Studies on thallium toxicity to Cyanobacterial strains isolated from a variety of natural habitats: implications for environmental biotechnology

Thesis

Submitted in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I hereby declare that the thesis entitled "*Studies on thallium toxicity to Cyanobacterial strains isolated from a variety of natural habitats: implications for environmental biotechnology*" has been carried out by me under the supervision of Dr A.K Singh and this work has not previously submitted for any degree or diploma of any other university.

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He has been very sincere and honest in his work and has successfully completed the work.

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LIST OF ABBREVIATIONS

ATP	Adenosine Tri phosphate
ADP - Na_2	Disodium Adenosine di phosphate
APC	Allophycocyanin
BG - 11_0	Blue green algae medium (Nitrogen free)
Bq	Bacqueral
Chl *a*	Chlorophyll *a*
CTAB	Cetyl Tri methyl acetyl bromide
C_2H_4	Ethylene
°C	Degree centrigrade
D	D medium for cultivation of *Mastigocladus* sp.
EDTA	Ethylene diamine tetra acetic acid
EPA	Environmental Protection Agency
Fd	Ferridoxin
g	Multiples of gravitional constant
GS	Glutamate synthetase (transferase)
hr	Hour (s)
HEPES	2, [4-Hydroxyethyl) 1- piperazinyl] – N' – [2-ethane sulphonic
LD	Lethal dose
LHC	Light Harvesting complex
ml	Millilitre (s)
min	Minute (s)
mg	Milli grams
MRL	Minumum Risk Level
Nm	Wavelength (nanometer)
µM	micro molar
µg	micro grams
NADP	Nicotinamide adenine Di nucleotide phosphate
NR	Nitrate reductase

NIR	Nitrite reductase
N	Nitrogen
nmole	nano moles
OD	Optical Density.
PBS	Phosphate Buffer Saline
PE	Phycoerythrin
PC	Phycocyanin
PEG	Poly Ethylene Glycol
Psi	Pounds per square inch
Ps	Pico seconds
PSI	Photo system I
PSII	Photo system II
ppb	Parts per billion
Ppm	Parts per million
PQ	Plastoquinone
%	Percentage
RC	Reaction centre
ROS	Reactive oxygen species
SD	Standard deviation
Tl	Thallium
$^{204}Tl^{2+}$	Radioactive Thallium
Tl-R	Thallium resistant
TCA	Tri chloro acetic acid
Tris	Tris – [hydroxymethyl] amino methane
TCA	Tri chloro acetic acid
UV	Ultraviolet
w/v	Weight by volume ratio
v/v	Volume by Volume ratio

Dedicated to

My parents, wife and my daughter

&

Teachers who have taught me

1. INTRODUCTION

CHAPTER – I

INTRODUCTION

The names "cyanobacteria" and "blue-green algae" (Cyanophyceae) are valid and compatible systematic terms. This group of micro-organisms comprises unicellular to multicellular prokaryotes that possess chlorophyll *a* and perform oxygenic photosynthesis associated with photosystems I and II (Castenholz & Waterbury, 1989). The majority of cyanobacteria are aerobic photoautotroph's and their life processes require only water, carbon dioxide, inorganic substances and light. Photosynthesis is their principal mode of energy metabolism however in the natural environment some species are able to survive long periods in complete darkness. Furthermore, certain cyanobacteria show a distinct ability for heterotrophic nutrition. Cyanobacteria are often the first plants to colonise bare areas of rock and soil. Many species are capable of living in the soil and other terrestrial habitats, where they are important in the functional processes of ecosystems and the cycling of nutrient elements (Whitton, 1992). The prominent habitats of cyanobacteria are limnic and marine environments. They flourish in water that is salty, brackish or fresh, in cold and hot springs, and in environments where no other microalgae can exist. Most marine forms (Humm & Wicks, 1980) grow along the shore as benthic vegetation in the zone between the high and low tide marks in oceans. The cyanobacteria comprise a large component of marine plankton with global distribution (Gallon *et al.,* 1996). A number of freshwater species are also able to withstand relatively high concentrations of sodium chloride. It appears that many cyanobacteria isolated from coastal environments tolerate saline environments (i.e. are halo tolerant) rather than require salinity (i.e. are halophytic). As frequent colonisers of eurhaline (very saline) environments, cyanobacteria are found in salt works and salt marshes, and are capable of

growth at combined salt concentrations as high as 3-4 molar mass (Reed *et al.*, 1984). Freshwater localities with diverse trophic states are the prominent habitats for cyanobacteria. Numerous species characteristically inhabit, and can occasionally dominate, both near-surface epilimnic and deep, euphoric, hypolimnic waters of lakes (Whitton, 1992). Others colonise surfaces by attaching to rocks or sediments, sometimes forming mats that may tear loose and float to the surface. Cyanobacteria have an impressive ability to colonise infertile substrates such as volcanic ash, desert sand and rocks (Dor & Danin, 1996), and are also found in extraordinary excavators, boring hollows of limestone and special types of sandstone. Another remarkable feature is their ability to survive extremely high and low temperatures. Cyanobacteria are inhabitants of hot springs (Castenholz, 1973), mountain streams (Kann, 1988), Arctic and Antarctic lakes (Skulberg, 1996a) and snow and ice (Laamanen, 1996). The cyanobacteria also include species that run through the entire range of water types, from polysaprobic zones to katharobic waters (Van Landingham, 1982).

Cyanobacteria also form symbiotic associations with animals and plants. Symbiotic relations exist with, for example, fungi, bryophytes, pteridophytes, gymnosperms and angiosperms (Rai, 1990). The hypothesis for the endosymbiotic origin of chloroplasts and mitochondria should be mentioned in this context. The evolutionary formation of a photosynthetic eukaryote can be explained by a cyanobacteria being engulfed and co-developed by a phagotrophic host (Douglas, 1994). Fossils of what were almost certainly prokaryotes are present in the 3,450 million year old Warrawoona sedimentary rock of north-western Australia. Cyanobacteria were among the pioneer organisms of the early earth (Brock 1973; Schopf, 1996). These photosynthetic micro-organisms were, at that time, probably the chief primary producers of organic matter, and the first organisms to release elemental oxygen into the primitive atmosphere.

The structure and organisation of cyanobacteria are studied using light and electron microscopes. The basic morphology comprises unicellular, colonial and multicellular filamentous forms. Unicellular forms, for example in the order *Chroococcales,* have spherical, ovoid or cylindrical cells. They occur singly when the daughter cells separate after reproduction by binary fission. The cells may aggregate in irregular colonies, being held together by the slimy matrix secreted during the growth of the colony. By means of a more or less regular series of cell division, combined with sheath secretions, more ordered colonies may be produced.

A particular mode of reproduction, which may supplement binary fission, distinguishes cyanobacteria in the order *Chamaesiphonales* and *Pleurocapsales.* Filamentous morphology is the result of repeated cell divisions occurring in a single plane at right angles to the main axis of the filament. The multicellular structure consisting of a chain of cells is called a trichome. The trichome may be straight or coiled. Cell size and shape show great variability among the filamentous cyanobacteria. Species in the order *Oscillatoriales*, with unseriated and unbranched trichomes, are composed of essentially identical cells. The other orders with a filamentous organization (orders *Nostocales* and *Stigonematales*) are characterised with trichomes having a heterogeneous cellular composition. Vegetative cells may be differentiated into heterocysts (having a thick wall and hyaline protoplast, capable of nitrogen fixation) and akinetes (large thick-walled cells, containing reserve materials, enabling survival under unfavourable conditions). In the order *Stigonematales*, the filaments are often multiseriated, with genuine branching. Both heterocyst and akinetes are present. The only means of reproduction in cyanobacteria is asexual. Filamentous forms reproduce by trichome fragmentation, or by formation of special hormogonia. Hormogonia are distinct reproductive segments of the trichomes. They exhibit active gliding motion upon their

liberation and gradually develop into new trichomes. Cyanobacteria do not posses membrane-bound sub-cellular organelles; they have no discrete membrane-bound nucleus; they possess a wall structure based upon a peptidoglycan layer; and they contain 70S rather than 80S ribosomes (Fay and Van Baalen, 1987).

The photosynthetic pigments of cyanobacteria are located in thylakoids that lie free in the cytoplasm near the cell periphery. Cell colours vary from blue-green to violet-red. The green of chlorophyll *a* is usually masked by carotenoids (e.g. beta-carotene) and accessory pigments such as phycocyanin, allophycocyanin and phycoerythrin (phycobiliproteins). The pigments are embodied in phycobilisomes, which are found in rows on the outer surface of the thylakoids (Douglas, 1994). All cyanobacteria contain chlorophyll *a* and phycocyanin, allophycocyanin and phycoerythrin. The basic features of photosynthesis in cyanobacteria have been well described (Ormerod, 1992). Cyanobacteria are oxygenic phototrophs possessing two kinds of reaction centres, PS I and PS II, in their photosynthetic apparatus. With the accessory pigments mentioned above, they are able to use effectively that region of the light spectrum between the absorption peaks of chlorophyll *a* and the carotenoids. The ability for continuous photosynthetic growth in the presence of oxygen, together with having water as their electron donor for CO_2 reduction, enables cyanobacteria to colonise a wide range of ecological niches (Whitton, 1992). Phycobiliprotein synthesis is particularly susceptible to environmental influences, especially light quality. Chromatic adaptation is largely attributable to a change in the ratio between phycocyanin and phycoerythrin in the phycobillisome. Thus, cyanobacteria are able to produce the accessory pigment needed to absorb light most efficiently in the habitat in which they are present. Cyanobacteria have a remarkable ability to store essential nutrients and metabolites within their cytoplasm. Prominent cytoplasmic inclusions for this purpose can be seen with the electron

microscope e.g. glycogen granules, lipid globules, cyanophycin granules, polyphosphate bodies, carboxysomes (Fay and Van Baalen, 1987).

Dinitrogen fixation is a fundamental metabolic process of cyanobacteria, giving them the simplest nutritional requirements of all living organisms. By using the enzyme nitrogenase, they convert N_2 directly into ammonium (NH_4^+ - a form through which nitrogen enters the food chain) and by using solar energy to drive their metabolic and biosynthetic machinery, only N_2, CO_2, water and mineral elements are needed for growth in the light. Nitrogen-fixing cyanobacteria are widespread among the filamentous, heterocyst forming like *Anabaena, Nostoc* (Stewart, 1973) and dinitrogen fixation among cyanobacteria not forming heterocysts like e.g. *Trichodesmium* sp. (Carpenter *et al.*, 1992). Under predominantly nitrogen limited conditions, but when other nutrients are available, nitrogen fixing cyanobacteria may be favoured and gain growth and reproductive success. Many species of cyanobacteria possess gas vesicles. These are cytoplasmic inclusions that enable buoyancy regulation and are gas-filled, cylindrical structures. Their function is to give planktonic species an ecologically important mechanism enabling them to adjust their vertical position in the water column (Walsby, 1987). To optimise their position, and thus to find a suitable niche for survival and growth, cyanobacteria use different environmental stimuli like photic, gravitational, chemical, thermal as clues. Gas vesicles become more abundant when light is reduced and the growth rate slows down. Increases in the turgor pressure of cells, as a result of the accumulation of photosynthetic pigments, cause a decrease in existing gas vesicles and therefore a reduction in buoyancy.

Although cyanobacteria probably evolved as a group of organisms about 2,000 million years before the advent of eukaryotes, they comprise fewer taxa than eukaryotic

microalgae (Rai, 1990). The concept of species in the cyanobacteria has, however, no distinct boundaries. The situation is similar for most organisms, except for those that are sexually reproductive. Depending on the classification system used, the number of species recognised varies greatly. Based on the International Code of Botanical Nomenclature the class *Cyanophyceae*, for example, contains about 150 genera and 2,000 species (Hoek *et al.*, 1995).

The cyanobacteria have both beneficial and detrimental properties when judged from a human perspective. Their extensive growth can create considerable nuisance for management of inland waters (water supply, recreation, fishing, etc.) and they also release substances into the water which may be unpleasant (Juttner, 1987) or toxic (Gorham & Carmichael, 1988). The water quality problems caused by dense populations of cyanobacteria are intricate, many and various (Skulberg, 1996a) and can have great health and economic impacts. As a consequence, the negative aspects of cyanobacteria have gained research attention and public concern. The properties that make the cyanobacteria generally undesirable are also the qualifications for possible positive economic use. Blue-greens are the source of many valuable products and also as suitable candidate for bioremediation and carry promising physiological processes, including light-induced hydrogen evolution by bio photolysis (Skulberg, 1994). Extensive research has taken place in the relevant fields of biotechnology. Cyanobacteria may be used for food or fodder because some strains have a very high content of proteins, vitamins and other essential growth factors and vital pigments of interest can also be produced (Borowitzka & Borowitzka, 1988). Cyanobacteria are also sources for substances of pharmaceutical interest such as antibiotics (Falch *et al.*, 1995). Due to ubiquitous occurrence & abundance in nature cyanobacteria offers possibility for exploitation for ample biotechnological methods.

Heavy metals are a general collective term which applies to the group of metals and metalloids with an atomic density greater than 4 g/cm^3. Although heavy metals differ in their chemical properties, they are used widely in electronic components, machinery and materials. Consequently, they are emitted to the environment from a variety of anthropogenic sources to supplement natural background geochemical sources. Although it is a loosely defined term (Duffus, 2002), it is widely recognized and usually applies to the widespread contaminants of terrestrial and freshwater ecosystems. The amounts of most heavy metals deposited to the surface of the earth are many times greater than depositions from natural background sources. Combustion processes are the most important sources of heavy metals, particularly, power generation, smelting, incineration and the internal combustion engine (Hutton & Symon 1986; Battarbee *et al.*, 1988; Nriagu & Pacyna 1988; Nriagu 1989).

The heavy metals include cadmium, chromium, copper, mercury, lead, zinc, arsenic, boron and the platinum group metal platinum, palladium, rhodium, ruthenium, osmium, and iridium. In the right concentrations, many metals are essential to life. In excess, these same chemicals can be poisonous. Similarly, chronic low exposures to heavy metals can have serious health effects in the long run. The main threats to human well-being are associated with lead, arsenic, cadmium and mercury, and it is these substances that are targeted by international legislative bodies (Fergusson, 1990) Lead poisoning in children causes neurological damage leading to a reduction in intelligence, loss of short term memory, learning disabilities and problems with coordination. Prenatal exposure can cause reduced birth weight and immune suppression or over sensitisation, which could explain why some children develop asthma and allergies (Day, 1998). High concentrations of arsenic in drinking water have been documented in specific parts of Argentina, Canada, Chile, China, Japan, Mexico, the Philippines and the USA. The

problem is particularly acute in West Bengal in India and Bangladesh, where an estimated 30 million people are drinking arsenic-poisoned water (WHO, 1997). Some 62% of wells supply arsenic-contaminated water above threshold limits with some containing as much as 400 times the limit (Bagla & Kaiser, 1996). The effects of arsenic include cardiovascular problems, skin cancer and other skin effects, peripheral neuropathy (WHO 1997) and kidney damage. Cadmium exposure occurs mainly through cereals and vegetables grown on soils contaminated by mining activities and use of phosphorus fertilizers. Shellfish and animal organs also contain high levels of toxic metals by bioaccumulation. Cadmium accumulation in the kidneys is implicated in a range of kidney diseases (WHO, 1997).

Mercury accumulates at the top of aquatic and marine food chains and fish is the major source of dietary exposure (WHO, 1997). The principal health risks associated with mercury are damage to the nervous system, with such symptoms as uncontrollable shaking, muscle wasting, partial blindness, and deformities in children exposed in the womb. At levels well below threshold limits, it can damage the foetal and embryonic nervous systems with consequent learning difficulties, poor memory and shortened attention spans (Jorgensen *et al.*, 1997). Low-level exposures can also adversely affect male fertility (Dickman *et al.*, 1998). Mercury pollution is a global problem. Most of the mercury found in high concentrations in the Everglades in Florida comes from thousands of miles away, travelling on trade winds from Europe and Africa. Although it appears that less mercury than previously thought is polluting Greenland (Boutron *et al.*, 1998), global transfers of mercury to the poles are still substantial, with base-levels three times what they were two centuries ago. Every spring, a toxic rain of mercury falls on the arctic, at the time when ecosystems are most active. (Pearce, 1997c). As a consequence,

one in six Greenlanders has potentially harmful blood-levels of mercury, from eating contaminated fish and whales.

Among heavy metal thallium is one of the most toxic compounds with very serious and deleterious effects on different ecosystems. It is a soft, bluish-white metal that is widely distributed in trace amounts in the earth's crust. In its pure form, it is odourless and tasteless. It can be found in pure form or mixed with other metals in the form of alloys (Environ Tools, 2002). It can also be found combined with other substances such as bromine, chlorine, fluorine, and iodine to form salts. These combinations may appear colourless to white or yellow. Thallium remains in the environment since it is a metal and cannot be broken down to simpler substances. Thallium exists in two chemical states (thallous and thallic). The thallous state is the most stable form and interacts with organisms when exposed (Ewers, 1988). Thallium is a non-volatile heavy metal, and if released to the atmosphere by anthropogenic sources, may exist as an oxide (thallium oxide), hydroxide (TlOH), sulfate (thallium sulfate), or as the sulfide Tl_2S (EPA 1988a). These thallium compounds are not volatile (Weast, 1970) and their oxides are less soluble in water, and may be subject to only atmospheric dispersion, and gravitational settling. Thallium precipitates in water as solid mineral phases. However, thallium chloride, sulfate, carbonate, bromide, and hydroxide are very soluble in water. The solubility of thallium sulfate at 0°C is about 27 g/L (EPA 1988a) and in extremely reducing water, thallium may precipitate as a sulfide (Tl_2S), and in oxidizing water, Tl^{3+} may be removed from solution by the formation of $Tl(OH)_3$ (Lee 1971, Callahan *et al.*, 1979a). Thallium is used mostly in the manufacture of electronic devices, switches, and closures and also used in the manufacture of special glasses and for medical procedures that evaluate heart disease, in infrared spectrometers, in crystals, in other optical systems, and for colouring glass (Zitko, 1975; Kazantzis, 1986). The

greatest use of thallium is in specialized electronic research equipment (EPA, 2002) and also in semiconductor and laser industry, in scintillographic imaging, in superconductivity, and as a molecular probe to emulate the biological function of alkali-metal ions (Nriagu, 2003). Up until 1972 thallium was being used as a rat poison, however, it was banned subsequently because of its potential harmful effects on human.

