed to lead.

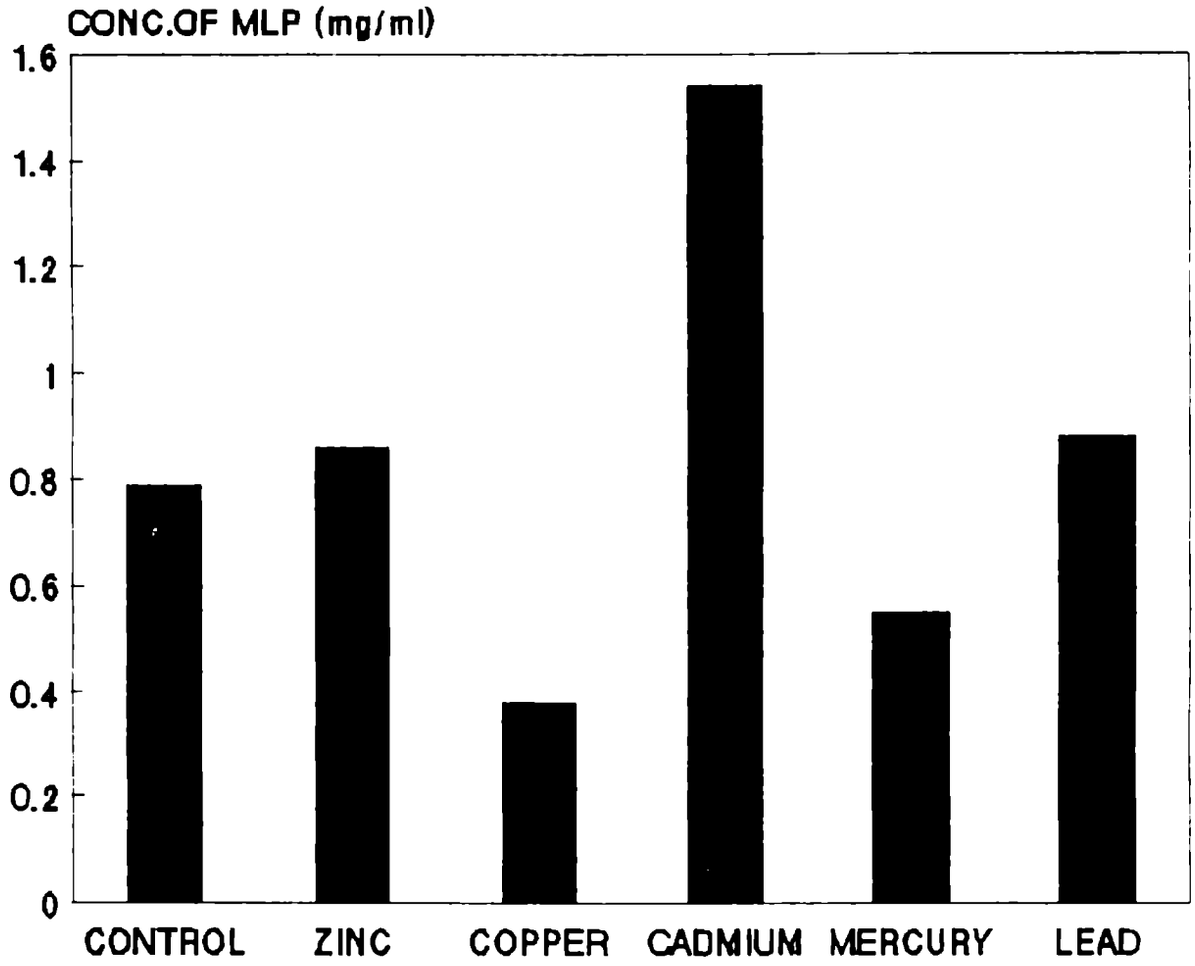


Fig. 47. Quantity of metallothionein produced after 24 hours of exposure to various heavy metals.