Thallium is present in air, water, and soil. Major releases of thallium to the environment are from processes such as coal-burning, cement and ore smelting operations and ferrous and nonferrous smelting operations and to a lesser extent from facilities producing or using thallium compounds (Sharma *et al.*, 1986; Ewers, 1988). Davison *et al.* (1974) reported increased concentrations of thallium on airborne fly ash emitted from a coal-burning power plant ranging from 29 to 76 μg/g ashes. The authors reported that these concentrations were representative of eight other United States power plants burning various types of coal. The highest thallium concentrations were also found on the smaller diameter (0.2 – 0.8 μM) particles of fly dust emitted from a West German cement plant (Ewers 1988). Additional sources of airborne thallium include manufacturers of alloys, artificial gems, electronics equipment, optical glass, and domestic heating plants (EPA 1980a; 1983c; Sharma *et al.*, 1986; EPA 1988a; Valerio *et al.*, 1988; Ewers, 1988). Thallium concentrations in raw or treated waste waters from these industries ranged up to 2 g/L (EPA 1983c). Thallium has been detected in urban waste waters, apparently from commercial and industrial sources (Callahan *et al.*, 1979a; Levins *et al.*, 1979). Thallium was detected at a geometric mean concentration of 1.7 ppm in positive soil samples from 3.5% of an unspecified number of hazardous waste sites (CLPSD 1989). Although direct soil releases are likely to be small, since thallium-containing wastes are subject to EPA land disposal restrictions, atmospheric thallium pollution may contribute to soil contamination in the vicinity of thallium emission

sources (Brockhaus *et al.*, 1981). A survey of tap water from 3,834 homes in the United States showed presence of thallium in 0.68% of samples only at an average thallium concentration of 0.89 µg/L (EPA 1980a, 1988a).

Thallium has been found in samples collected from seawater at 0.01-14.00 µg/L (Sharma *et al.*, 1986) and in earth's crust at a range from 0.3 to 0.7 ppm (EPA 1988a), . Thallium concentrations in lake sediments ranged from 0.13 to 0.27 µg/g in four remote Rocky Mountain lakes (Heit *et al.* 1984) to 2.1-23.1 mg/kg (mean value 13.1 mg/kg) in a Michigan lake reportedly polluted by airborne particulate matter (EPA 1988a). Mathis and Kevern (1975) presented indirect evidence suggesting thallium absorption by lake sediments. The absorption of thallium by other sources such as micaceous clays in solution has been reported (Frantz & Carlson, 1987). Partition coefficients such as adsorption constants describe the tendency of a chemical to partition between solid phases from water. Thallium adsorption capacity for hectorite clay (a rare montmorillonite clay mineral) at pH 8.1 has been found to be 19 L/g (Magorian *et al.* 1974).

In addition to non living materials thallium is absorbed by plants from soil as well thereby entering the terrestrial food chain. Several research findings have revealed significant increase in intracellular thallium levels in food and vegetable crops grown in thallium-polluted soils (Sharma *et al.*, 1986; Ewers 1988). A study on the thallium content accumulated in different varieties of food in the United Kingdom has reported elevated levels of thallium in meat, fish, fats, and green vegetables (Sherlock & Smart 1986).

Ingestion, inhalation, or dermal absorption is the major routes of entry on thallium in the body of humans. Inhalation of thallium laden air near emission sources or in the workplace contributes to thallium exposure of personals working near various thallium emission sources (Barnes *et al.* 1988). Several cases of thallium poisoning have been reported. Acute toxicity studies on people ingesting large amounts of thallium over a short time period have reported vomiting, diarrhoea, temporary hair loss, and effects on the nervous system, lungs, heart, liver, kidneys gastroenteritis, polyneuropathy and alopecia (Kazantzis, 1986), palmar erythema, acne, anhydrosis and dry scaly skin (Mulkey & Oehme, 1993) and finally death (Enviro Tools, 2002). In case of acute intoxication by exposure to a large single dose, there is usually an initial hypotension and bradycardia owing to direct effects of the sinus node and cardiac muscle (Marcus, 1985), followed by hypertension and tachycardia, failure of central and peripheral nervous system (Kemper & Bertram, 1991; Ramsden, 2002). Chronic toxicity of thallium poisoning includes anorexia (Grunfeld & Hinostroza, 1964) headache, pains in abdomen, upper arms and thighs and even all over the body (Zhang *et al.*, 1998). Studies carried out on the effect of thallium due to human occupation behaviour in US has revealed that people dwelling in the industrial belts exhibited adverse nervous defect followed by paresthesia, numbness of toes and fingers, the "burning feet" phenomenon, and muscle cramps (Ludolph *et al.*, 1986) in the age groups between 5-44 years. These patients also exhibited fiber necrosis, central nucleation, and fiber splitting as revealed by the histopathological examination of muscles (Limos *et al.* 1982, Olsen & Jonsen 1982), elevated levels of serum glutamic oxaloacetic transaminase, serum pyruvic oxaloacetic transaminase, and alkaline phosphatase (Stoltz *et al.*, 1986).

Until the middle 1960s, therapy for thallium poisoning was generally unsuccessful. Since then successful method for treating thallium poisoning have been

developed (Schoer, 1984) which induces thallium excretion via the kidney upon dosage of potassium chloride or upon administration of diuretics. Furthermore activated charcoal, British antilewsite (BAL), calcium salts, cystine, dithiocarb, dithizone, histamine and theophylline were recommended as antidotes against acute and chronic thallium poisoning (Zitko, 1975; Hoffman *et al.*, 1999).

The exact mechanism of thallium toxicity is unclear but reports suggest that like other heavy metals, thallium binds to sulfhydril groups of proteins and mitochondrial membranes thereby inhibiting a range of enzyme reactions and leading to a generalized poisoning (Dolgner *et al.*,1983; Ramsden, 2002), interaction with riboflavin and riboflavin-based cofactors by affecting distribution of calcium homeostasis (Mulkey & Oehme, 1993), interference with the vital potassium-dependent processes, substitution of potassium in the (Na^+/K^+)- ATPase and other potassium involving biochemical activities.

Owing to acute and chronic toxicity of thallium poisoning to mankind EPA, USA (2002) has set up environmental standards for thallium. In drinking water the safe threshold limit was fixed to be 2 ppb concentrations whereas in ground water to be 0.5 ppb respectively. Although there are various methods of thallium removal which EPA (2002) has approved e.g. activated alumina ion exchange method, electrolytic manganese dioxide (EMD) method, DOWA iron powder method and CABSORB Zeolite method. Although all these methods being very effective to remove thallium, they are highly expensive and less cost effective. Therefore development of cost effective methods to detoxify thallium polluted environments warrants immediate attention. Microbes including cyanobacteria are one of the most suitable candidates that can be used for bioremediation of thallium. However, this should be preceded by evaluation of toxicity and kinetics of thallium uptake in cyanobacteria. Cyanobacteria have developed natural

methods of responding to metals such as copper, lead and cadmium through passive accumulation in cells and through surface binding to various functional groups (Torresdey *et al.*, 1998) and remove harmful metals from the environment (Slotton *et al.*, 1989). Since these organisms are primary producers in the food chains, the metabolic and morphological effects on them by environmental toxicants play an important role in assessing aquatic ecosystems responses to heavy metal contamination (Lustigman *et al.*, 2000). Toxic effects of different heavy metal cations on cyanobacteria have been studied in detail (Conway, 1978; Strattion & Huber, 1979; Laube *et al.*, 1980; Singh & Pandey, 1981; Massalski *et al.*, 1981; Wurtsbaugh & Horne, 1982; Rai & Raizada, 1986; Rai & Dubey, 1989; Rai, *et al.*, 1990; De Filippis & Pallaghy, 1994; Gupta & Singhal, 1996). To the best of our information, no study has been performed involving thallium toxicity in cyanobacteria which is prerequisite and should form a basis for using cyanobacteria as a potential tool for thallium bioremediation purposes. In view of this we have conducted a comparative study on the effect of thallium in *Nostoc muscorum*, *Anabaena cycadae*, *Nostoc* ANTH, *Anabaena variabilis* and *Mastigocladus* sp. isolated in our laboratory from different free living areas, hot springs and symbiotic associations based on the following parameters:

- Regulation of thallium toxicity to the cyanobacteria in response to organic and inorganic nitrogen sources.
- Effect of thallium on the activities of enzymes GS, NR, NIR, and intracellular proline level in cyanobacterial strains *Nostoc muscorum*, *Anabaena cycadae, Anabaena variabilis* and *Nostoc* ANTH.
- Characterization of thallium uptake activity and its regulation by cationic elements and nitrogen sources in *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Mastigocladus* sp.

2. MATERIAL AND METHODS

CHAPTER II

Materials and Methods

2.1 Organism Used

The cyanobacterial strains *Nostoc* ANTH, *Anabaena cycadae, Nostoc muscorum, Anabaena variaiblis* and *Mastigocladus* sp. were used in the present study.

Nostoc ANTH was isolated from the under surface of the gametophytic thalli of the bryophyte *Anthoceros* whereas *Anabaena cycadae* was isolated from coralloid roots of *Cycas* from the campus of North Eastern Hill University, Shillong. Both of these cyanobacteria were found in symbiotic associations. They were cultured and maintained in free living medium. *Nostoc muscorum and Anabaena variabilis* which are free living photoautotrophs, heterocystous, unbranched, filamentous and diazotrophic cyanobacteria were also isolated from the campus of North Eastern Hill University, Shillong. Axenic clonal cultures of these strains were grown luxuriantly in modified BG-11_0 medium (Rippka *et al.*, 1979) and were maintained on agar slants which were prepared by adding 1.5% SISCO agar to the liquid medium before autoclaving.

Mastigocladus sp. was originally isolated from hot springs of Jakrem in Meghalaya. Axenic cultures of *Mastigocladus* sp. was grown and maintained in D-medium (Castenholz, 1969) with slight modifications. Clonal cultures were maintained on agar slants which were prepared by adding 1.5% SISCO agar to the liquid medium before autoclaving.

2.2 Culture medium

The culture medium used in the present study has the following composition:

Modified BG-11_0 medium

Macronutrients stock	**gms/100mL**
$NaNO_3$	1.5 g
K_2HPO_4	0.04g
$MgSO_4 \cdot 7H_2O$	0.075g
$CaCl_2 \cdot 2H_2O$	0.036g
Citric acid	0.006g
Ferric ammonium citrate	
EDTA (disodium salt)	0.001g
$NaCO_3$	0.02g
Micronutrient stock	**gms/1000mL**
H_3BO_3	2.86g
$MnCl_2 \cdot 4H_2O$	1.81g
$ZnSO_4 \cdot 7H_2O$	0.222g
$NaMoO_4 \cdot 2H_2O$	0.39g
$CuSO_4 \cdot 5H_2O$	0.079g
$Co(NO_3)_2 \cdot 6H_2O$	49.4mg

1mL of each macronutrient and 1mL of micronutrient was added in a final volume of 1000 mL with Millipore Distilled water. The pH was adjusted to 7.5-8.0 before autoclaving.

Modified D-medium (for *Mastigocladus* sp.)

Macronutrients	**gms/1000 mL**
$NaNO_3$	0.7 g
Na_2HPO_4	0.11 g
KNO_3	0.10 g
$MgSO_4 \cdot 7H_2O$	0.10 g
Nitrilotriacetic acid	0.10 g
$CaSO_4 \cdot 2H_2O$	0.06 g
NaCl	8.0 mg
$FeCl_3$ solution	1.14 mg
Micronutrient	**gms/1000mL**
$MnSO_4.H_2O$	2.28 g
H_3BO_3	0.5 g
$ZnSO_4 \cdot 7H_2O$	0.5 g
$CoCl_2 \cdot 6H_2O$	0.025 g
$CuSO_4 \cdot 5H_2O$	0.025 g
$Na_2MoO_4\ 2H_2O$	0.025 g
H_2SO_4	0.5 ml

1mL of each macronutrient and 1mL of micronutrient was added in a final volume of 1000 mL with Millipore Distilled water. The pH was adjusted to 7.5-8.0 before autoclaving

Combined nitrogen sources stock:

Sodium nitrate	5mM
Ammonium chloride	2mM
Glycine	1mM
Alanine	1mM
Valine	1mM
Theorine	1mM

Glutamine	1mM
Glutamic acid	1mM
Aspartic acid	1mM
Asparagine	1mM
Methionine	1mM
Lysine	1mM
Cystein	1mM
Serine	1mM
Proline	1mM
Leucine	1mM
Isoleucine	1mM
Arginine	1mM
Phenylalanine	1mM
Tryptophan	1mM
Tyrosine	1mM
Histidine	1mM

BG-11_0 medium without any N (nitrogen) source was represented as N_2-medium, D-medium for culturing *Mastigocladus* sp., without any N source was referred to as D-medium under present investigations. The combined nitrogen sources were added to N_2 medium or D-medium as when required with final concentration of 5mM $NaNO_3$ (nitrate medium) or 2mM NH_4Cl (ammonium medium) as inorganic source or 1mM of 20 amino acids as organic sources respectively. All media supplemented with combined nitrogen sources- organic or inorganic were buffered with equimolar concentration of HEPES (2, [4-Hydroxyethyl) 1- piperazinyl] – N' – [2-ethane sulphonic acid) and pH was adjusted to 7.5 – 8.0 before autoclaving. The thallium salt used for the present study was thallium

sulphate – Tl_2SO_4. The experiments were conducted in sets of two independent experiments each with two replicates.

2.3 Sterilization

The culture medium for all the cyanobacterial strains was sterilized prior to inoculation by autoclaving at 15 lbs/sq inch pressure for 15 minutes at 121°C.

2.4. Chemicals

All glassware used for the present investigations were procured from Schott Duran Ltd, Germany. Thallium sulphate (Tl_2SO_4) was purchased from Fluka chemicals, Germany. All biochemicals for various physiological activities were purchased from Sigma Aldrich Company, USA of analytical grade. The chemicals were dissolved in deionized distilled water using Millipore distillation system (Millipore India Ltd, Bangalore). General chemicals and solvent were from Qualigens or Glaxo or SRL, alcohol from Bengal Chemicals, Kolkata. Gases used were of highest purity from Sigma Gases & Services, New Delhi (helium) and Assam Air Products, Assam (N_2, O_2, H_2). Ethylene was obtained from Eurasian associates, West Bengal. The radioisotopes were procured from Board of Radiation and Isotope Technology (BRIT), Mumbai in the form of $^{204}Tl_2SO4$ (specific activity 643 mCi g^{-1}). Metal solutions and working standards were prepared afresh for every experiment by diluting stock solutions. To eliminate contamination all glassware and plasticware were cleaned before use with cedepol detergent, followed by overnight soaking in 50% nitric acid.

2.5. Culture growth conditions:

Axenic clonal cultures of *Nostoc* ANTH, *Anabaena cycadae*, *Anabaena variabilis* and *Nostoc muscorum* were grown in liquid media or solid medium (with 1.5%

agar) in an air conditioned chamber at 24 ± 2°C with a photon fluence rate of 50µmol m^{-2} s^{-1} and control illumination of 12 hours light and 12 hours dark. For *Mastigocladus* sp. cultures were incubated in a BOD incubator at 45 ± 1°C with a photon fluence rate of 50µmol m^{-2} s^{-1} and control illumination of 12 hours light and 12 hours dark.

2.6. Determination of lethal dose concentration.

The lethal dose determination of cyanobacterial strains in response to increasing concentration of thallium sulphate were measured as changes in the chlorophyll *a* content in N_2-medium or D-medium and as when required supplemented with 5mM $NaNO_3$ (NO_3^- medium) or 2mM NH_4Cl (NH_4^+ medium) as inorganic source. The exponentially growing cyanobacterial cells of *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum*, *Anabaena variabilis* and *Mastigocladus* sp. were centrifuged at 6000 g and washed thrice with sterile distilled water. Such cells were inoculated into 100 mL of fresh N_2 or D medium (containing thallium sulphate in varying concentrations of 0.1 µM – 1.0 µM) in 250 ml Erlenmeyer flask with initial day Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ respectively. A control tube was prepared without the addition of thallium along with cultures. The cultures inoculated in N_2, NO_3^-, and NH_4^+ medium were incubated at 24 ± 2°C and 45 ± 1°C (for *Mastigocladus* sp.) with a photon fluence rate of 50µmol m^{-2} s^{-1} for 6 days with control illumination of 12 hours light and 12 hours dark.

Method of Mackinney (1941) was followed for Chl *a* determination on the 6th day of incubation with increasing concentration of thallium. After a requisite period of incubation the samples were harvested in a centrifuges tube at 6000 g for 5 mins. After centrifugation the supernatant was discarded and the pellet was washed with $BG\text{-}11_0$ twice with subsequent centrifugation. After washing, 5ml of methanol was added to all the tubes and subjected to boiling in a water bath for 10 – 15 mins. After subsequent

cooling, tubes were centrifuged at 2000 g for 5 minutes and Chl *a* was estimated at 663 nm with an extinction coefficient of 74.5 g^{-1} cm^{-1} and expressed µg Chl *a*.ml^{-1}.

2.7. Measurement of growth in medium supplemented with organic nitrogen sources

The effect of increasing concentration of thallium on the growth measured as changes in the chlorophyll *a* content of cyanobacterial strains *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Anabaena* variabilis in N_2-medium containing amino acids as organic source was determined. The exponentially growing cyanobacterial cells were centrifuged at 6000 g and washed thrice with sterile distilled water. Such cells were inoculated into 100 mL of N_2 supplemented with 1mM of Glycine, alanine, valine, lysine, theorine, glutamine, glutamic acid, aspartic acid, asparagine, methionine, cysteine, serine, proline, leucine, isoleucine, arginine, phenylalanine, tryptophan, tyrosine, histidine (containing thallium sulphate in varying concentrations of 0.1 µM – 1.0 µM) in 250 ml Erlenmeyer flask with initial day Chl *a* concentration of 0.4 µg.ml^{-1} respectively. A control tube was prepared without the addition of thallium along with cultures. The cultures were incubated at 24 ± 2°C with a photon fluence rate of 50µmol m^{-2} s^{-1} for 6 days with control illumination of 12 hours light and 12 hours dark. After 6^{th} day of inoculation Chl *a* was measured by the method as described by Mackinney (1941) and expressed as µg Chl *a*.ml^{-1}.

2.8. Measurement of growth in medium supplemented with potassium

The effect of thallium on the growth measured as changes in the chlorophyll *a* content of cyanobacterial strains *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Anabaena* variabilis in N_2-medium increasing concentration of KCl as K^+ source was determined. The exponentially grown cyanobacterial strains was centrifuged at 6000 g

and washed thrice with sterile distilled water. Such cells with initial day Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ were inoculated into 100 mL of N_2-medium (containing KCl in varying concentrations of 1mM – 25 mM) supplemented with 0.5µM thallium respectively. A control tube containing thallium was prepared without the addition of potassium chloride along with culture.

The inoculated cultures were incubated at 24 ± 2°C with a photon fluence rate of 50µmol m^{-2} s^{-1} for 6 days with control illumination of 12 hours light and 12 hours dark. After 6th day of incubation Chl *a* content was measured by the method as described by Mackinney (1941) and expressed as µg Chl *a*.ml^{-1}.

2.9. Measurement of phycobilisome contents

The phycobilisome content - phycocyanin, allophycocyanin and phycoerythrin was determined as per method described by Benett and Bogorad (1976) with slight modifications. The exponentially growing cyanobacterial cells was centrifuged at 6000 g and washed thrice with sterile distilled water. Such cells were inoculated into 100 mL of fresh N_2 or D medium (containing thallium sulphate in varying concentrations of 0.1 µM – 1.0 µM) in 250 mL Erlenmeyer flask with initial day Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ respectively. A control tube was prepared without the addition of thallium along with culture. The cultures in N_2 or D-medium were incubated at 24 ± 2 °C and 45 ± 1 °C for *Mastigocladus* sp. with a photon fluence rate of 50µmol m^{-2} s^{-1} for 6 days with control illumination of 12 hours light and 12 hours dark.

After a requisite period of incubation the samples were harvested in a centrifuge tube and centrifuged at 6000 g for 5 mins. After centrifugation the supernatant was discarded and the pellet was washed with PBS (concentration of 1x with a pH 7.2) twice with subsequent centrifugation. The cells were resuspended in the same buffer. This

resuspended material was disrupted by ultrasonication at 4°C using an ultrasonicator (Sonics & Materials Inc., USA) fitted with a microprobe for 10 minutes and then centrifuged for 30 mins at 8000 g. The supernatant obtained was used for the estimation of phycocyanin (PC) at 652 nm, allophycocyanin (APC) at 615 nm and phycoerythrin (PE) at 562 nm. . The readings were expressed in $\mu g\ .ml^{-1}$.

$$[PC] = OD615 - 0.475\ (OD\ 652) / 5.34 \quad mg/ml$$

$$[APC] = OD652 - 0.208\ (OD615) / 5.09\ mg/ml$$

$$[PE] = OD652 - 2.41\ [PC] - 0.849\ (APC) / 9.62\ mg/ml$$

2.10. Measurement of phycobilisome contents in medium supplemented with organic nitrogen sources

The exponentially growing cyanobacterial cells was centrifuged at 6000 g and washed thrice with sterile distilled water. Such cells were inoculated into 100 mL of N_2-medium supplemented with 1mM of alanine, glutamine, asparagine and proline (containing thallium sulphate concentration of 0.5μM) with initial day Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ respectively. A control tube was prepared without the addition of thallium along with culture. The cultures were incubated at 24 ± 2 with a photon fluence rate of 50$\mu mol\ m^{-2}\ s^{-1}$ for 6 days with control illumination of 12 hours light and 12 hours dark.

After a requisite period of incubation the changes in the PC, APC and PE were determined as described in section 2.8 and values were expressed in percentage (%) protection taking control cultures phycobilisome content as 100%.

2.11. Estimation of Protein.

Protein content was estimated as method depicted by Lowry *et al.*, (1951) in cyanobacterial cultures grown in different combined nitrogen sources medium with

thallium at 0.5μM concentration and without thallium. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells grown in N_2 -medium. The protein content was determined 6 days after inoculation. Cyanobacterial cells after requisite period of incubation were harvested by centrifugation in a centrifuge tube at 6000 g for 5 mins. After centrifugation the supernatant was discarded and the pellet was washed with sterile water twice followed by subsequent centrifugation and resuspended in the same. The cells were sonicated at 4°C using an ultrasonicator (Sonics & Materials Inc., USA) fitted with a microprobe for 10 mins. The suspension was centrifuge at 5000 g for 10 mins and supernatant was used as extract for protein estimation. The reagents used were

Reagent A= 1.0 N NaOH

Reagent B = (i) 5.0% Na_2CO_3

(ii) 0.5% $CuSO_4$. $5H_2O$ in 1% sodium potassium tartarate

Solution (i) and (ii) were mixed in the ratio of 25:1 (v/v)

Reagent C= 1.0N Folin- Phenol reagent.

To 0.5 mL of cyanobacterial samples, 0.5 ml of reagent A was added and kept in boiling water bath for 5 min followed by cooling under running water. 2.5 mL of reagent B was added to it and incubated for 10 min at room temperature followed by addition of 0.5 mL of reagent C. The intensity of blue colour developed after incubation in dark for 15-20 mins was measured at 650nm in a spectrophotometer (Beckman, UV/VIS). A standard curve was prepared by using Bovine serum albumin (BSA) as standard and expressed as $mg.ml^{-1}$ of protein.

2.12. Measurement of Heterocyst frequency:

Heterocyst frequency per 100 vegetative cells in thallium treated (0.5μM) and untreated cultures was calculated by light microscope observation of the filaments of the cyanobacterium.

2.13. Measurement of electron transport activities:

Cyanobacterial cells grown in different nitrogen sources with 0.5μM thallium or without thallium were harvested and washed twice with harvesting buffer consisting of 20 mM HEPES – NaOH buffer, pH 7.5; 1 mM $CaCl_2$ and 7.5% PEG – 4000 and resuspended in the same buffer. The cell suspension was centrifuge at 3000 g for 10 mins. Then the cells were suspended in reaction buffer of 25 mM HEPES-NaOH, pH 7.5, 20 mM NaCl. These cells were used for measuring oxygen evolution and oxygen consumption as described by Robinson *et al* (1982). The assay of electron activities were carried out using the thermostated (28 ± 1°C) glass cuvette fitted within a Clark type O_2 electrode installed in a 3 ml plexi glass container with magnetic stirring (Rank Brothers, USA). Measurement involved adding 3 ml cyanobacterial cultures to the sample chamber of non polarised electrode and each sample were allowed to equilibrate for 5 minutes while stirring. The electrode was then polarized and the linear of O_2 evolution was obtained in light supplied by tungsten filament bulb, which was shielded from the sample by water bath acting as heat filter (photon fluence at the surface of the sample chamber: 50μmol m^{-2} s^{-1}). O_2 consumption was measured in darkness with sample chamber wrapped with aluminium foil. The change in the oxygen level in dark and light supply was recorded in an electrograph. Photochemical activity estimated in terms of O_2 exchange expressed as nmoles O_2 evolved or consumed μg^{-1} Chl *a*.hr^{-1}.

2.14 Glutamine synthetase (transferase) activity (EC 6.3.1.2)

The enzyme was assayed using the method of Sampaio *et al* (1979). Cyanobacterial cells grown in different nitrogen sources in thallium treated (0.5μM) and untreated medium were harvested and washed twice with harvesting buffer Tris – HCl (pH 7.0) and resuspended in the same buffer. The cell suspensions were disrupted at 4°C by ultrasonication using an ultrasonicator (Sonics & Materials Inc., USA) fitted with a microprobe for 10 minutes and this extract was used as the enzyme extract. The reaction mixture in addition to enzyme extract in a total volume of 1 ml contained

40 mM	Tris – HCl (pH 7.0)
3 mM	MnCl2
K – arsenate	20 mM
0.4 mM	Na – ADP.
60 mM	Hydroxylamine neutralize with 2N NaOH.
30 mM	Glutamine.

The reaction was allowed to proceed for 10 mins at 37°C in dark. Finally reaction was terminated by adding 2.0 ml of stop mixture (4 ml of 10 % $FeCl_3$ + 1 ml of 24 % TCA + 0.5 ml of 6 N HCl + 6.5 ml of double distilled water) and the γ - glutamyl hydroxymate formed was estimated by measuring the absorbance at 540 nm. The amount of γ - glutamylhydroxymate formed was determined from the standard curve. The transferase activity was expressed as nmol of γ - glutamylhydroxymate formed.min^{-1}.mg $^{-1}$.protein.

2.15. Nitrogenase activity (EC 1.8.6.1).

Nitrogenase activity was measured as per method described by Stewart *et al.* (1967). Assay was done in test tube fitted with serum stoppers. Air was removed from the tubes containing thallium treated (0.5μM) and untreated cyanobacterial cells by evacuation and replaced by the desired gas phase which contained 10 % (v/v) acetylene. These tubes were incubated with intermittent shaking at 24 ± 2°C with a photo fluence rate of 50μmol m^{-2} s^{-1}. After 1 hr of incubation, 1 ml gas sample was analyzed for ethylene produced by using a Chemito gas chromatograph fitted with a poropak – T – Column (80-100 mesh size; 1/8″ x 2m stainless steel) and a flame ionization detector. The oven temperature of the GC was maintained at 60°C during the operation. The nitrogenase activity was expressed in terms of nmol C_2H_4 formed.mg^{-1}.Chl *a*.hr^{-1}.

2.16. Nitrate reductase activity (E C 1.7.1.1)

Cells of diazotrophic cyanobacteria grown in different nitrogen media containing 0.5μM) or without thallium after 6th day of inoculation were harvested by centrifugation at 5000g for 10 min and washed twice with 50 mM Tris –HCl (pH 7.5) buffer containing

NaCl	0.1 M
Sucrose	0.3 M
EDTA	1 mM

and resuspended in the same buffer. This suspension was sonicated at 4°C in an ultrasonicator (Sonics & Materials Inc., USA) fitted with a microprobe for 10 mins. The broken material was centrifuged at 3000 g for 30 mins and the resulting supernatant was used as crude enzyme.

The method of Manzano *et al.* (1976) as described by Bagchi & Singh (1984) was followed for estimating nitrate reductase activity. The enzyme activity was

measured colorimetrically following the appearance of nitrite using methyl viologen, chemically reduced by dithionite as electron donor. The reaction mixture contained in a final volume of 1.0 ml. Glycine-KOH buffer pH 10.5, 100 µmol; 20 µmol KNO_3; 4 µmol methyl viologen; 10 µmol of $Na_2S_2O_4$ in 0.1 ml of 0.25M $NaHCO_3$ and an appropriate amount of enzyme. This mixture was incubated at 30°C for 10 mins and the reaction was terminated by addition of 0.2 ml of 1.0 M Zinc acetate. The amount of nitrite formed was determined from the standard curve and was expressed as nmol NO_2^- formed.min^{-1}.mg^{-1}.protein.

2.17. Nitrite reductase activity (E C 1.7.1.4)

Nitrite reductase (NIR) activity was measured as per methods described by Arizmendi & Serra (1990). The cells were harvested (both thallium treated with 0.5µM and untreated cells) in their exponential phase of growth by centrifugation, washed twice with 0.5 mM Tris –HCl (pH 7.5) buffer and resuspended in the same. This suspension was sonicated at 4°C using an ultrasonicator (Sonics & Materials Inc., USA) fitted with a microprobe for 10 mins. The broken material was centrifuged at 3000 g for 30 mins and the resulting supernatant was used as crude enzyme.

The reaction mixture in addition to enzyme extract in a total volume of 1 ml contained

0.5 M	Tris – HCl (pH 7.05
1.6 mM	$NaNO_2$
4 µmol	Methyl Viologen

The reaction was allowed to proceed for 15 mins at room temperature with the addition of 250 mM of sodium dithionite & bi carbonate buffer. Finally reaction was terminated by vigorously shaking the reaction mixture in a vortex

shaker until blue colour developed and the amount of nitrite formed was estimated by measuring the absorbance at 540 nm. Subsequently, remaining nitrite was determined by the method of Snell & Snell (1949). The amount of nitrite formed was determined from the standard curve and expressed as nmol NO_2^- consumed.min^{-1}.mg^{-1}.protein.

2.18. Proline estimation

Estimation of intracellular level of free proline was performed as per method described by Bates *et al.*, (1975) with slight modifications. Exponential grown cyanobacterial cells grown in different nitrogen sources with thallium treatment at 0.5μM) or without thallium were centrifuged and harvested in 3% sulphosalicylic acid and cells disrupted by ultrasonication at 4°C using an ultrasonicator (Sonics & Materials Inc., USA) fitted with a microprobe. To about 2ml of the extract, 2 ml of glacial acetic acid and 2 ml of acid ninhydrin were added. The mixture was boiled in a water bath for 1 hour. The reaction was terminated by placing the tube in the ice bath. To the ice cold reaction mixture, 4 ml of toluene was added and shaken vigorously for 30 seconds. The toluene layer was removed the red colour intensity was measured at 520 nm. A standard curve was prepared with proline and the results were expressed as μmol.proline.mg^{-1}.protein.

2.19 Thallium uptake activity:

The method of Scott & Nicholls (1980) developed by Singh *et al* (1997) was used for the uptake of thallium in cyanobacterial cells grown in N_2, nitrate, ammonium or amino acid supplemented media. Exponentially grown cyanobacterial cells were harvested by centrifugation, washed twice with 10 mM HEPES – NaOH buffer (pH 7.0), resuspended in the same and equilibrated for 30 mins at 24±2 °C under photoautotrophic growth conditions. $^{204}Tl^{2+}$ labelled thallium was then added to the cell suspension to a

final concentration of 20 μM (Specific activity of 194.1 Bq μmol^{-1}). At different times 400 μl of samples were withdrawn at a specific time intervals and separated from the bathing medium by micro centrifugation through silicon oil / dinonyl phthalate (45/55, v/v) into perchloric acid/water (15/85, v/v) as previously described by Scott & Nicholls (1980) and counted for cellular level of radio labelled compound in a BECKMAN LS 1801 liquid scintillation counter. Non specific binding of radioactive was determined by measuring its radioactivity incorporated in toluene treated cells (Rai *et. al.*, 1984). This value was always subtracted from radioactivity incorporated in toluene-treated cells. The uptake of thallium in the cyanobacterial cells was subsequently followed by the addition of 2mM KCl as potassium source.

2.20. Isolation of thallium resistant mutants

As a first step towards isolation of Tl-R mutants, the survival of all the cyanobacterium was checked at increasing concentration of thallium as developed by Singh *et al* (1997). A concentration of 0.5μM was found as lethal dose for all the five cyanobacterial cells both in liquid as well as solid media. Spontaneously occurring Tl-R mutants were obtained by plating approximately 2.7 X 10^8 colony forming units on solid N_2 medium containing 0.8 μM thallium. After 4 weeks of incubation the few surviving colonies that remained were picked up and transferred to plates containing 0.8 μM thallium. The resistant phenotype of mutant cells was checked and ascertained by growing mutant cells in 0.8 μM thallium containing medium after seven generation of growth in thallium deficient media. The mutant strain was always maintained in N_2-medium containing 0.8μM thallium sulphate.

The comparative effect of growth as measure of changes in chlorophyll *a* content in mutant and wild type cells were measured in N_2, nitrate and ammonium medium containing 0.5μM thallium. The mutant strain treated with 0.5μM thallium and without

treatment was further characterized for various physiological activities viz. heterocyst frequency, Glutamine synthetase activity (transferase), Nitrate reductase activity, nitrite reductase activity, O_2 consumption and evolution activities in N_2, nitrate and ammonium medium. The uptake of thallium in N_2- medium was investigated in the mutant strain

2.21. Statistical analysis

Mean, standard deviation has been calculated for the data obtained in different experiments and the variation found to be in the range of 5-10%. The values presented are means of two independent experiments each with two replicates unless otherwise specified. The standard deviation (SD) of the data on specific observation was calculated as

$$SE = SD/\sqrt{n}$$

Where SD = standard deviation

N= no. of variants.

The level of significance (p value) was also determined for some experiments and values ($p<0.001$) indicates statistically significant values.

3. RESULTS

CHAPTER 3

RESULT

Thallium is a heavy metal which belongs to group five in periodic table. The toxicity of thallium to various categories of organisms is well known (Delvalls *et al.*, 1999, Ewers, 1998). When accumulated in the body, it affects functioning of different organs by influencing their metabolic behaviour. It is an analogue of potassium and in general affects the potassium dependent processes. Therefore strategies are needed to be developed for bioremediation of thallium from polluted environment. Many physical and chemical methods are in use for this purpose. However they are all very expensive and most of them are not very efficient. The potential of cyanobacteria for removal of thallium as a tool for thallium bioremediation has not been assessed in detail. Cyanobacteria are photoautotrophic organism and are known to grow efficiently in complete inorganic media and therefore can be exploited for bioaccumulation of thallium and other toxic metal cations from metal contaminated environments. However, this will require a better understanding of thallium toxicity to various cyanobacterial strains. Therefore the present study is focussed to understand the effect of thallium on cyanobacterial growth and different metabolic activities related to nitrogen metabolism. Growth of cyanobacterial strains was measured in terms of changes in photosynthetic pigments. Further its effects on metabolic activities were determined by measuring nitrogenase activity, glutamine synthetase (transferase) activity, nitrate and nitrite reductase activity, heterocyst frequency, photosynthetic oxygen evolution and respiratory oxygen consumption activity, and accumulation of intracellular proline in thallium stress conditions. Finally thallium uptake activity of wild type and Tl-R strains was determined

in cyanobacterial strains grown in different nitrogen (inorganic and organic) sources supplemented media.

3.1 Effect of thallium on growth of cyanobacterium:

Growth of cyanobacterium was determined as changes in their chlorophyll *a* content or phycobilisome contents in response to thallium treatment in different nitrogen sources supplemented BG-11_0 media.