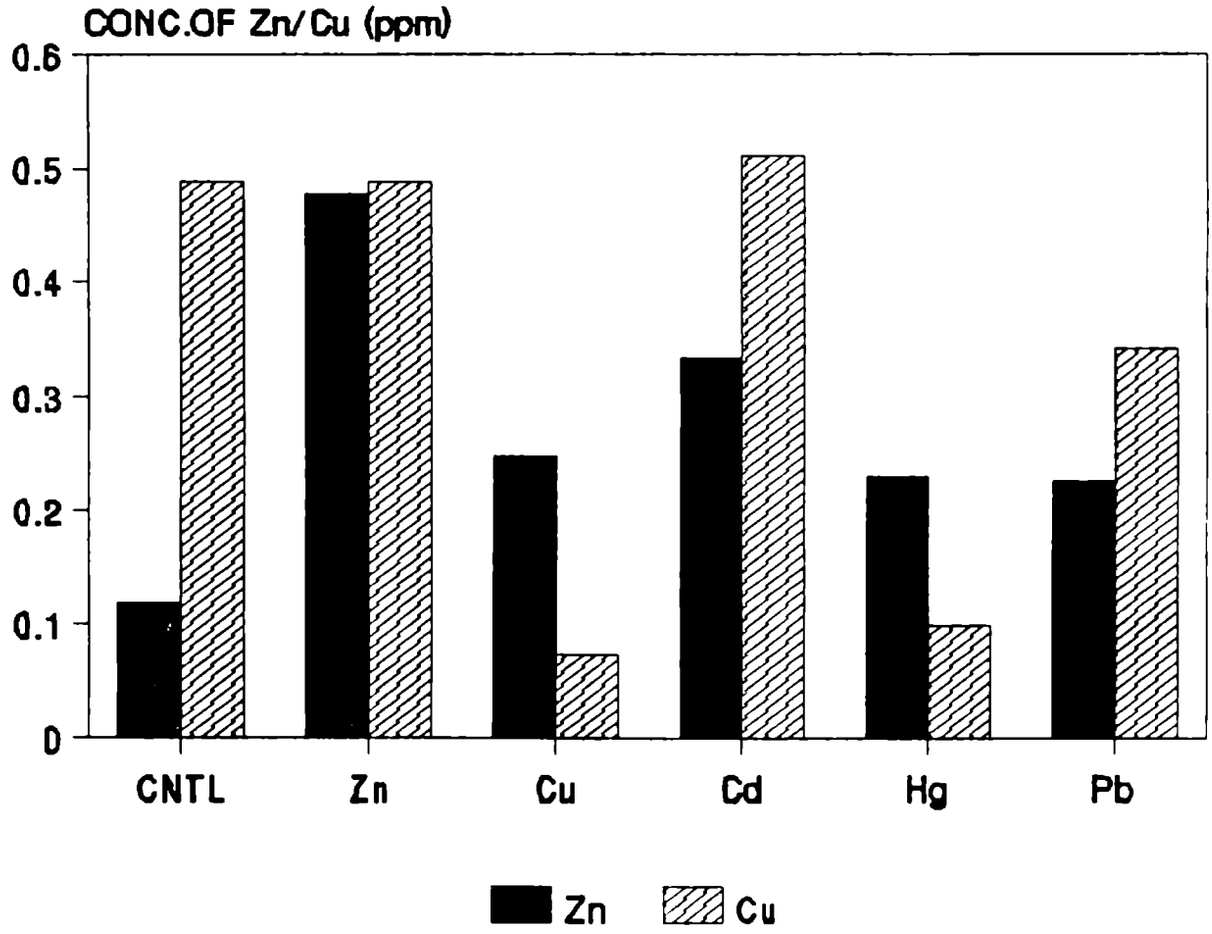


Fig. 48. Relative distribution of Zn and Cu in metallothionein fractions obtained from various cultures exposed to different heavy metals.

CHAPTER 6

6. ANTIBACTERIAL PRINCIPLES FROM SYNECHOCYSTIS SALINA WISLOUCH

6.1 INTRODUCTION

It is known that certain cyanobactria can produce and excrete a wide variety of biologically active organic substances. In addition to toxins cyanobacterial exudates have also been shown to have antibiotic effects (Bloor & England, 1989). Early work reported activities against bacteria and algae (Flint & Moreland, 1946; Lefevre & Nisbet, 1948; Lefevre et al., 1950; Jakob, 1957). The number of reports of antibiotic and other pharmacological effects from cyanobacteria has increased recently. The activities vary from antiviral (Starr et al., 1962), antifungal (Welch, 1962; Kellam et al., 1988), antibacterial (Flint and Moreland 1946; Hornsey, 1985; Cannell et al., 1988), growth promoting (Decaire et al., 1977 and 1979), algicidal (Mason et al., 1982) enzyme inhibitory (Cannell et al., 1987; Schwartz et al., 1990), antiinflammatory, anti amphetamic (Baker, 1984), and antineoplastic (Reinhart et al., 1981; Gerwick et al., 1989; Patterson et al., 1984, 1991). Each of these screening tests has established that the blue green algae are rich sources of novel bioactive agents. The rate at which activity is discovered is comparable to rates observed for more traditional sources of pharmaceuticals while the rate of rediscovery of known compounds which can exceed 95% of confirmed activities from actinomycete screening is significantly lower among micro algae (Patterson et al., 1991). Rao (1994) suggested that the cyanobacteria possess antimicrobial properties which possibly help to prevent bacterial colonisation of the live filaments.