3.1.1 Effect of thallium on growth as changes in chlorophyll *a* content in inorganic nitrogen source supplemented media:

In an effort to examine the toxicity of thallium to cyanobacterium, five cyanobacterial strains namely *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum*, *Anabaena variabilis* and *Mastigocladus* sp., were inoculated and grown at various concentration of thallium sulphate ranging from 0.0 µM to 1 µM at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ in a 250 ml Erlenmeyer flask containing 100 mL of BG – 11_0 (N_2-medium) or D medium (for *Mastigocladus* sp.). The culture media were supplemented with either 5mM KNO_3 (as nitrate source), or 2 mM NH_4Cl (as ammonium source). The chlorophyll *a* contents of different cultures were measured 6 days after inoculation. The chlorophyll *a* content of all the cyanobacterial strains decreased with increasing thallium concentration. Due to its toxic nature thallium inhibited growth of all the cyanobacterial strains with a minimum growth inhibition of 74% at 0.5µM thallium concentration. However, growth was arrested completely 10 days after inoculation as revealed by complete loss of chlorophyll *a* content. Therefore 0.5µM was treated as the lethal dose concentration (LD) for the cyanobacteria used in the present study.

Among the five cyanobacterial strains *Nostoc* ANTH in nitrate supplemented medium showed higher Chl *a* content (3.65 μg ml^{-1}) in thallium untreated culture medium as compared to its growth in similar medium with 0.5μM thallium concentration. At this concentration the growth inhibition was found to be 83% (Fig 3.1). Similarly inhibition of growth was 89% in *Anabaena variabilis* (Fig 3.4), 88% in *Mastigocladus* sp. (Fig 3.5), 84% in *Anabaena cycadae* (Fig 3.2) and 83% in *Nostoc muscorum* (Fig 3.3) as compared to their respective controls in thallium deficient medium.

The next best growth of cyanobacterium after nitrate medium was subsequently observed in N_2 (combined nitrogen free) medium. Apparently, among all the five cyanobacterium, all exhibited more or less equal Chl *a* content in the thallium untreated cells whereas thallium treated cells at 0.5μM showed significant decrease of 90%, 89 %, 88 %, 84 % and 74 % in *Nostoc muscorum* (fig 3.3), *Anabaena variabilis* (fig 3.4), *Mastigocladus* sp. (fig 3.5), *Anabaena cycadae* (fig 3.2) and *Nostoc* ANTH (fig 3.1) respectively.

In the ammonium supplemented medium, growth was always found to be less than the growth in N_2 or nitrate medium. In ammonium supplemented medium at 0.5μM thallium concentration, inhibition in Chl *a* content was found to be 78% in *Nostoc* ANTH (fig 3.1), 75% in *Anabaena cycadae* (fig 3.2), 76% in *Nostoc muscorum* (fig 3.3), 80% in *Anabaena variabilis* (fig 3.4) and 74% in *Mastigocladus* sp. (fig 3.5) as compared with their respective controls. Interestingly none of the inorganic nitrogen sources exhibited protection to cyanobacterial strains against thallium toxicity.

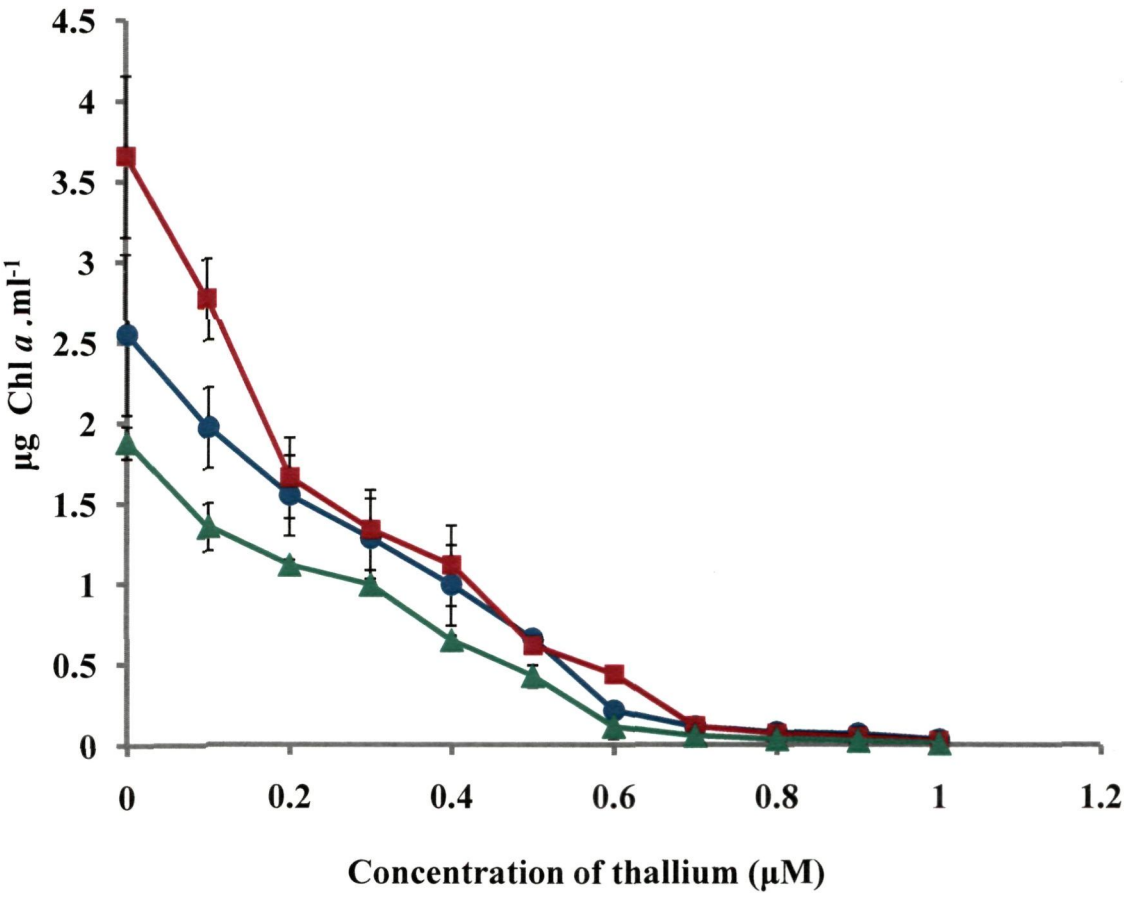


Fig 3.1: Effect of increasing concentrations of thallium sulphate on the growth of *Nostoc* ANTH cells grown in different nitrogen sources at 25°C. Growth in terms of µg Chl *a*.ml^{-1} was determined after an interval of 6 days. The initial day inoculum was N_2 grown exponential cells inoculated at a Chl *a* concentration of 0.4 µg.ml^{-1}. N_2 refers to nitrogen free medium (●), NO_3^- to medium N_2 with 5mM $NaNO_3$ (■), NH_4^+ to medium N_2 with 2mM NH_4Cl (▲) as N source. The values represent means ± standard deviation (SD) from two independent experiments with two replicates each.

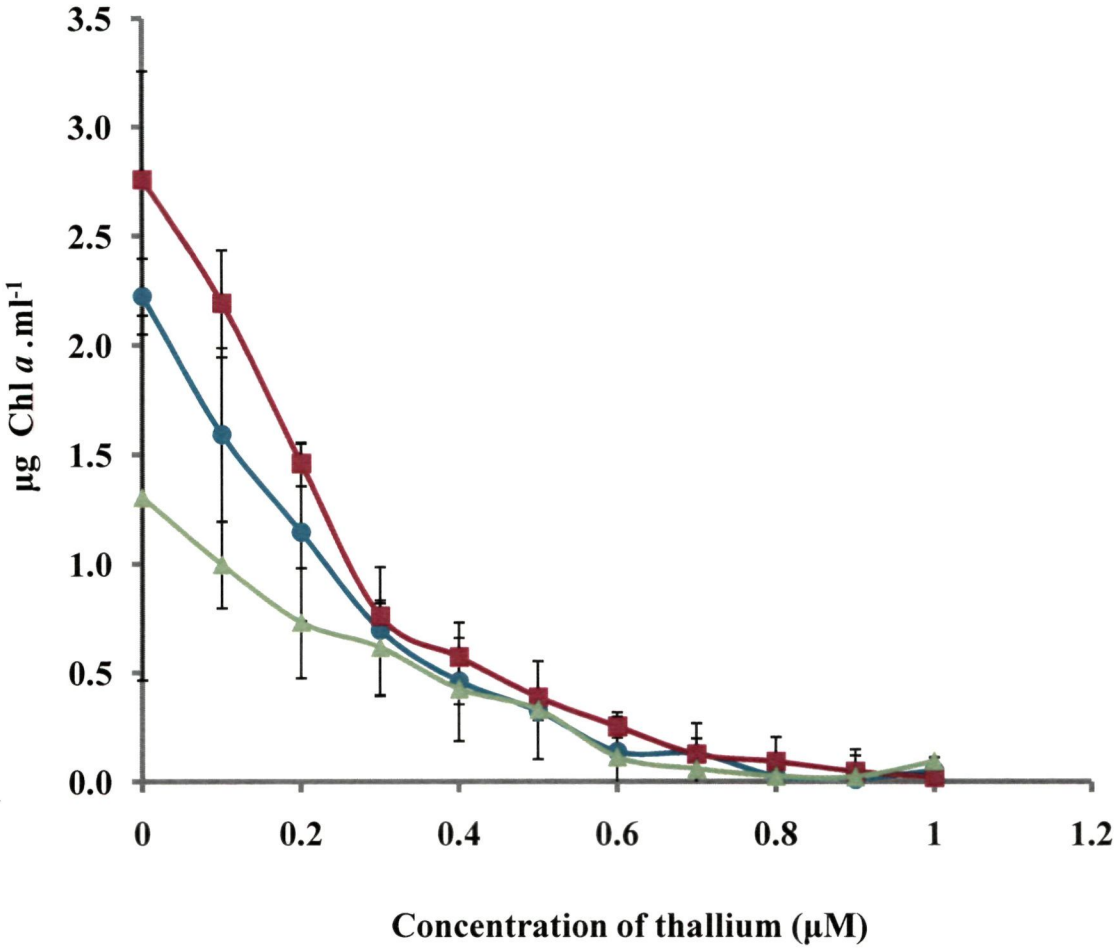


Fig 3.2: Effect of increasing concentrations of thallium sulphate on the growth of *Anabaena cycadae* cells grown in different nitrogen sources at 25°C. Growth in terms of µg Chl *a* .ml^{-1} was determined after an interval of 6 days. The initial day inoculum was N_2 grown exponential cells inoculated at a Chl *a* concentration of 0.4 µg .ml^{-1}. N_2 refers nitrogen free medium (●), NO_3^- to medium N_2 with 5mM $NaNO_3$ (■), NH_4^+ to medium N_2 with 2mM NH_4Cl (▲) as N source. The values represent means ± standard deviation (SD) from two independent experiments with two replicates each.

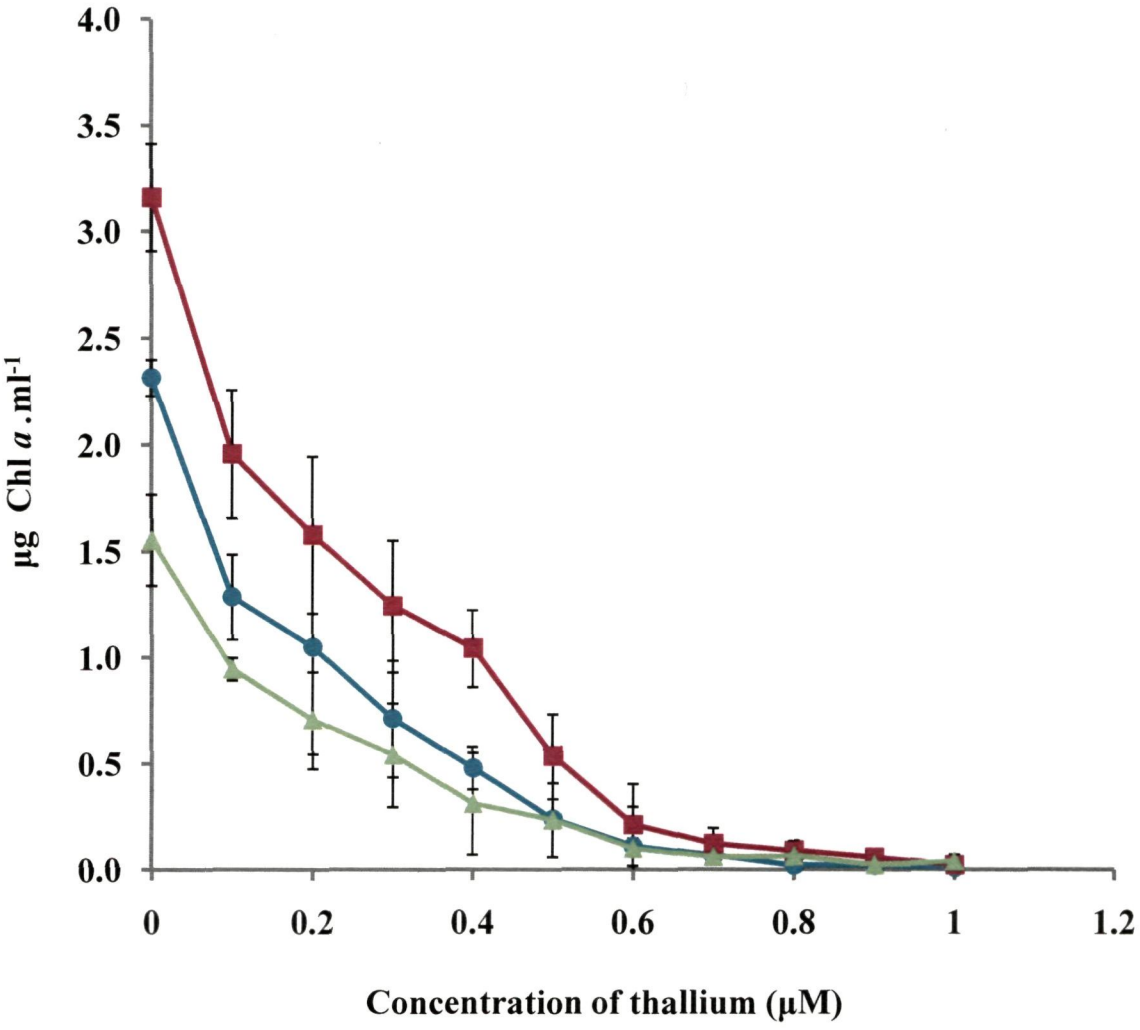


Fig 3.3: Effect of increasing concentrations of thallium sulphate on the growth of *Nostoc muscorum* cells grown in different nitrogen sources at 25°C. Growth in terms of µg Chl *a* .ml^{-1} was determined after an intervals of 6 days. The initial day inoculum was N_2 grown exponential cells inoculated at a Chl *a* concentration of 0.4 µg.ml^{-1}. N_2 refers to nitrogen free medium (●), NO_3^- to medium N_2 with 5mM $NaNO_3$ (■), NH_4^+ to medium N_2 with 2mM NH_4Cl (▲) as N source. The values represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

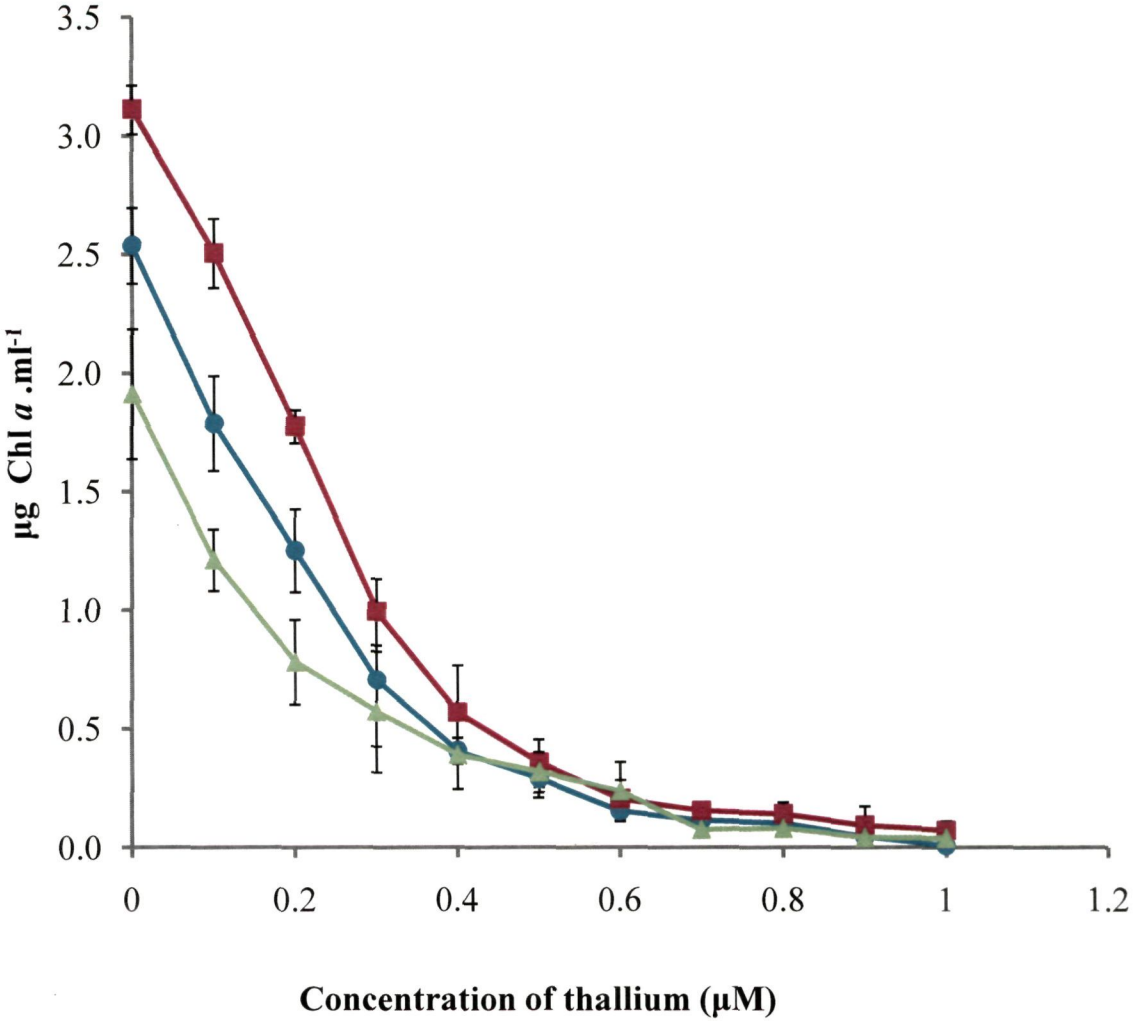


Fig 3.4: Effect of increasing concentrations of thallium sulphate on the growth of *Anabaena variabilis* cells grown in different nitrogen sources at 25°C. Growth in terms of µg Chl *a* .ml^{-1} was determined after an intervals of 6 days. The initial day inoculum was N_2 grown exponential cells inoculated at a Chl *a* concentration of 0.4 µg .ml^{-1}. N_2 refers to nitrogen free medium (●), NO_3^- to medium N_2 with 5mM $NaNO_3$ (■), NH_4^+ to medium N_2 with 2mM NH_4Cl (▲) as N source. The values represent means ± standard deviation (SD) from two independent experiments with two replicates each.

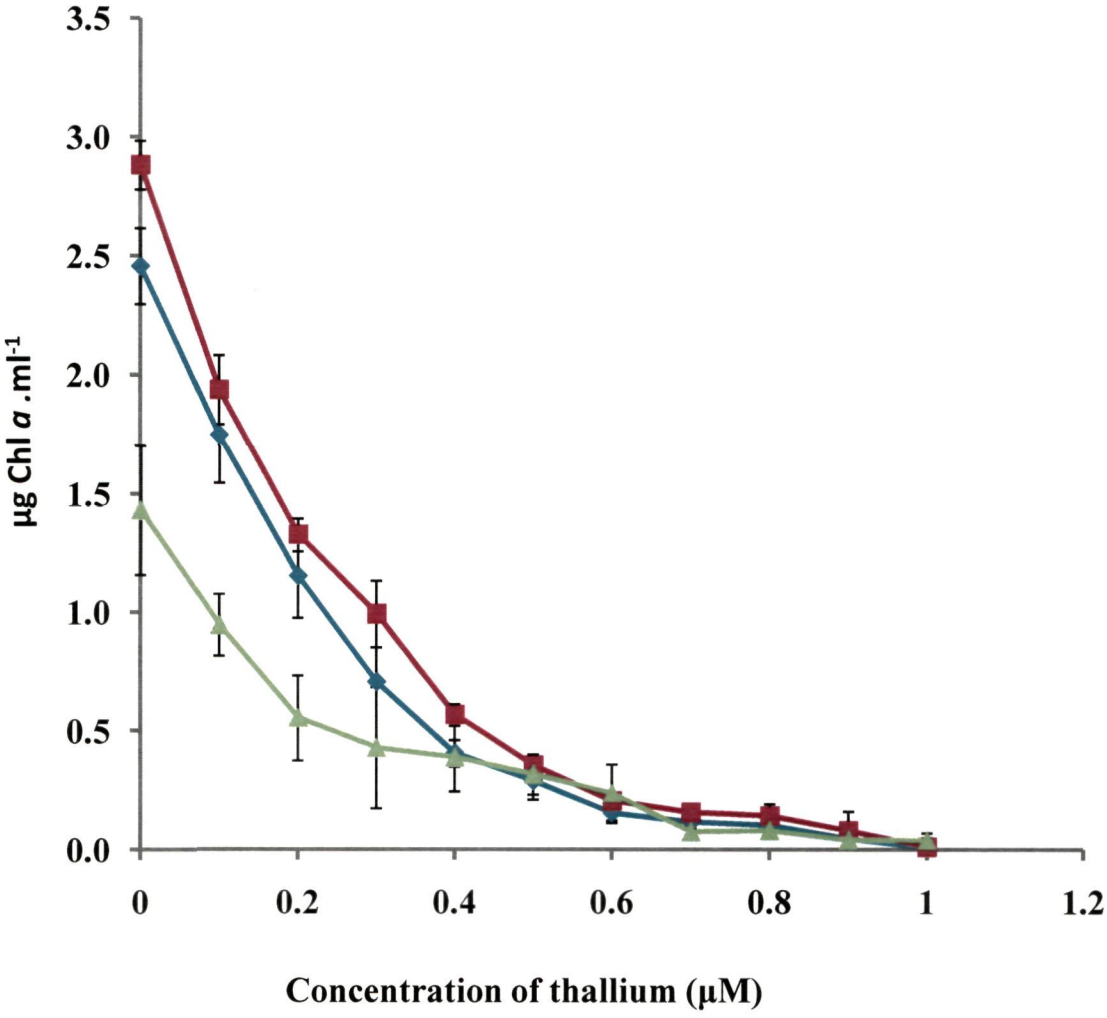


Fig 3.5: Effect of increasing concentrations of thallium sulphate on the growth of *Mastigocladus* sp. cells grown in different nitrogen sources at 45°C. Growth in terms of µg Chl *a* .ml^{-1} was determined after an intervals of 6 days. The initial day inoculum was D-medium grown exponential cells inoculated at a Chl *a* concentration of 0.4 µg .ml^{-1}. D- medium refers to nitrogen free medium (●), NO_3^- to D- medium with 5mM $NaNO_3$ (■), NH_4^+ to D-medium with 2mM NH_4Cl (▲) as N source. The values represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

3.1.2 Effect of thallium on the phycobilisome contents

Since thallium was found to be toxic to all cyanobacterial strains grown in different inorganic nitrogen sources supplemented media equally, thallium effect on phycobilisome content: phycocyanin [PC], allophycocyanin [APC] and phycoerythrin [PE] was measured in cultures grown in nitrogen free BG-11_0 medium and combined nitrogen free D-medium for *Mastigocladus* sp. For this study exponentially grown cells were inoculated at an initial chlorophyll *a* concentration of 0.4 $\mu g.ml^{-1}$ in 100 mL culture medium containing increasing concentration of thallium sulphate. Like chlorophyll *a* content, phycobilisome pigments showed decreases in their cellular level in response to thallium treatment. The percentage of decrease in phycobilisome contents 6 day after treatment ranges from 68 % to 94 %. The decrease in [PC] content was 94%, 89%, 86%, 84% and 68% for *Mastigocladus* sp. (fig 3.10), *Anabaena variabilis* (fig 3.9), *Nostoc* ANTH (fig 3.6), *Anabaena cycadae* (fig 3.7) and *Nostoc muscorum* (fig 3.8) respectively as compared to their respective control cultures grown in thallium deficient medium. The respective values for decrease in [APC] was found to be 95% in *Mastigocladus* sp. (fig 3.10) followed by 89% inhibition in *Anabaena variabilis* (fig 3.9), 87% in *Anabaena cycadae* (fig 3.7), 81% in *Nostoc* ANTH (fig 3.6) and 71% in *Nostoc muscorum* (fig 3.8) respectively. Similarly measurement of [PE] content in thallium treated cells revealed similar decreasing trends with inhibition of 93% in *Nostoc* ANTH (fig 3.6) and *Mastigocladus* sp. (fig 3.10), 94% in *Anabaena cycadae* (fig3.7), 91% in *Nostoc muscorum* (fig3.8) and 85% in *Anabaena variabilis* (fig 3.9). These results suggest the toxic nature of thallium to cyanobacteria as revealed by its inhibitory effects on chlorophyll *a* content and phycobilisome contents in cyanobacteria.

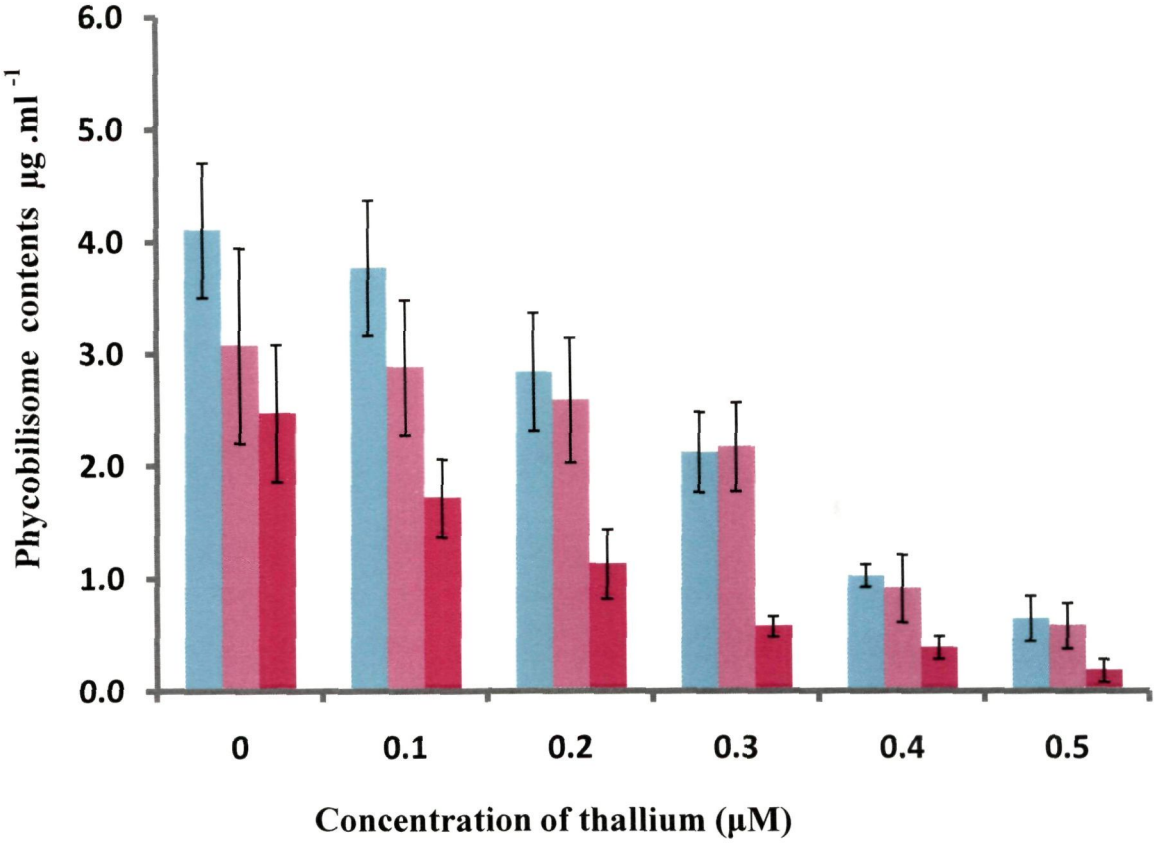


Fig. 3.6: Effect of increasing concentrations of thallium sulphate on the phycobilisome contents – Phycocyanin (■), Allophycocyanin (■) and Phycoerythrin (■) of *Nostoc* ANTH cells grown in N_2-medium at 25°C. Phycobilisome contents in terms of µg .ml $^{-1}$ was determined after an interval of 6 days. The initial day inoculum was N_2 grown exponential cells inoculated at a Chl *a* concentration of 0.4 µg .ml^{-1}. N_2 refers to nitrogen free medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

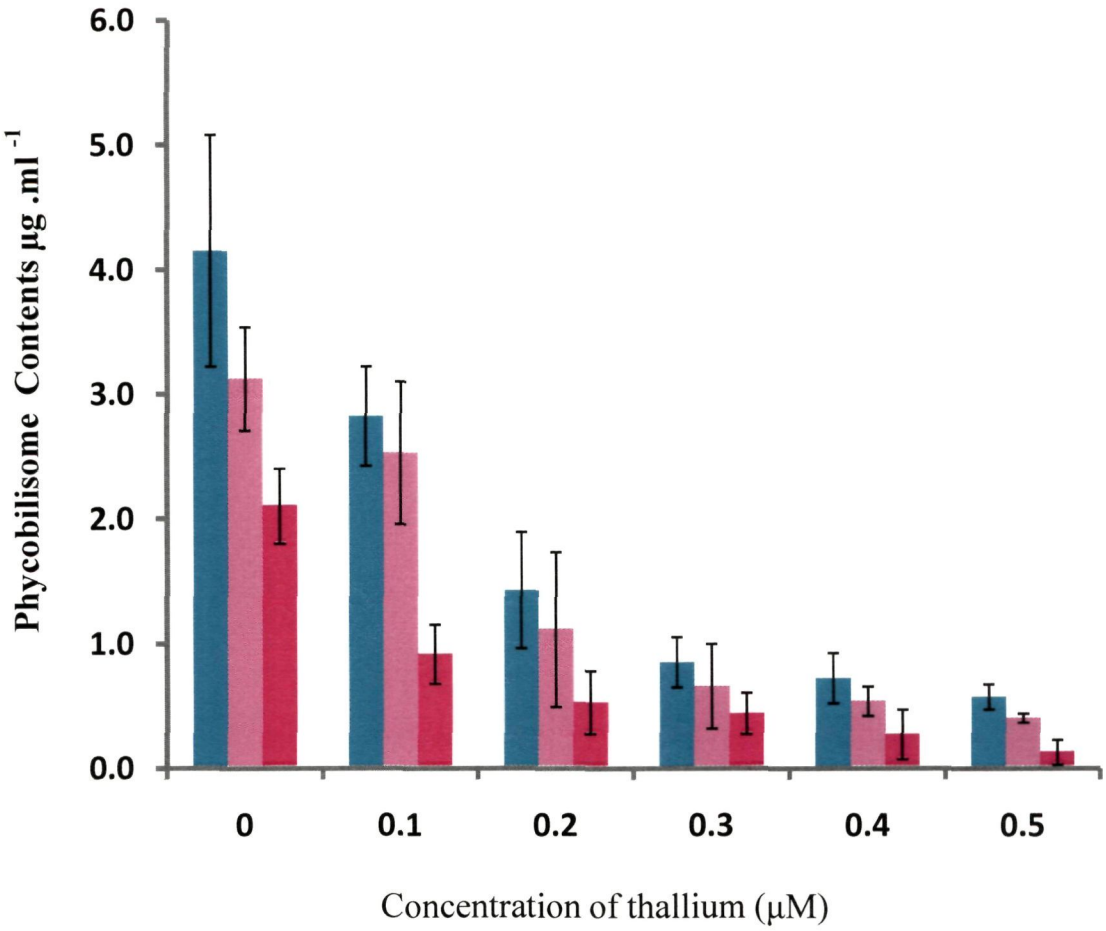


Fig. 3.7: Effect of increasing concentrations of thallium sulphate on the phycobilisome contents – Phycocyanin (■), Allophycocyanin (■) and Phycoerythrin (■) of *Anabaena cycadae* cells grown in N_2-medium at 25°C. Phycobilisome contents in terms of µg .ml $^{-1}$ was determined after an interval of 6 days. The initial day inoculum was N_2 grown exponential cells inoculated at a Chl *a* concentration of 0.4 µg .ml^{-1}. N_2 refers to nitrogen free medium. The values represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

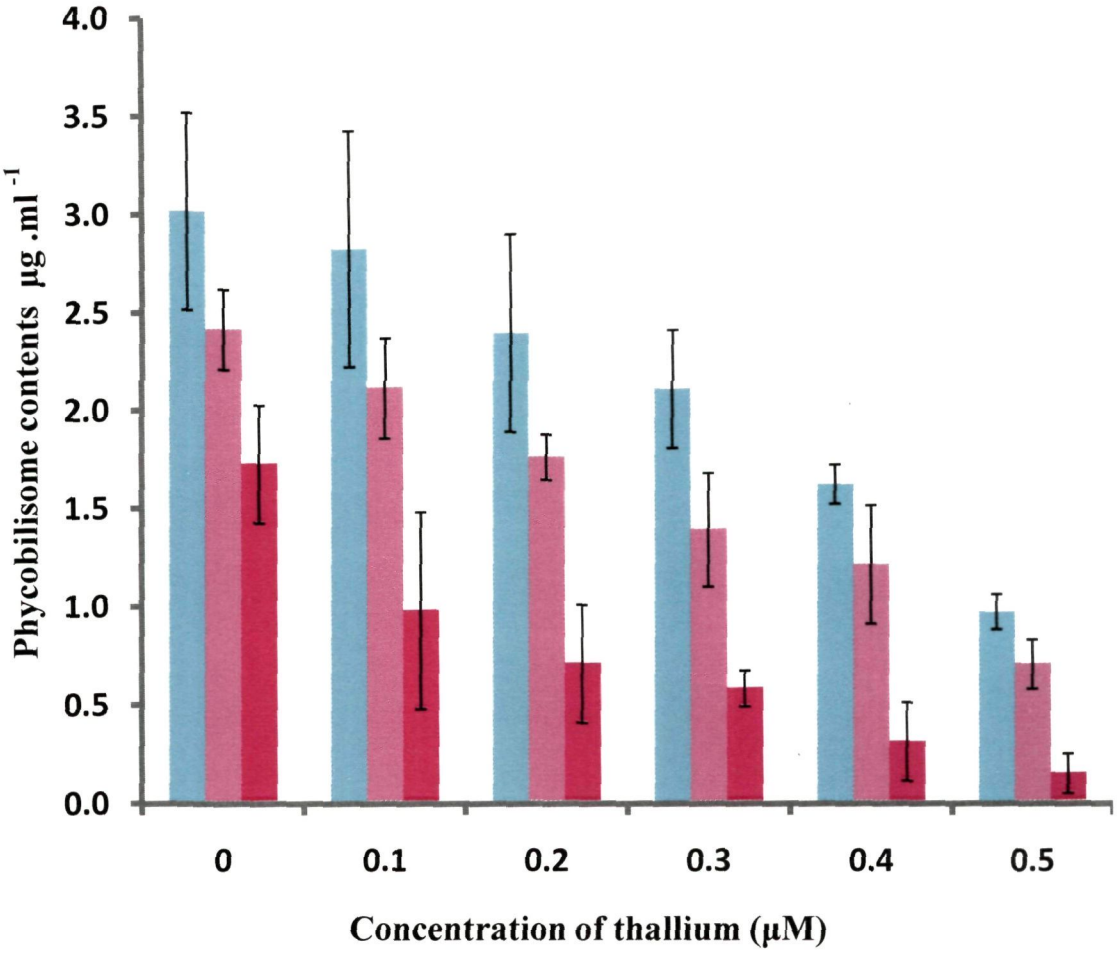


Fig. 3.8: Effect of increasing concentrations of thallium sulphate on the phycobilisome contents – Phycocyanin (■), Allophycocyanin (■) and Phycoerythrin (■) of *Nostoc muscorum* cells grown in N_2-medium at 25°C. Phycobilisome contents in terms of µg .ml $^{-1}$ was determined after an interval of 6 days. The initial day inoculum was N_2 grown exponential cells inoculated at a Chl *a* concentration of 0.4 µg .ml^{-1}. N_2 refers to nitrogen free medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