There are currently a large number of antibiotic compounds available for the treatment of pathogenic conditions, the vast majority of these being produced by the actinomycetes. However with the ever increasing incidence of antibiotic resistance, the search for new agents has widened into previously neglected biological phyla (Kellam et al., 1988). The majority of work concerned with the isolation, screening and microbial physiology of antibiotic producing micro organisms has focussed on chemoheterotrophs. Very little attention has been paid to other groups such as the cyanobacteria, some of which are able to grow under diverse nutrient conditions; photo autotrophically, photoheterotrophically or chemoheterotrophically and are amenable to controlled fermenter studies (Bloor and England, 1989). If algae are to be used for the large scale production of pharmacologically active compounds, it will be necessary to carry out laboratory studies to optimize growth and production under controlled culture conditions. Micro algae are much more amenable to this type of study, and to immobilization and in this respect show greater potential for industrial development (Cannel et al., 1988). The search and discovery of antibiotics and other bioactive compounds cannot be performed satisfactorily by chemical means alone. The biological activity of specific compounds cannot be predicted by the identification and examination of their structures. Therefore the search for novel bioactive compounds utilizes biologically based screening systems (Bloor & England, 1989).

6.2 MATERIALS AND METHODS

S. salina cultures were grown aseptically in 100ml medium in 250ml. conical flasks and incubated at 25±2°C, in a 8:16 light-dark photoperiod with a luminosity of 3000 lux.

A 20ml sample of culture was removed aseptically from the flask about 10 days after inoculation and the remainder of the culture harvested after 6 weeks. This allowed testing of filtrates from cultures which were presumably in both exponential and stationary phases. The cells were harvested by centrifugation. The resultant cyanobacterial pellet was extracted by shaking with 5ml methanol for 2 hours, centrifuged and the supernatant collected. The remaining pellet was then further extracted in the same manner with acetone and then hexane. Similarly, harvested algal cells were suspended in 5ml sterile distilled water and the cells were subjected to repeated freezing and thawing. Broken cells were removed by centrifugation and the resultant extract was lyophilised. Algal cells were harvested by centrifugation. The supernatant was lyophilised and the sample prepared thus is tested for extracellular antibacterial principles. Culture broth was directly applied on the bacterial culture medium to test the axenic nature of the cyanobacteria. Streak plates were also prepared on soft agar to confirm the unialgal nature of the culture (Fig. 49).

Bacterial test organisms were grown overnight in 100ml nutrient broth. The organisms tested against were Bacillus subtilis,

Escherichia coli and Salmonella sp. Petridishes containing nutrient agar were seeded with the test organisms using swabs. Filter paper discs dipped in different extracts were placed on the test organisms. Lyophilized sample was applied directly. The petridishes were incubated at 37°C for 24 hours. At the end of the incubation period, confluent or semiconfluent bacterial growth could be seen throughout the plate except for clear zones around the filter paper disc. A clear zone of radius greater than 1mm was taken as a positive result. Control was also kept for different solvents such as methanol, acetone and hexane and culture medium. Cultures maintained at salinities 25 ‰ 32 ‰ and 38 ‰ were further extracted in acetone and the effect of salinity on the synthesis of antibacterial principles was further verified.

6.3 RESULTS AND DISCUSSION

The results are given in table 10. All the positve results were found in acetone extracts. No activity was found in the hexane extract, lyophilised samples or in the supernatant. Methanol itself could bring inhibition of bacterial growth while hexane and acetone are nontoxic. Out of the three organisms tested Salmonella can tolarate methanol. Acetone extract is found to be effective against all the three bacteria (both gram +ve and -ve) and it is making an inhibition zone of >3mm radius. No bacterial colonies were developed after 24 hours, when the culture broth containing S. salina was inoculated in to the bacterial culture medium.

One of the important findings in this study is that cells harvested during the exponential as well as stationary phase exhibit antibacterial activity. This is contradictory to the previous reports (Cannell et al., 1988). One or more nutrients must be limiting or depleted before the antibiotic is released in to the culture broth (Bloor & England, 1989). According to them whether nutrient limitation acts by inhibition or repression of necessary biosynthetic enzymes or whether the specific growth rate is important, is unclear. Antibacterial activity is unequivocally clear with acetone extract. This is in confirmation with the findings of Cannell et al. (1988). According to them all the inhibition was found in the organic solvent extracts and this may indicate that the inhibitory compounds tend to be hydrophobic compounds, retained on the algal cell surface as has been found to be the case with many pharmacologically active compounds produced by streptomyces.