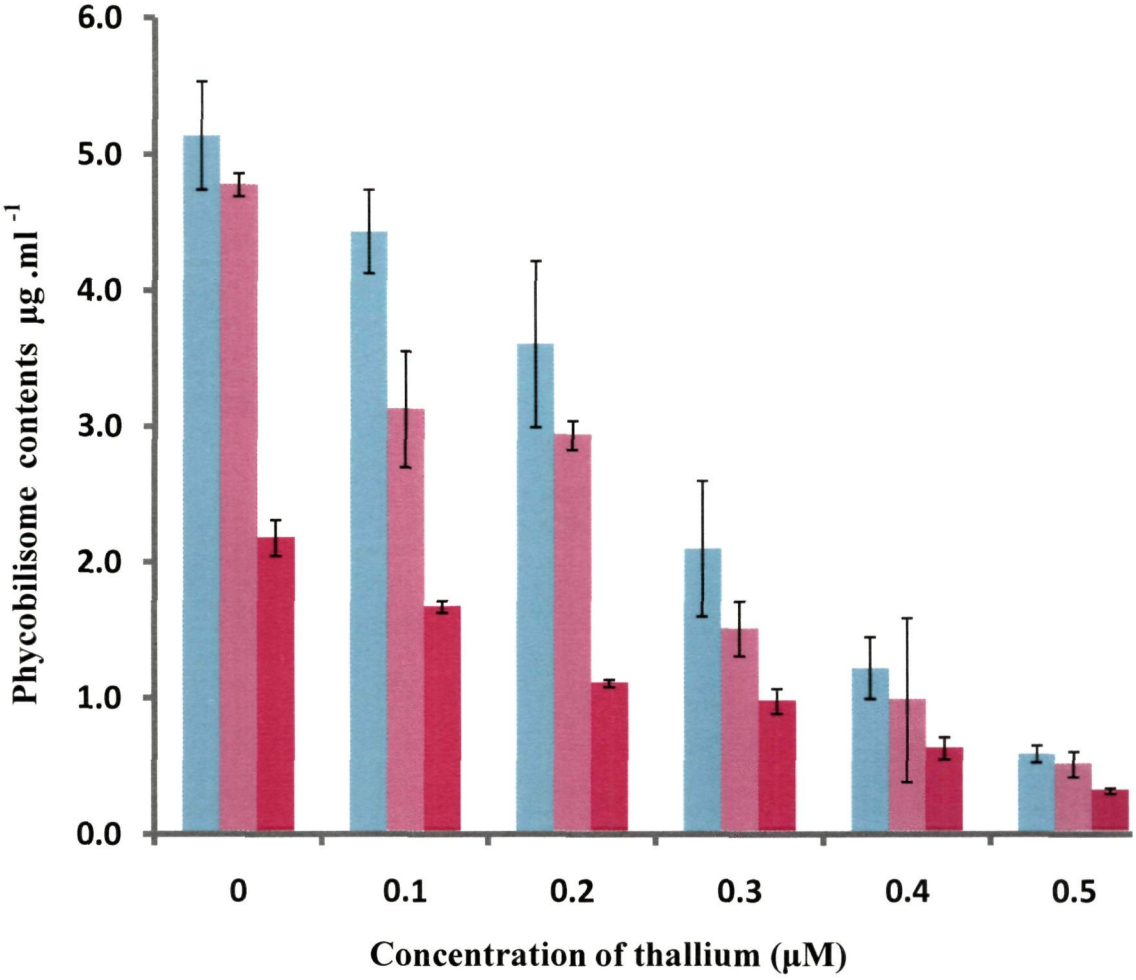


Fig 3.9: Effect of increasing concentrations of thallium sulphate on the phycobilisome contents – Phycocyanin (■), Allophycocyanin (■) and Phycoerythrin (■) of *Anabaena variabilis* cells grown in N_2-medium at 25°C. Phycobilisome contents in terms of µg .ml $^{-1}$ was determined after an interval of 6 days. The initial day inoculum was N_2 grown exponential cells inoculated at a Chl *a* concentration of 0.4 µg .ml^{-1}. N_2 refers to nitrogen free medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

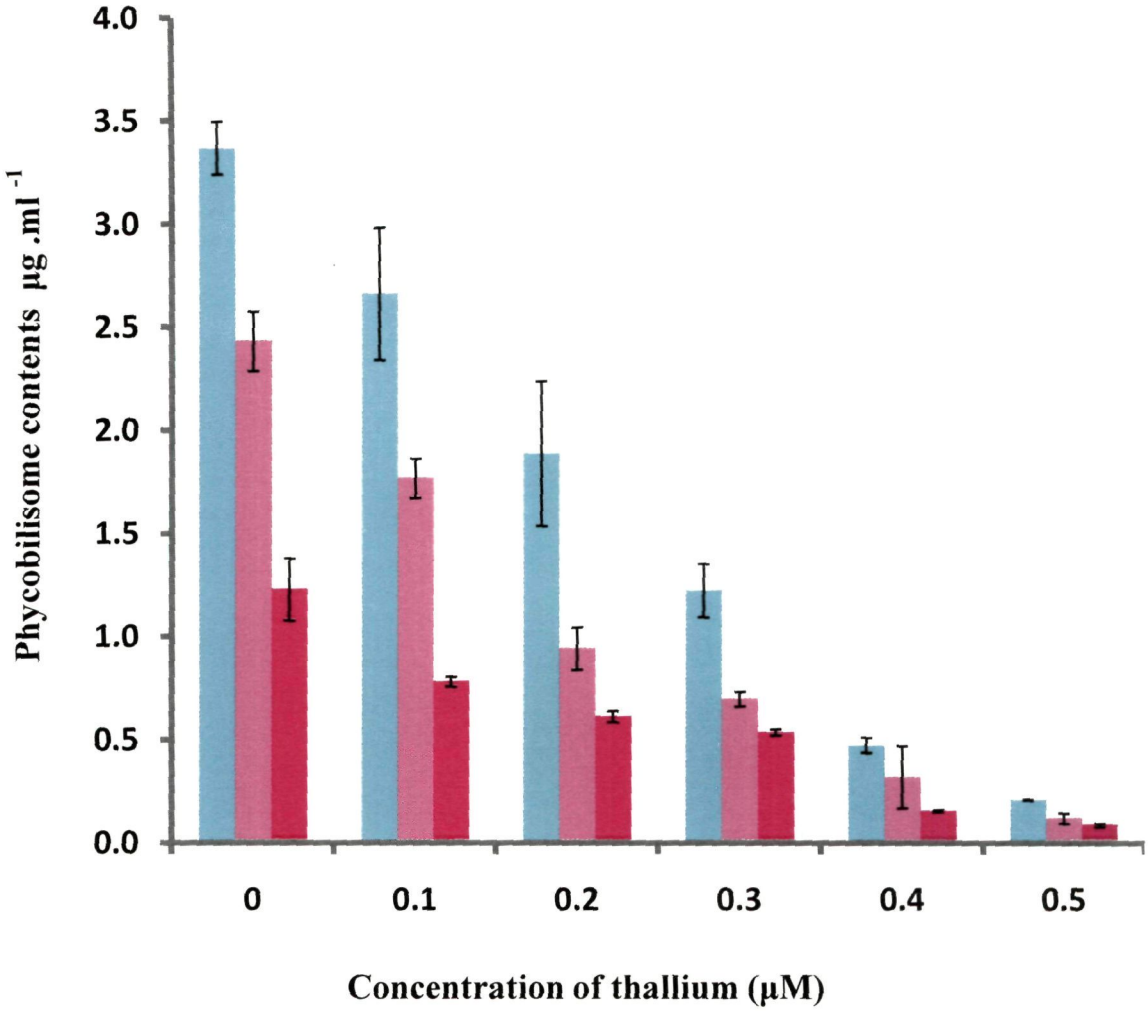


Fig 3.10: Effect of increasing concentrations of thallium sulphate on the phycobilisome contents – Phycocyanin (■), Allophycocyanin (■) and Phycoerythrin (■) of *Mastigocladus* sp. cells grown in D-medium at 45°C. Phycobilisome contents in terms of µg .ml $^{-1}$ was determined after an interval of 6 days. The initial day inoculum was D-medium grown exponential cells inoculated at a Chl *a* concentration of 0.4 µg .ml^{-1}. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

3.2. Effect of thallium on the heterocyst frequency and activities of enzymes namely nitrogenase, nitrate reductase, nitrite reductase, and glutamine synthetase (transferase), and O_2 evolution, O_2 consumption activities and free proline accumulations:

Cyanobacteria like any other living organism respond to various physical and chemical treatments by changing their physiological and biochemical activities. Since inorganic combined nitrogen sources did not protect cyanobacteria from thallium toxic effects, effect of thallium on heterocyst frequency and intracellular activities related to nitrogen metabolism and photosynthesis were determined in order to assess physiological basis of thallium toxicity.

3.2.1 Effect of thallium on the heterocyst frequency and nitrogenase activities:

The effect of thallium on heterocyst frequency and nitrogenase activity in N_2 grown cells was measured in *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Anabaena variabilis* respectively. The exponentially grown cells were inoculated at an initial chlorophyll *a* concentration of 0.4 μg ml^{-1} in 100 mL culture medium containing 0.5μM of thallium sulphate. Heterocyst frequency was counted per 100 vegetative cells, nitrogenase activity was expressed as nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1} measured 6 days after inoculation in thallium containing media.

In heterocystous cyanobacteria heterocyst provide a site for nitrogen fixation. Cultures grown in thallium untreated condition showed heterocyst frequency ranging from 8 - 9%. However, heterocyst frequency was observed to be decrease significantly at 0.5 μM thallium concentrations. The frequency of heterocyst (%) in thallium grown filaments was 9±0.71, 9±0.9, 8±0.9 and 9±0.3 for *Nostoc* ANTH, *Anabaena cycadae*,

Nostoc muscorum, and *Anabaena variabilis* respectively. Therefore per cent decrease in heterocyst frequency compared to their respective control filaments was 78% in *Anabaena cycadae* (table 4.4) & *Nostoc* ANTH (table 4.1) followed by 75% in *Nostoc muscorum* (table 4.7) and 67% in *Anabaena variabilis* (table 4.10) as compared with the control cultures in the thallium deficient N_2 -medium.

Maximum level of nitrogenase activity (12±0.6 nmol C_2H_4 formed .μg^{-1} .Chl *a* .hr^{-1}) was shown by *Nostoc* ANTH, followed by *Anabaena cycadae* (10±0.8 nmol C_2H_4 formed μg^{-1} Chl·*a*.hr^{-1}), *Nostoc muscorum* (9±0.5 nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}) and *Anabaena variabilis* (8.6±1.8 nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}) in thallium untreated cultures. In contrast 0.5µM thallium treated cultures showed significant decline in their nitrogenase activity. The nitrogenase activity inhibition was 90% in *Nostoc* ANTH (table 4.1), 78% in *Nostoc muscorum* (table 4.7), 75% in *Anabaena cycadae* (table 4.4) and 74% in *Anabaena variabilis* (table 4.10). The heterocyst formation activity and nitrogenase activity was not detected in combined nitrogen (NO_3^- & NH_4^+) sources supplemented media thus indicating the capacity of cyanobacteria to use nitrate and ammonium as nitrogen sources. The result further confirms the ability of fixed nitrogen sources to repress heterocyst formation activity and nitrogenase activity in filamentous heterocystous cyanobacteria.

3.2.2 Effect of thallium on the Glutamine synthetase (transferase) [GS] activity

Effect of thallium on glutamine synthetase (transferase) activity now referred to as GS activity was determined in cyanobacterial strains namely *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Anabaena variabilis* grown in N_2, NO_3^- and NH_4^+ media supplemented with 0.5µM thallium. Their respective control cultures were

grown in thallium deficient media. Six days old exponentially grown cells were used for GS activity determination after washing with HEPES-NaOH as described in materials and methods. GS activity has been expressed as nmol- γ - glutamylhydroxamate formed .min^{-1}.mg^{-1}.protein.

The GS activity in N_2 grown cells and thallium untreated cells of *Anabaena variabilis* was 1048.5±22 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein followed by 1018.2±21 nmol - γ - glutamylhydroxamate formed min^{-1} mg^{-1}.protein , 965.5±37 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein and 981.5±26 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein in *Anabaena cycadae*, *Nostoc* ANTH & *Nostoc muscorum* respectively. Determination of GS activity in 0.5μM thallium treated cells showed a significant decrease. The level of reduction in activity was 67% in *Anabaena variabilis* (table 4.10) and *Anabaena cycadae* (table 4.4), 68% and 76% in *Nostoc muscorum* (table 4.7) and *Nostoc* ANTH (table 4.1) as compared with the control cells grown in thallium deficient medium.

Similarly thallium treated cells grown in NO_3^- supplemented medium showed drastic decrease in GS activity with 77% decrease in *Anabaena cycadae* (table 4.5) cells as compared to its control cultures in thallium deficient media showing activity of 948.6±11 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein. Similar patterns were observed for decrease in GS activity in *Anabaena variabilis* which showed 74% (table 4.11) decrease, followed by decreases of 70% in *Nostoc* ANTH (table 4.2) and 69% in *Nostoc muscorum* (table 4.8) in comparison to their respective control cultures grown in thallium deficient medium. The respective cultures in thallium deficient medium showed GS activity of 828±18 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein in *Anabaena variabilis,* 1053.4±30 nmol - γ – glutamylhydroxamate

formed.min^{-1}.mg^{-1}.protein in *Nostoc* ANTH and 828.6±17 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein in *Nostoc muscorum.*

Similarly thallium revealed its inhibitory effects on GS activity in NH_4^+ grown cells of cyanobacteria. However, in case of ammonium grown cells activity was always less than that observed in N_2 and NO_3^- grown cells. The decrease in enzyme activity in thallium treated cells was found to be 72% in *Nostoc muscorum* (table 4.9), 66% in *Nostoc* ANTH (table 4.3), 62% in *Anabaena variabilis* (table 4.12) and 60% in *Anabaena cycadae* (table 4.6) as compared with GS activity of their respective controls grown in thallium deficient medium. The respective controls exhibited GS activity of 489.1±19 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein in *Nostoc muscorum* (table 4.9), 663.89±16 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein in *Nostoc* ANTH (table 4.3), 489.1±19 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein in *Anabaena variabilis* (table 4.12) and 539.3±13 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein in *Anabaena cycadae* (table 4.6). The toxicity of thallium to cultures grown in combined nitrogen sources supplemented media suggests that nitrate or ammonium may not inhibit thallium movement across the cyanobacterial cells. Thus the probable increase in intracellular thallium concentration might be the reason for thallium toxicity by affecting potassium dependent metabolic processes

3.2.3. Effect of thallium on the Nitrate reductase [NR] activity

Effect of thallium on Nitrate reductase activity was measured in cyanobacterial strains namely *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Anabaena variabilis* grown in N_2, NO_3^- or NH_4^+ media supplemented with 0.5μM thallium. Their

respective control cultures were grown in thallium deficient media. Six days old grown cells were used for NR activity determination as described in materials and methods. NR activity has been expressed as nmol NO_2^- formed.min^{-1}.mg^{-1}.protein.

The NR activity in thallium deficient N_2-medium of *Nostoc* ANTH cells was found to be 1.73±0.16 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein followed by 1.36±0.46 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein, 1.27±0.46 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein and 1.32±0.23 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Anabaena cycadae, Nostoc muscorum* and *Anabaena variablis* respectively. Determination of NR activity with 0.5 µM thallium treated cells was found to be 0.77±0.16 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Anabaena cycadae*, 0.47±0.19 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Nostoc muscorum*, 0.57±1.2 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein and 0.51±0.12 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Nostoc* ANTH. The level of reduction in activity was 70% in *Nostoc* ANTH (table 4.1), 56% in *Anabaena variaiblis* (table 4.10), 62% in *Nostoc muscorum* (table 4.7) and 43% in *Anabaena cycadae* (table 4.4) as compared to their respective control cultures grown in thallium deficient medium.

Similarly in NO_3^- grown thallium treated cells, decrease in NR activity was 81% in *Nostoc* ANTH (table 4.2) cells as compared to its control culture showing a activity of 4.8±0.16 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein. Similar patterns of decrease was observed in *Anabaena cycadae* which showed 77% (table 4.5) decrease, followed by decreases of 76% in *Nostoc muscorum* (table 4.8) and 68% in *Anabaena variabilis* (table 4.11) in comparison to their respective control cultures in thallium deficient medium showing NR activity of 5.8±1.6 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Anabaena cycadae*, 4.2±0.15 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Nostoc muscorum*, 4.8

±0.16 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Nostoc* ANTH and 5.1±0.14 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Anabaena variabilis.*

Like N_2 and nitrate grown cell, estimation of NR activity of cells grown in ammonium containing medium supplemented with thallium or without thallium exhibited similar pattern of inhibition in NR activity. However, in case of ammonium grown cells activity was always less than that observed in N_2 grown cells and NO_3^- grown cells. This is in agreement with the earlier finding that NR activity is nitrate inducible and ammonium repressible (Manzano *et al.,* 1976; Losada & Guererro 1979). The decrease in enzyme activity in thallium treated cells was found to be 72% in *Nostoc muscorum* (table 4.9), 73% in *Nostoc* ANTH (table 4.3) and *Anabaena cycadae* (table 4.6) and 50% decrease in *Anabaena variabilis* cells (table 4.12) as compared with NR activity in their respective thallium deficient control cultures which was found to be 0.41 ±0.05 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Nostoc* ANTH, 0.63±0.12 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Anabaena variabilis*, 0.67±0.18 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Anabaena cycadae* and 0.57±0.19 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in *Nostoc muscorum.* These results suggest the inability of combined nitrogen sources such as nitrate or ammonium to protect cyanobacterial cells against thallium toxicity.

3.2.4 Effect of thallium on the Nitrite reductase [NiR] activity

Effect of thallium on NiR activity was determined in cyanobacterial strains namely *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Anabaena variabilis* cells grown in N_2, NO_3^- and NH_4^+ media supplemented with 0.5μM thallium. Their respective control cultures were grown in thallium deficient media. Six days old

cells were used for NiR activity determination as described in materials and methods. The activity has been expressed as nmol NO_2^- consumed.min^{-1}.mg^{-1}.protein.

In N_2 grown cells in thallium deficient medium, NiR activity was found to be 887.6±22 nmol NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Anabaena variabilis* (table-4.10), followed by 787.2±26 nmol NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Nostoc muscorum* (table-4.7), 678.1±27 nmol NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Anabaena cycadae* (table 4.4) and 517.5±18 nmol NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Nostoc* ANTH (table 4.1). On the other hand NiR activity in thallium treated cells showed a significant decrease. The NiR activity values in thallium treated cells were 217.1±19 nmol NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Anabaena variabilis*, 337.1±18 nmol NO_2^- consumed .min^{-1}.mg^{-1}.protein in *Nostoc muscorum*, 229.8±17 nmol NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Anabaena cycadae* and 192.8±11 nmol NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Nostoc* ANTH. The level of reduction in activity of thallium treated cells was 75% in *Anabaena variabilis* (table 4.10), 66% *Anabaena cycadae* (table 4.4), 62% in *Nostoc* ANTH (table 4.1) and 57% in *Nostoc muscorum* (table 4.7) as compared with their respective controls grown in thallium deficient N_2-medium

Similarly thallium treated cells grown in NO_3^- supplemented medium showed drastic decrease in NiR activity with 73% decrease in *Anabaena variabilis* (table 4.11) cells as compared to its control culture in thallium untreated cells showing a activity of 814.2±22 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein. Similar patterns of reduction in NiR activity was observed in *Anabaena cycadae* with 67% (table 4.) decrease, followed by 57% decrease in *Nostoc* ANTH (table 4.2) and 75% decrease in *Nostoc muscorum* (table 4.8) in comparison to their respective thallium untreated cultures. NiR activity in

thallium untreated nitrate grown cells were 498±14 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Nostoc* ANTH, 435±21 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Anabaena cycadae* and 794.2±21 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Nostoc muscorum.*

Thallium treatment also led to inhibition of NiR activity in NH_4^+ grown cells of cyanobacteria as well. The decrease in enzyme activity in thallium treated NH_4^+ grown cells was found to be 58% both in *Nostoc muscorum* (table 4.9) and *Anabaena variabilis* (table 4.12), 52% in *Nostoc* ANTH (table 4.3), and 54 % in *Anabaena cycadae* (table 4.6) as compared with NiR activity of their respective controls grown in thallium deficient medium. The NiR activity in thallium untreated control cells were 349.7±16 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Nostoc mus*corum, 312.7±11 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Anabaena variabilis*, 269.7±12 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Nostoc* ANTH and 271.6±11 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein in *Anabaena cycadae.*

3.2.5 Effect of thallium on the electron transport activities.

Since heterocyst frequency, nitrogenase activity, GS activity, nitrate reductase and nitrite reductase activity of cyanobacteria were inhibited in response to thallium treatment, effect of thallium on electron transport activities (O_2 evolution/consumption) were determined as well. For this cyanobacterial cells grown in different combined nitrogen sources supplemented media with and without thallium were used. The measurement was performed by using Clark type O_2 electrode installed in a 3 ml plexi glass container with magnetic stirrer as described by Robinson *et al.* (1982) in 6 days old cultures. The activities has been expressed as nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} for

photosynthetic O_2 evolution and for respiratory O_2 consumption activities as nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}.

Determination of O_2 evolution/consumption activity in thallium treated cells grown in different nitrogen media was carried out. Like its inhibitory effects on above mentioned intracellular activities, electron transport activity of cyanobacteria was found to decrease in response to thallium treatment as well. The values of O_2 evolution rates were 135.3±18 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Anabaena variabilis*, 126.3±19 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Anabaena cycadae*, 125.3±19 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Nostoc muscorum* and 116.3±15 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Nostoc* ANTH in N_2 medium. However in presence of thallium O_2 evolution rates were found to be 15.2±3.3 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Anabaena variabilis*, 18±0.15 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Anabaena cycadae*, 21.2±4 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Nostoc muscorum* and 22±2.3 nmoles O_2 evolved μg^{-1} Chl *a* .hr^{-1} in *Nostoc* ANTH. The decrease in activity was 89% in *Anabaena variabilis* (table 4.10), 84% in *Anabaena cycadae* (table 4.4), 83% in *Nostoc muscorum* (table 4.7) and 81% in *Nostoc* ANTH (table 4.1) respectively

In NO_3^- supplemented medium also significant decrease in O_2 evolution activity at 0.5 µM thallium concentrations was observed. The percent inhibition in comparison to respective controls in thallium untreated cells was 91% in *Anabaena variabilis* (table-4.11), 90 % in *Nostoc* ANTH (table-4.2) 85% inhibition in *Nostoc muscorum* (table-4.8), and 80% in *Anabaena cycadae* (table-4.5). The values of activity in control cells grown without thallium treatment was 174.1±12 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Anabaena variabilis*, 169.4±17 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Nostoc* ANTH, 124.1±22

nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Nostoc muscorum* and 109.4±19 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Anabaena cycadae*.

In ammonium supplemented medium the O_2 evolution rates were observed to be inhibited by 98% in *Anabaena cycadae* (table-4.6), 86% in *Nostoc* ANTH (table-4.3), 83 % in *Nostoc* muscorum (table4.9) and 72% in *Anabaena variabilis* (table-4.12) at 0.5 μM thallium concentration as compared with their respective controls showing activity of 91.1±11 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Anabaena variabilis*, 88.8±19 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Nostoc* ANTH, 98.8±18 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Anabaena cycadae* and 99.5±18 nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1} in *Nostoc muscorum*.

Like O_2 evolution, O_2 consumption capabilities of the cyanobacterial cells were significantly affected showing decreases in respiratory activity by 83% in *Nostoc muscorum* (table- 4.7), 76% in *Nostoc* ANTH and *Anabaena cycadae* (table-4.1 & table-4.4) and 54% in *Anabaena variabilis* (table-4.10) in comparison with their respective controls showing O_2 consumption rates of 82.7±14 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Anabaena variabilis*, 71.7±13 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Nostoc muscorum*, 65.4±3 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Nostoc* ANTH and 55.4±11 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Anabaena cycadae*.

In nitrate supplemented medium, the O_2 consumption rate in thallium untreated cells was found to be 79.4±13 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Nostoc muscorum*, 69.3±16 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Anabaena variabilis*, 69.4±16 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Anabaena cycadae* and 59.4±7.5 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Nostoc* ANTH. Similarly thallium treatment of cells in nitrate medium showed decrease in O_2 consumption rate to the value of 10.1±0.07 nmoles O_2 consumed

μg^{-1} Chl *a*.hr^{-1} in *Nostoc muscorum*, 18.1±3.5 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Anabaena variabilis*, 10.1±2.9 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Anabaena cycadae* and 7.1±0.13 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Nostoc* ANTH. The percent decrease in in O_2 consumption in response to thallium treatment in NO_3^- medium was 87 % in *Nostoc muscorum* and *Nostoc* ANTH (table-4.8 & table-4.2), 85% in *Anabaena cycadae* (table-4.5) and 73% in *Anabaena variabilis* (table-4.11) in comparison with the cells grown in thallium deficient conditions.

Cyanobacterial cells grown in ammonium supplemented medium with thallium showed a decreasing pattern in O_2 consumption rates when compared to their respective control cells grown in thallium deficient medium. The level of decrease in activity was 91% *Nostoc* ANTH (table-4.3), followed by 84% in *Nostoc muscorum* (table 4.9), 81% in *Anabaena cycadae* (table 4.6) and 79% in *Anabaena variabilis* (table 4.12). The activity in their respective control cultures grown in thallium deficient medium was found to be 61.8±17 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Nostoc muscorum*, 67.8±13 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Anabaena variabilis*, 49.4±13 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Anabaena cycadae* and 49.4±5.7 nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1} in *Nostoc* ANTH.

3.2.6. Effect of thallium on the free Proline accumulation.

Amino acid proline acts as intracellular protectants against heavy metal stress, osmotic stress, temperature stress in prokaryotic and eukaryotic organisms by quenching ROS-reactive oxygen species (Farago & Mullen, 1979). A general increase in the level of heavy metals to microbial cultures poses a pervasive threat resulting in production of ROS. The toxic effects of ROS thus produced are prevented by the production of proline that accumulates heavily in several cyanobacteria under stress, providing the cells

protection against oxidative damages (Ahmad & Hellebust, 1988). Accumulation of proline under stress conditions has been reported profusely (Smirnoff & Cumbes, 1989; Chang, 1991; Bassi & Sharma, 1993; Costa & Morel, 1994; Kavi Kishor *et al.*, 1995; Singh *et al.*, 1996; Schat *et al.*, 1997; Wu *et al.*, 1998). Therefore changes in intracellular level of free proline in response to thallium exposure was also determined in cells and expressed as μmol.proline .mg^{-1}.protein.

Increase in the proline content was found to be induced by thallium treatment to cells. Proline content in N_2 grown cells without thallium was found to be 9.45±1.4 μmol.proline.mg^{-1}.protein in *Nostoc muscorum*, 11.4±1.7 μmol.proline.mg^{-1}.protein in *Anabaena variabilis*, 10.06±2 μmol.proline.mg^{-1}.protein in *Anabaena cycadae* and 9.76±1.3 μmol.proline.mg^{-1}.protein in *Nostoc* ANTH whereas the proline content in 0.5μM thallium stressed cells was found to be 10.3±1.9 μmol.proline.mg^{-1}.protein in *Nostoc muscorum*, 12.3±1.3 μmol.proline.mg^{-1}.protein in *Anabaena variabilis*, 11.2±1.7 μmol.proline.mg^{-1}.protein in *Anabaena cycadae* and 14.19±1.0 μmol.proline.mg^{-1}.protein in *Nostoc* ANTH. The increase in intracellular proline content in thallium stressed cells was 45%, 9.3%, 9.1% and 12% in *Nostoc* ANTH (table-4.1), *Nostoc muscorum* (table-4.7), *Anabaena variabilis* (table-4.10) and *Anabaena cycadae* (4.4) as compared with the respective control cultures grown in thallium deficient medium.

Increase in intracellular proline level was observed in nitrate grown cells as well. In nitrate supplemented medium, increase in the proline levels was 18% in *Nostoc* ANTH (table-4.2), followed by 14 % in *Anabaena cycadae* (table-5) and 12% in both *Nostoc muscorum* (table-8) & *Anabaena variabilis* (table-11) as compared with their controls in thallium deficient cells. The proline content in the thallium deficient cells were 6.77±2 μmol.proline.mg^{-1}.protein in *Nostoc* ANTH (table-4.2), followed by 9.2±2.1 μmol

.proline.mg^{-1}.protein in *Anabaena cycadae* (table-4.5), 6.5±1.1 µmol.proline.mg^{-1}.protein in *Nostoc muscorum* (table-4.8) and 12.5±1.9 µmol.proline.mg^{-1}.protein in *Anabaena variabilis* (table-4.11).

In ammonium supplemented medium, there was no significant difference between the intracellular levels of proline. The thallium untreated controls showed proline content of 5.52±3 µmol.proline.mg^{-1}.protein and 0.5 µM thallium stressed cells showed proline content of 5.37±3 µmol.proline.mg^{-1}.protein in *Nostoc* ANTH (table-4.3). The intracellular proline level in thallium treated cells of *Anabaena variabilis*, *Anabaena cycadae* and *Nostoc muscorum* was found to be 8.1±1.6 µmol.proline.mg^{-1}.protein, 6.21 ±1.8 µmol.proline.mg^{-1}.protein and 5.1±1.6 µmol.proline.mg^{-1} protein respectively. The intracellular level of proline content in their respective control cells grown in thallium deficient medium was found to be 7.2±1.6 µmol.proline.mg^{-1}.protein in *Anabaena variabilis*, 5.23±1.23 µmol.proline.mg^{-1}.protein in *Anabaena cycadae* and 4.2±1.2 µmol.proline.mg^{-1}.protein in *Nostoc muscorum*. This results indicates that under thallium stressed conditions proline content was increased by 12.5% in *Anabaena variabilis* (table 4.12), 14% in *Anabaena cycadae* (table 4.6) and 21% in *Nostoc muscorum* (table 4.9) as compared with the proline content in their respective controls cultures grown in thallium deficient media.

Table 4.1:

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmoles C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}), Glutamine synthetase activity (transferase) (nmol -γ–glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a* .hr^{-1}), O_2 consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Nostoc* ANTH** cells grown in N_2 media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	N_2	$N_2 + Tl^{2+}$	*p*
Heterocyst frequency (%)	9 ± 0.71	2 ± 0.5	< 0.001
Nitrogenase	12 ± 0.6	1.2 ± 0.2	< 0.001
Glutamine synthetase (transferase)	965.2 ± 37	230.5 ± 19	< 0.001
Nitrate reductase	1.73 ± 0.16	0.51 ± 0.12	NS
Nitrite reductase	517.5 ± 18	192.8 ± 11	< 0.001
O_2 evolution	116.3 ± 15	22 ± 2.3	< 0.001
O_2 Consumption	65.4 ± 03	15.01 ± 1.3	< 0.001
Proline levels	9.76 ± 1.3	14.19 ± 1.0	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2-medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each. N_2 refers to nitrogen free medium; $N_2 + Tl^{2+}$ refers to N_2-medium with 0.5 μM thallium sulphate, '*p*' indicates level of significance, the values (p <0.001) indicates statistically significant and NS not significant values.

Table 4.2

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a* hr^{-1}), Glutamine synthetase activity (transferase) (nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a* hr^{-1}), O_2 consumption (nmoles O_2 consumed μg^{-1} Chl *a* hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Nostoc*** **ANTH** cells grown in NO_3^- supplemented media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	**NO_3^-**	**NO_3^- + Tl^{2+}**	***p***
Heterocyst frequency (%)	0.0	0.0	-
Nitrogenase	0.0	0.0	-
Glutamine synthetase (transferase)	1053.4 ± 30	316.71 ± 12	< 0.001
Nitrate reductase	4.8 ± 0.16	0.9 ± 0.30	< 0.001
Nitrite reductase	498.8 ± 14	213.8 ± 09	< 0.001
O_2 evolution	169.4 ± 17	16 ± 3.8	< 0.001
O_2 Consumption	59.4 ± 7.5	7.1 ± 0.13	NS
Proline levels	6.77 ± 2.0	8.75 ± 1.8	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each. NO_3^- refers to N_2 medium supplemented with 5mM $NaNO_3$; NO_3^- + Tl^{2+} refers to NO_3^- medium with 0.5 μM thallium sulphate, '*p*' indicates level of significance, the values (p <0.001) indicates statistically significant and NS not significant values.

Table 4.3

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a* hr^{-1}), Glutamine synthetase activity (transferase) (nmoles - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a* hr^{-1}), O_2 Consumption (nmoles O_2 consumed μg^{-1} Chl *a* hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Nostoc* ANTH** cells grown in NH_4^+ supplemented media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	NH_4^+	$NH_4^+ + Tl^{2+}$	*p*
Heterocyst frequency (%)	0.0	0.0	-
Nitrogenase	0.0	0.0	-
Glutamine synthetase (transferase)	663.89 ± 16	225.8 ± 9.4	< 0.001
Nitrate reductase	0.41 ± 0.05	0.11 ± 0.03	NS
Nitrite reductase	269.7 ± 12	128.5 ± 17	NS
O_2 evolution	88.8 ± 19	12 ± 2.2	NS
O_2 Consumption	49.4 ± 5.7	4.4 ± 0.86	< 0.001
Proline levels	5.52 ± 3	5.37 ± 1.6	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values represents are mean ± standard deviation (SD) from two independent experiments with two replicates each.NH_4^+ refers to N_2 medium supplemented with 2mM NH_4Cl; $NH_4^+ + Tl^{2+}$ refers to NH_4^+ medium with 0.5μM thallium sulphate, '*p*' indicates level of significance, the values ($p < 0.001$) indicates statistically significant and NS not significant values.

Table 4.4

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}), Glutamine synthetase activity (transferase) (nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 Consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Anabaena cycadae*** cells grown in N_2- media at 24±2°C and determined 6 days after inoculation

Characteristics	N_2	$N_2 + Tl^{2+}$	*p*
Heterocyst frequency (%)	9 ± 0.9	2 ± 0.2	< 0.001
Nitrogenase	10 ± 0.8	2.5 ± 0.4	< 0.001
Glutamine synthetase (transferase)	1018.2 ± 21	331.8 ± 19	< 0.001
Nitrate reductase	1.36 ± 0.46	0.77 ± 0.16	NS
Nitrite reductase	678.1 ± 27	229.8 ± 17	< 0.001
O_2 evolution	126.3 ± 16	18 ± 0.15	< 0.001
O_2 Consumption	55.4 ± 11	13.3 ± 0.14	NS
Proline levels	10.06 ± 2	11.2 ± 1.7	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values presented are mean ± standard deviation (SD) from two independent experiments with two replicates each. N_2 refers to nitrogen free medium; $N_2 + Tl^{2+}$ refers to N_2-medium with 0.5 μM thallium sulphate, '*p*' indicates level of significance, the values ($p < 0.001$) indicates statistically significant and NS not significant values.

Table 4.5

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}), Glutamine synthetase activity (transferase) (nmol - γ - glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 Consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Anabaena cycadae*** cells grown in NO_3^- media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	NO_3^-	NO_3^- + Tl^{2+}	*p*
Heterocyst frequency (%)	0.0	0.0	-
Nitrogenase	0.0	0.0	-
Glutamine synthetase (transferase)	948.6 ± 11	213.7 ± 15	< 0.001
Nitrate reductase	5.8 ± 1.6	1.3 ± 0.3	NS
Nitrite reductase	435.8 ± 21	141.1 ± 11	< 0.001
O_2 evolution	109.4 ± 19	21.1 ± 0.12	NS
O_2 Consumption	69.4 ± 16	10.1 ± 2.9	NS
Proline levels	9.2 ± 2.1	10.5 ± 2.5	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each. NO_3^- refers to N_2 medium supplemented with 5mM $NaNO_3$; NO_3^- + Tl^{2+} refers to NO_3^- medium with 0.5 μM thallium sulphate, '*p*' indicates level of significance, the values (p <0.001) indicates statistically significant and NS not significant values.