It is also found that in this case salinity is playing an important role in the neogenesis of the antibacterial principle. Out of the three salinites tried (25‰, 32‰, 38‰) antibacterial activity was noticed only at 38 It is possible that since S. Salina is a euryhaline cyanobacterium, it may produce new biochemical molecule(s) or amplify the production of already existing biochemical molecule(s) as osmoticum in order to nullify the external concentration and to keep the structural integrity of

the cell. Production of bacterial and fungal antibiotics usually takes place in the stationary phase (Egorov, 1985). This may be due to the depletion of a particular chemical, repression of necessary biosynthetic enzymes or the particular growth rate (Bloor and England, 1989). However in the present study the derivation of antibacterial principle appears to be because of the salinity stress. This is evident because, even extract taken from exponentially growing cells at higher salinity tend to be antibacterial. So this parameter may be taken as an important environmental factor for the production/screening of antibacterial principles especially for euryhaline species.

Table - 10

	Meth-anol	Methanol Extract	Acet-one	Acet-one Ext.	Hexane	Hexane Extract	Lyoph-ilised	Super-natant	Medium
Bacillus Subtilis	+	+	-	++	-	-	-	-	-
E.coli	+	+	-	++	-	-	-	-	-
Salmonella Sp.	-	-	-	++	-	-	-	-	-

Antibacterial activity exhibited by various solvent extracts of Synechocystis salina

(+ less than 3mm radius)
++ more than 3mm radius)

Different solvents were also kept as control

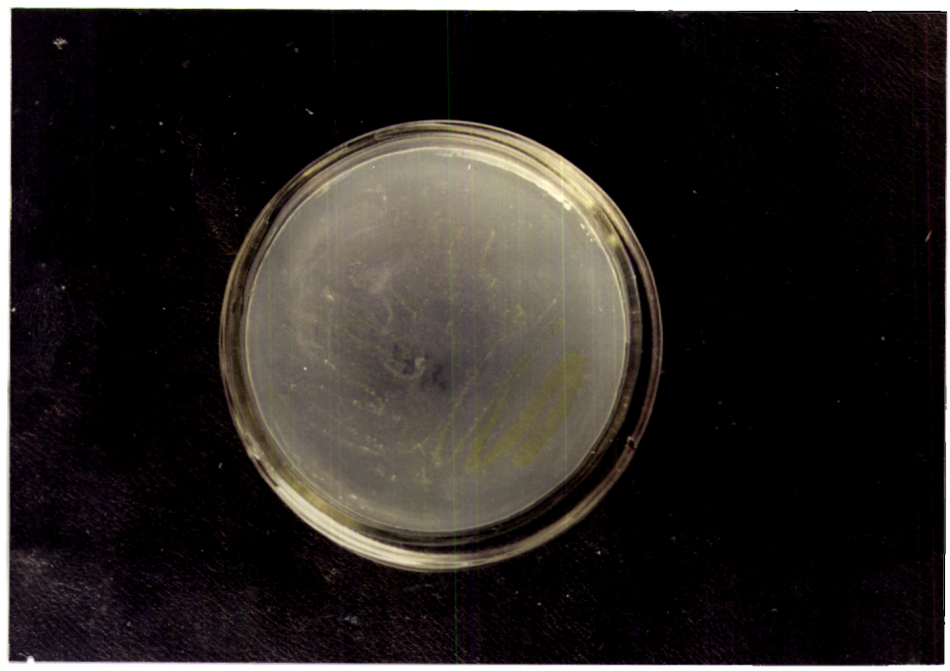


Fig. 49. Streak plate showing unialgal nature of the S. salina culture.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The group cyanobacteria includes a large number of organisms characterised by a low state of cellular organization. Their cells lack a well defined nucleus. Cell division is by division of the protoplast by an ingrowth of the septum. These organisms are characterised generally by a blue green colouration of the cell, the chief pigments being chlorophyll-a, carotenes, xanthophylls, C phycocyanin and C phycoerythrin. The product of photosynthesis is glycogen. These organisms lack flagellate reproductive bodies and there is a total lack of sexual reproduction. They are also unique because of the presence of murein in the place of cellulose (cell wall) and the absence of chloroplast, mitochondria and endoplasmic reticulum. Just like bacteria some of them possess plasmids and can fix atmospheric nitrogen.

In the present study growth kinetics, heavy metal tolerance, tolerance mechanisms, heavy metal intake, and antibacterial activity of Synechocystics salina Wislouch - a nanoplanktonic, euryhaline, Cyanobacterium present in Cochin back waters has been carried out for the potential biotechnological application of this organism. S. salina occur as small spherical cells of 3μ diameter (sometimes in pairs) with bluish green colour. The species is characterised by jerky movement of the cells and is structrually similar to other cyanobacteria.

A comparison of the growth of S. salina in 5 different media under 3 different set of conditions revealed the superiority of Allen and Nelson's medium for promoting growth at 34°C - 36°C temperature, 3000 lux light intensity and an exposure period 8 h light 16 h dark. The

superiority of Allen and Nelson's medium is believed to be because of the abundance of NO_3^- in it. Similarly higher temperature and light intensity to a certain level are also factors favouring increased rate of growth. Cyanobacterial growth is generally favoured by higher temperature as evidenced by their abundance in tropics. Photosynthesis and nitrogen metabolism are integrally coupled as assimilation of N in to aminoacids occurs primarily via the GS/GOGAT pathway. It is also found that slightly acidic pH and a salinity of 20‰ - 25‰ can promote the growth of S.salina. This might be because of the better speciation of ions in the above conditions, there by making more ions available to the growing cells. At higher salinities there may be decreased intake of ions from the medium, owing to the increased binding of ions already present in the sea water on to the cell wall. This might have reduced the intake of different ions required for the growth of cyanobacterial cells. Similarly at higher pH the cyanobacteria also might be spending a quantum of its energy for keeping the osmotic potential of the cell, to avoid a collapse. This channelisation of the energy might have a negative impact on the quantum yield of the cells. The initial pH of the medium is around 8. But eventually it may reach as high as 9.5 and get stabilised around 9. This is because of the absorption of NO_3^- ion from the medium by the growing cells accompanied by an increased up take of CO_2 which results in a buffering action.

The term heavy metal has been generally used to describe those metals having an atomic number greater than that of iron or having a density greater than 5g/ml. Some heavy metals like Cu, Zn, Mn, Mg etc are important as trace nutrients while others are toxic. Heavy metals are found in the aquatic environment as inorganic cations and they may be

concentrated by the primary trophic levels and thus incorporated in to the food chain. A study of 5 heavy metals (Hg, Cu, Pb, Cd & Zn)at 3 sublethal concentrations (0.1 ppb, 1ppb and 10 ppb) is found to enhance the growth over control. Copper and zinc are essential micronutrients while other 3 heavy metals are not essential and usually toxic. The former is a component of plastocyanin while both are cofactors for several biochemical conversions. It is believed that the actual concentration of heavy metals is far below the dosed concentration because of higher salinity (32‰), higher pH, heavy metal adsorption on borosilicate glass, algal products capable of complexing heavy metals, "biological dilution", abundance of NO_3^- etc. There are also previous reports saying that binding of metallic cations to enzymes could alter their activity not only by inhibiting but also by stimulating their catalytic function.

Dosing the cells with higher concentration (1ppm) of metals revealed a toxicity index Hg > Cu > Cd > pb > Zn. S. salina is capable of concentrating both lead and cadmium. Initially the metals are adsorbed to the cell surface by displacing the cations already occupying the binding sites of the cells which is a mosaic of interspersed cationic and anionic exchange sites provided by carboxylic, sulphydryl, phosphatidic, amino and other groups. The metal adsorbed thus is further translocated into the cell. Due to the smaller size (3 μ) a large number of cells can be present in limited volume. In the present study, cells are found to be packed to the extent of 8 million cells per ml. This vast surface area and a higher surface area: volume ratio may help in an increased adsorption of the metal. Present study also revealed that physiology of the cell is a major factor governing the intake of heavy metals. Generally cyanobacteria are sensitive to Cu and Cd. However S. salina is usually resistant against Pb and Cd and it can concentrate both metals.

Heavy metal uptake is directly proportional to the available surface areas. So Inorder to increase the cell number in a limited volume as well as to utilise the physiological peculiarity of the cells at definite growth period, immobilization technique (Ca-alginate) has been employed. 25ml algal suspension of 15-20 days old, when immobilised and incubated in 25ml of lppm solutions of Pb and cadmium reduced the quantity of metals in each medium as low as 0.1 ppm within 24 hrs. For a living organism, the major chunk of the energy is spent for molecular mechanisms associated with cell division and sexual reproduction. But when an autotrophic unicellular organism is immobilised, the energy associated with the above processes is saved. This may result in the increased longivity of cells. It is also found that Cd can extend the longivity further (6 months) over Pb (4 months) and control (3 months).

The heavy metal uptake and concentration is eventually associated with metallothinein or metallothinein like proteins. They are low molecular weight cysteine rich polypeptides that complex soft metal ions in thiol clusters. Plant metallothioneins are structurally diverse - some are gene products while others are secondary metabolites. A common feature of all metallothioneins is an abdunance of cys-xaa-cys sequences, where xaa is an aminoacid other than cysteine. Synthesis of metallothionein increases in organisms exposed to elevated concentrations of certain trace metals. Metallothionein or metallothionein like protein was extracted from the S. salina after repeated freezing and thawing accompanied by grinding in a mortar in 0.9% Nacl. The protein mixture thus obtained was partitioned in a sephadex G75 column. The protein concentration and metal content of each fraction was analysed using a UV-

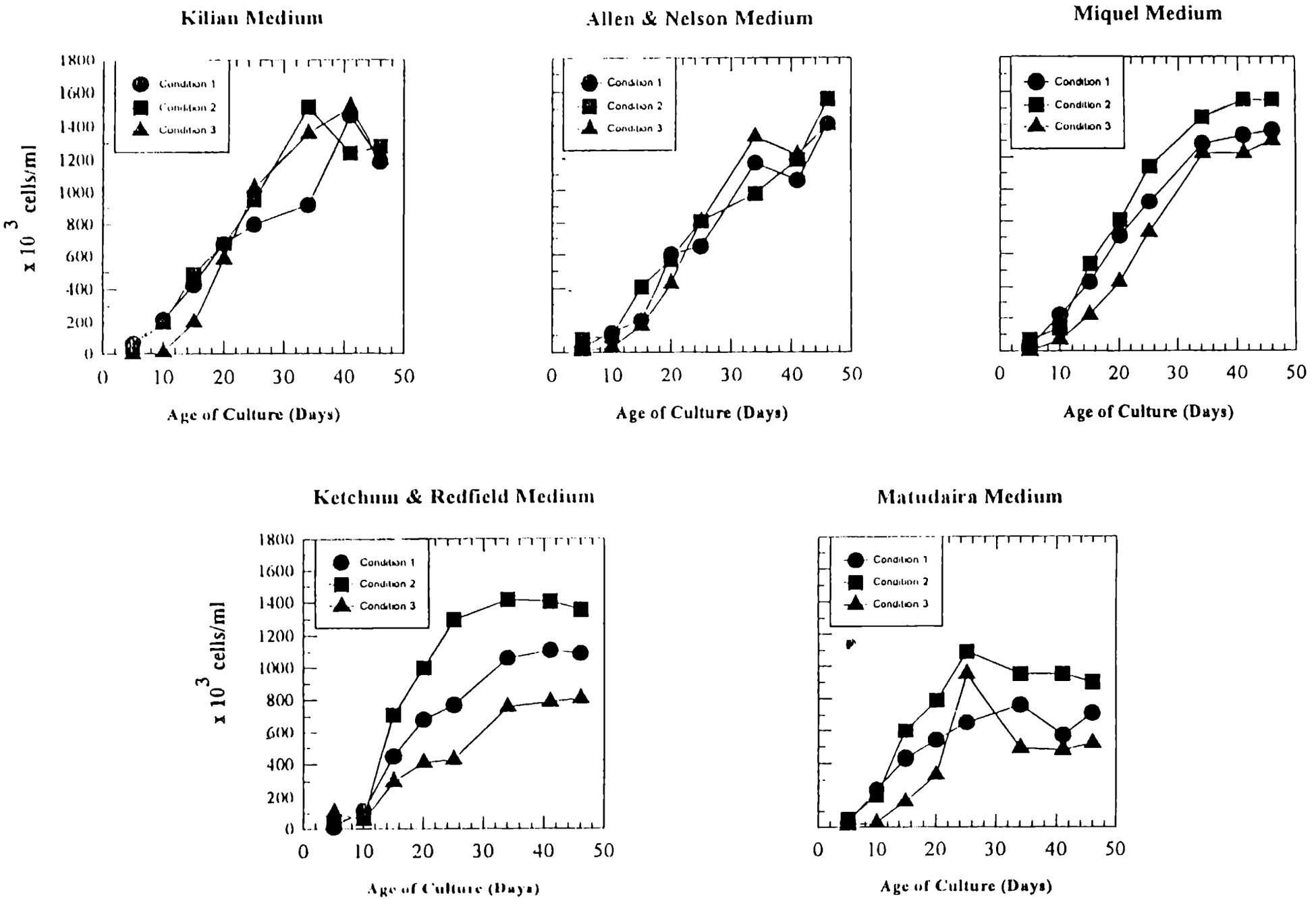
visible spectrophotometer and atomic absorption spectrophotometer respectively. A screening of eluant fractions for the metals revealed that the metallothionein or metallothionein like protein of S. salina is predominantly copper bound, zinc being present in lesser amounts. Metallothionein or metallothionein like protein from S. salina exposed to zinc was significantly increased. This sporadic increase in the concentration of metallothionein or metallothionein like protein signifies their role in zinc homeostasis. When S. salina cells were exposed to copper, there was a reduction in metallothionein or metallothionein like protein. However, there was an increased synthesis of high molecular weight protein to accommodate the excess Cu. When dosed with Cd, there was a sharp increase in the production of metallohionein or metallothionein like protein, nevertheless cadmium is not found to be appreciably bound with it. It is also noticed that when S. salina cells grown in a medium containing cadmium were subcultured into another medium containing cadmium, instead of the predicted growth lag, they grew immediately. Behaviour of cells dosed with Pb was similar to that of Cd. Incubation of cells in a medium containing mercury (Hg^{++}) reduced the production of metallothionein or metallothionein like protein. Invariably in all the heavy metal exposed cultures, the metallothionein fraction contains more than double the quantity of zinc, compared to the control while the concternation of copper fluctuates. Considering all these facts in addition to the binding to the toxic heavy metals, it could be speculated that metallothionein or metallothionein like protein might be playing a major role in the detoxification mechanism by metallothionein mediated ion antagonism. It is also possible that metallothionein induces some other molecules that could sequester the heavy metal, at least in the case of Cadmium.

There are currently a large number of antibiotic compounds available for the treatment of pathogenic conditions, the majority of these being produced by actinomycetes. However, with the ever increasing evidence of antibiotic resistance, the search for new agents has widened in to previously neglected biological phyla. Very little attention has been paid to other groups such as the cyanobacteria, some of which are able to grow under diverse nutrient conditions. S. salina cells from exponential and stationery phases were harvested and extracted in hexane, methanol and acetone and tested for antiobacterial activity on Bacillus subtilis, Escherichia coli and salmonella sp. Similarly cells grown in different salinities were extracted in the above solvents and the extracts were tested for antibacterial activity on the same bacteria. Results indicates the presence of an intracellular broad spectrum antiobiotic in acetone extracts from cultures grown at higher salinities. The axenic nature of the cyanobacterial cultures was proved by plating them on nutrient agar medium. Unilke the antibiotics accumulated in the actinomycetes, which are secondary metabolites accumulated in the stationery period, here the antibacterial principle seems to be a product produced at higher salinities without affecting the growth phase of S. salina.

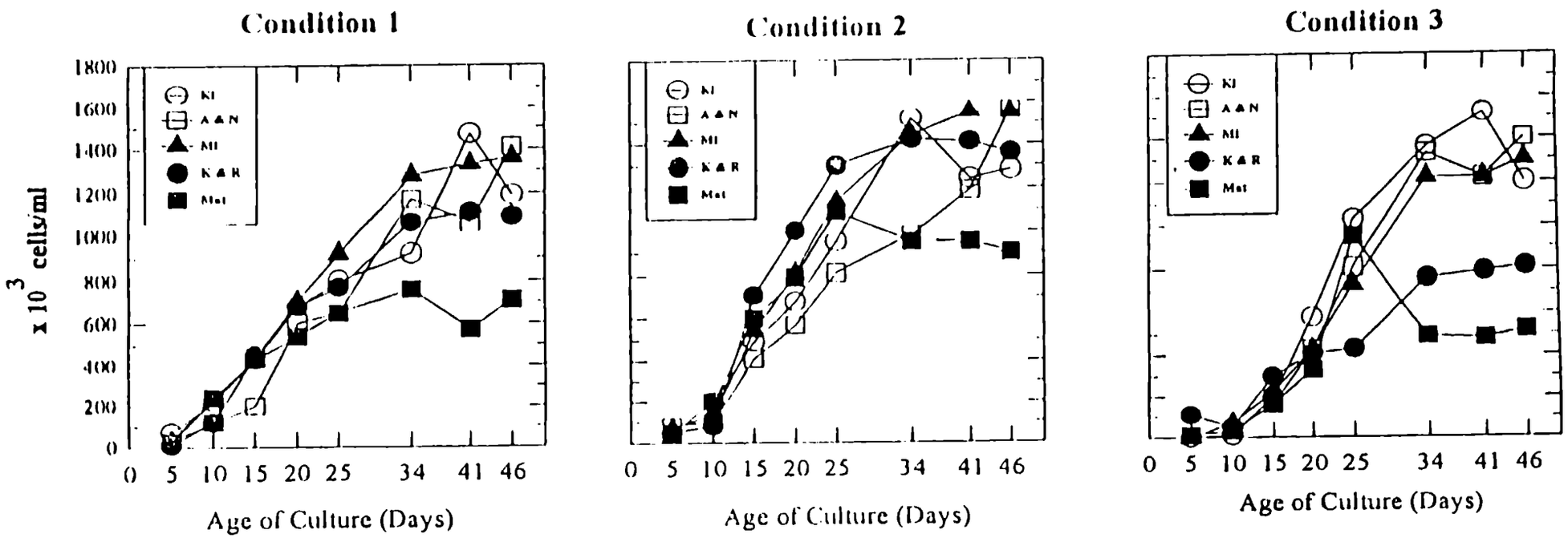
Experiments conducted in our laboratory divulged the biotechnological potential of S. salina. At present they are used as a feed in aquacultue hatcheries due to their high protein content, smaller size and easy digestibility. But the present investigations indicate that the cells grown at higher salinities may be successfully used as nutrient-rich natural antiobiotic agents capable of preventing the origin and spread of primary and secondary bacterial infections. Further purification and characterisation of this antibiotic principle would enable its extraction on a large scale.

Similarly the ability of S. salina to concentrate Pb and Cd may be exploited to cleanup the industrial effluents containing these heavy metals. However, the commercial utilization of this information is much depends upon designing a cost effective bioreactor with standardised optimum parameters with a device for recharging the immobilised cells.

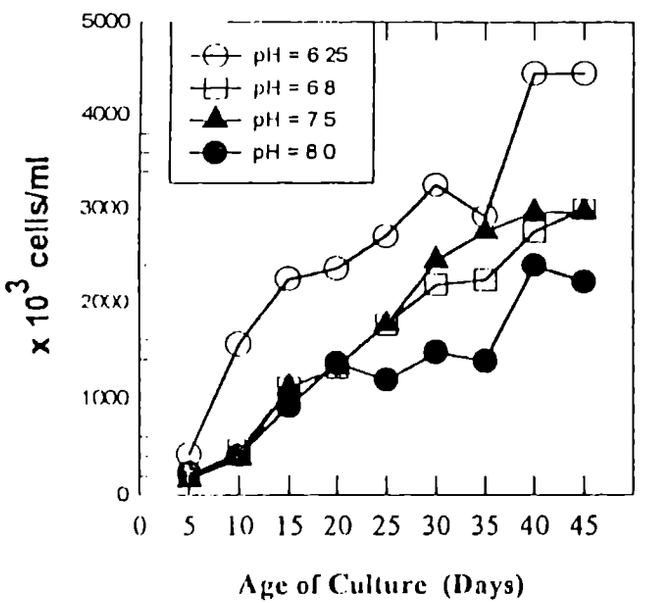
Growth of *S. salina* in Five Different Media



Growth of *S. salina* in Five Different Media Under Three Different Sets of Conditions



Effect of pH on the Growth of *S. salina*



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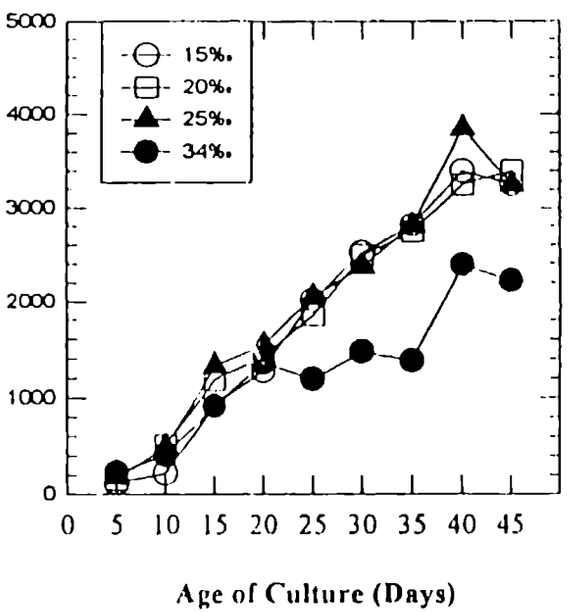
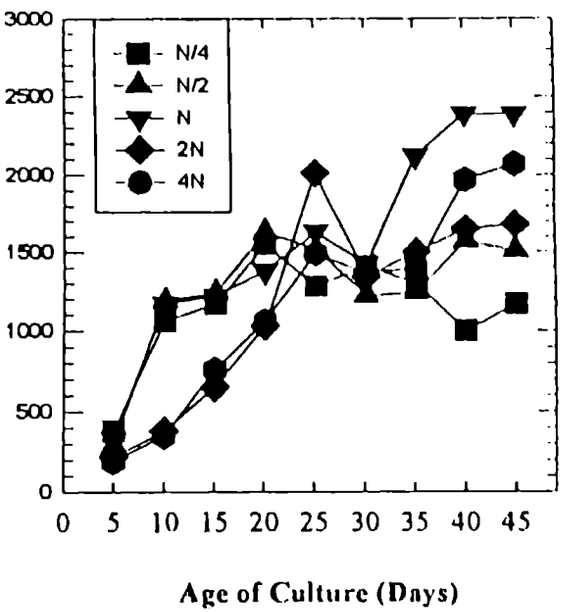


Effect of NO^-_3 Concentration on the Growth of *S. salina*



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* Originals not seen.