Table 4.6

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}), Glutamine synthetase activity (transferase) (nmol - γ - glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 Consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in *Anabaena cycadae* cells grown in NH_4^+ media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	NH_4^+	$NH_4^+ + Tl^{2+}$	*p*
Heterocyst frequency (%)	0.0	0.0	-
Nitrogenase	0.0	0.0	-
Glutamine synthetase (transferase)	539.3 ± 13	215.86 ± 17	< 0.001
Nitrate reductase	0.67 ± 0.18	0.18 ± 0.2	NS
Nitrite reductase	271.6 ± 11	123.9 ± 16	NS
O_2 evolution	98.8 ± 18	1.5 ± 0.12	NS
O_2 Consumption	49.4 ± 13	9.4 ± 1.1	NS
Proline levels	5.43 ± 1.2	6.21 ± 1.8	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each. NH_4^+ refers to N_2 medium supplemented with 2mM NH_4Cl; $NH_4^+ + Tl^{2+}$ refers to NH_4^+ medium with 0.5 μM thallium sulphate, '*p*' indicates level of significance, the values ($p < 0.001$) indicates statistically significant and NS not significant values..

Table 4.7

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}), Glutamine synthetase activity (transferase) (nmol - γ - glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 Consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Nostoc muscorum*** cells grown in N_2 media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	N_2	$N_2 + Tl^{2+}$	*p*
Heterocyst frequency (%)	8 ± 0.9	2 ± 0.2	NS
Nitrogenase	9 ± 0.5	2 ± 0.3	< 0.001
Glutamine synthetase (transferase)	981.5 ± 26	311.8 ± 14	< 0.001
Nitrate reductase	1.27 ± 0.46	0.47 ±0.19	NS
Nitrite reductase	787.2 ± 26	337.1 ± 18	< 0.001
O_2 evolution	125.3 ± 19	21.2 ± 4	NS
O_2 Consumption	71.7 ± 13	12.11 ± 2	NS
Proline levels	9.45 ± 1.4	10.3 ± 1.9	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each. N_2 refers to nitrogen free medium; $N_2 + Tl^{2+}$ refers to N_2-medium with 0.5 μM thallium sulphate, '*p*' indicates level of significance, the values ($p < 0.001$) indicates statistically significant and NS not significant values.

Table 4.8

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}), Glutamine synthetase activity (transferase) (nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 consumption (nmoles O_2 consumed μg^{-1}.Chl *a*.hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Nostoc muscorum*** cells grown in NO_3^- media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	NO_3^-	$NO_3^- + Tl^{2+}$	*p*
Heterocyst frequency (%)	0.0	0.0	-
Nitrogenase	0.0	0.0	-
Glutamine synthetase (transferase)	828.6 ± 17	253.7 ± 13	< 0.001
Nitrate reductase	4.2 ± 0.15	0.97 ± 0.3	< 0.001
Nitrite reductase	794.2 ± 21	196.6 ± 16	< 0.001
O_2 evolution	124.1 ± 22	18.5 ± 5.1	NS
O_2 Consumption	79.4 ± 13	10.1 ± 2.2	NS
Proline levels	6.5 ± 1.1	7.3 ± 1.8	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each. NO_3^- refers to N_2 medium supplemented with 5mM $NaNO_3$; $NO_3^- + Tl^{2+}$ refers to NO_3^- medium with 0.5μM thallium sulphate, '*p*' indicates level of significance, the values ($p < 0.001$) indicates statistically significant and NS not significant values.

Table 4.9

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}), Glutamine synthetase activity (transferase) (nmol - γ - glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Nostoc muscorum*** cells grown in NH_4^+ media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	NH_4^+	$NH_4^+ + Tl^{2+}$	*p*
Heterocyst frequency (%)	0.0	0.0	-
Nitrogenase	0.0	0.0	-
Glutamine synthetase (transferase)	489.1 ± 19	135.8 ± 12	< 0.001
Nitrate reductase	0.57 ± 0.18	0.18 ± 0.08	NS
Nitrite reductase	349.7 ± 16	145.8 ± 14	< 0.001
O_2 evolution	99.5 ± 18	16.5 ± 4.2	NS
O_2 Consumption	61.8 ± 17	9.4 ± 4.3	NS
Proline levels	4.2 ± 1.2	5.1 ± 1.6	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each. NH_4^+ refers to N_2 medium supplemented with 2mM NH_4Cl; $NH_4^+ + Tl^{2+}$ refers to NH_4^+ medium with 0.5μM thallium sulphate, '*p*' indicates level of significance, the values ($p < 0.001$) indicates statistically significant and NS not significant values.

Table 4.10

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}), Glutamine synthetase activity (transferase) (nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 Consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Anabaena variablis*** cells grown in N_2 media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	N_2	$N_2 + Tl^{2+}$	*p*
Heterocyst frequency (%)	9 ± 0.3	3 ± 0.6	< 0.001
Nitrogenase	8.6 ± 1.8	2.2 ± 0.7	NS
Glutamine synthetase (transferase)	1048.5 ± 22	341.8 ± 14	< 0.001
Nitrate reductase	1.32 ± 0.23	0.57 ± 1.2	NS
Nitrite reductase	887.6 ± 22	217.1 ± 19	< 0.001
O_2 evolution	135.3 ± 18	15.2 ± 3.3	NS
O_2 Consumption	82.7 ± 14	17.13 ± 6.1	NS
Proline levels	11.4 ± 1.7	12.3 ± 1.3	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each. N_2 refers to nitrogen free medium; $N_2 + Tl^{2+}$ refers to N_2-medium with 0.5μM thallium sulphate, '*p*' indicates level of significance, the values ($p < 0.001$) indicates statistically significant and NS not significant values

Table 4.11

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}), Glutamine synthetase activity (transferase) (nmol - γ - glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Anabaena variablis*** cells grown in NO_3^- media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	NO_3^-	$NO_3^- + Tl^{2+}$	*p*
Heterocyst frequency (%)	0.0	0.0	-
Nitrogenase	0.0	0.0	-
Glutamine synthetase (transferase)	828.6 ± 18	213.7 ± 11	< 0.001
Nitrate reductase	5.1 ± 0.14	1.6 ± 0.5	NS
Nitrite reductase	814.2 ± 22	212.2 ± 16	< 0.001
O_2 evolution	174.1 ± 12	14.5 ± 2.1	< 0.001
O_2 Consumption	69.3 ± 16	18.1 ± 3.5	NS
Proline levels	12.5 ± 1.9	14.1 ± 2.1	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each. NO_3^- refers to N_2 medium supplemented with 5mM $NaNO_3$; $NO_3^- + Tl^{2+}$ refers to NO_3^- medium with 0.5μM thallium sulphate, '*p*' indicates level of significance, the values ($p < 0.001$) indicates statistically significant and NS not significant values.

Table 4.12

The frequency of heterocyst (per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a*.hr^{-1}), Glutamine synthetase activity (transferase) (nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}), Proline content (μmol.proline.mg^{-1}.protein) in ***Anabaena variablis*** cells grown in NH_4^+ media at 24 ± 2°C and determined 6 days after inoculation.

Characteristics	NH_4^+	$NH_4^+ + Tl^{2+}$	*p*
Heterocyst frequency (%)	0.0	0.0	-
Nitrogenase	0.0	0.0	-
Glutamine synthetase (transferase)	489.1 ± 19	185.86 ± 12	< 0.001
Nitrate reductase	0.63 ± 0.12	0.31 ± 0.09	NS
Nitrite reductase	312.7 ± 11	131.8 ± 15	< 0.001
O_2 evolution	91.1 ± 12	25.5 ± 2.2	< 0.001
O_2 Consumption	67.8 ± 13	14.3 ± 5.1	NS
Proline levels	7.2 ± 1.6	8.1 ± 1.6	NS

Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each. NH_4^+ refers to N_2 medium supplemented with 2mM NH_4Cl; $NH_4^+ + Tl^{2+}$ refers to NH_4^+ medium with 0.5μM thallium sulphate, '*p*' indicates level of significance, the values (p <0.001) indicates statistically significant and NS not significant values.

3.3 Effect of thallium on the growth of cyanobacterium in medium supplemented with organic sources.

The effect of toxicity of thallium to cyanobacterium with amino acids as organic source was measured in four cyanobacterial strains namely *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Anabaena variabilis*. The cultures were inoculated and grown at various concentration of thallium sulphate ranging from 0.0 μM to 1 μM at an initial Chl *a* concentration of 0.4 μg ml^{-1} in a 250 ml Erlenmeyer flask containing 100 mL of BG - 11_0 medium supplemented with 20 amino acids individually at 1mM concentrations. The chlorophyll *a* contents of different cultures were measured 6 days after inoculation. Out of all the 20 amino acids, glutamine, proline, asparagine and alanine showed the best growth and the study strongly suggested that these amino acids protect the cells from thallium toxicity.

In glutamine supplemented medium, *Nostoc* ANTH showed a higher Chl *a* content of 5.8 $\mu g.ml^{-1}$, followed by 4.18 $\mu g.ml^{-1}$ in *Anabaena cycadae*, 3.97 $\mu g.ml^{-1}$ in *Nostoc muscorum* and 3.32 $\mu g.ml^{-1}$ in *Anabaena variabilis* in thallium untreated cells. However in the presence of 0.5μM thallium, cyanobacterial growth measured in terms of Chl *a* content was found to be 2.92 $\mu g.ml^{-1}$ in *Nostoc* ANTH, 1.97 $\mu g.ml^{-1}$ in *Anabaena cycadae*, 1.98 $\mu g.ml^{-1}$ in *Nostoc muscorum* and 1.82 $\mu g.ml^{-1}$ in *Anabaena variabilis*. These results indicated that at 0.5μM thallium in glutamine supplemented medium, cells continued their growth though at a slower rate. The protection level decreased with increase in thallium concentration in medium. The percent of protection levels were 52% in *Anabaena cycadae* (fig 3.12), 51% in *Nostoc* ANTH (fig 3.11) & *Nostoc muscorum* (fig 3.13) and 49% in *Anabaena variabilis* (fig 3.14) as compared with their respective thallium untreated controls.

The protection against thallium toxicity was also observed in N_2-medium supplemented with 1mM proline. Similarly like glutamine supplemented medium, Chl *a* content in proline supplemented medium was found to be 2.91 $\mu g.ml^{-1}$ in *Anabaena variabilis*, 2.11 $\mu g.ml^{-1}$ in *Nostoc muscorum*, 2.54 $\mu g.ml^{-1}$ in *Anabaena cycadae* and 2.44 $\mu g.ml^{-1}$ in *Nostoc* ANTH at 0.5μM thallium concentration in the medium. The Chl *a* content in the respective controls of the proline supplemented medium was found to be 5.56 $\mu g.ml^{-1}$ in *Anabaena variabilis*, 4.16 $\mu g.ml^{-1}$ in *Nostoc muscorum*, 4.62 $\mu g.ml^{-1}$ in *Anabaena cycadae* and 4.33 $\mu g.ml^{-1}$ in *Nostoc* ANTH. The percent protection levels by proline against thallium toxicity was 52% in *Anabaena variabilis* (fig 3.18) followed by 51% in *Nostoc muscorum* (fig 3.17), 55% in *Anabaena cycadae* (fig 3.16) and 56% in *Nostoc* ANTH (fig 3.15) as compared with their respective controls grown in thallium deficient media.

Similar significant increase in levels of protection was observed in media supplemented with asparagine and alanine at 0.5μM thallium concentration. The percent protection levels by asparagines was found to be 52% in *Nostoc* ANTH (fig 3.19) and *Anabaena cycadae* (fig 3.20), 51% in *Nostoc muscorum* (fig 3.21) and 55% in *Anabaena variabilis* (fig 3.22) as compared to their respective controls cultures in thallium deficient medium. The thallium deficient controls cultures amended with asparagines showed Chl *a* content of 4.06 $\mu g.ml^{-1}$ in *Nostoc* ANTH, 5.17 $\mu g.ml^{-1}$ in *Anabaena cycadae*, 6.14 $\mu g.ml^{-1}$ in *Nostoc muscorum* and 3.52 $\mu g.ml^{-1}$ in *Anabaena variabilis* respectively.

In alanine supplemented medium, the extent of protection level was higher in *Nostoc muscorum* with 53% protection (fig 3.25) as compared with the thallium untreated control which showed Chl *a* content of 3.97 $\mu g.ml^{-1}$. Similarly percent protection of 52%, 51% and 50% against thallium toxicity was observed in *Anabaena cycadae* (fig 3.24), *Anabaena variabilis* (fig 3.26) and *Nostoc* ANTH (fig 3.23) as

compared with their respective controls cells grown in thallium deficient medium. The thallium deficient controls cultures amended with alanine showed Chl *a* content of 3.24 $\mu g.ml^{-1}$ in *Anabaena cycadae*, 4.39 $\mu g.ml^{-1}$ in *Anabaena variabilis* and 3.2 $\mu g.ml^{-1}$ in *Nostoc* ANTH. Rest of the 16 amino acids tested did not give protection to these four cyanobacterial strains namely *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Anabaena variabilis* against thallium toxicity.

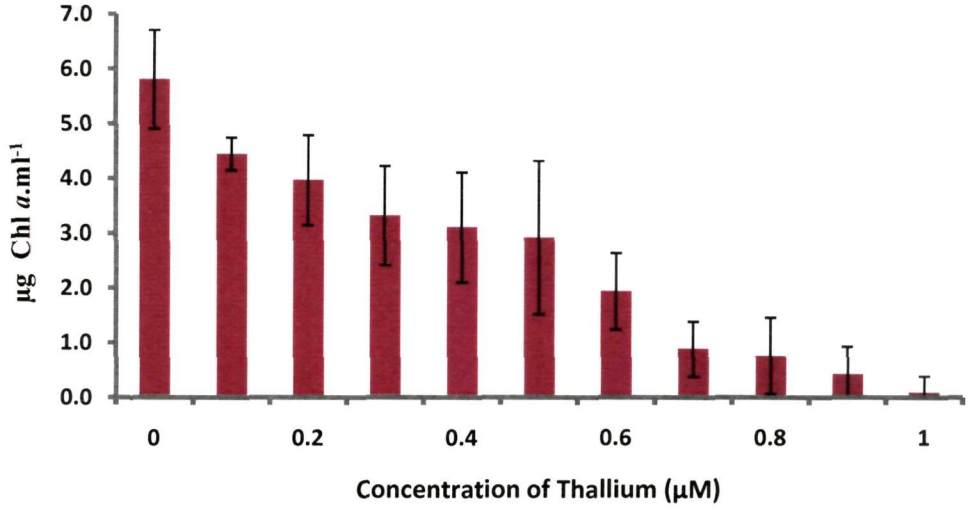


Fig 3.11: Effect of increasing concentrations of thallium sulphate in *Nostoc* ANTH cells grown in 1mM glutamine supplemented BG-11$_0$ medium at 24±2°C. Growth in terms of µg Chl *a* .ml^{-1} was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2-medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

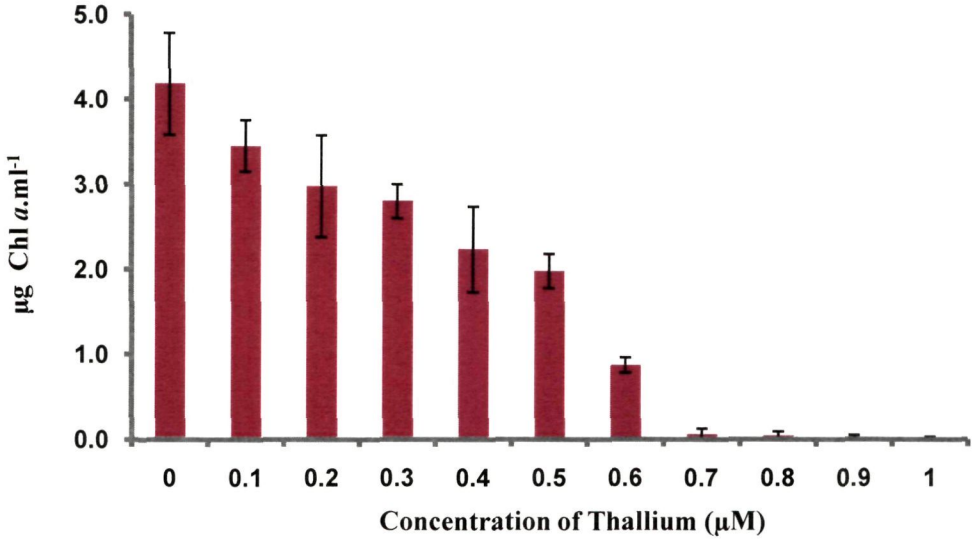


Fig 3.12: Effect of increasing concentrations of thallium sulphate in *Anabaena cycadae* cells grown in 1mM glutamine supplemented BG-11$_0$ medium at 24±2°C. Growth in terms of µg Chl *a* .ml^{-1} was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2-medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each

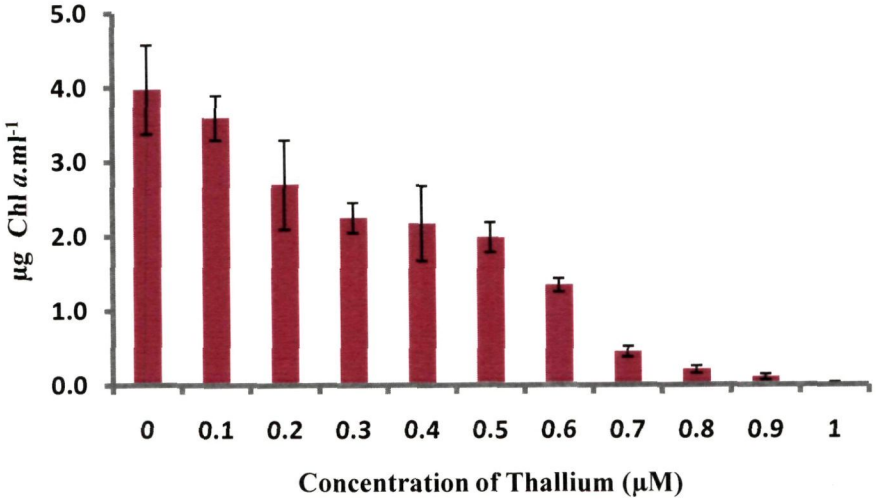


Fig 3.13: Effect of increasing concentrations of thallium sulphate in *Nostoc muscorum* cells grown in 1mM glutamine supplemented BG-11_0 medium at 24 ± 2°C. Growth in terms of µg Chl *a* .ml^{-1} was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2-medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

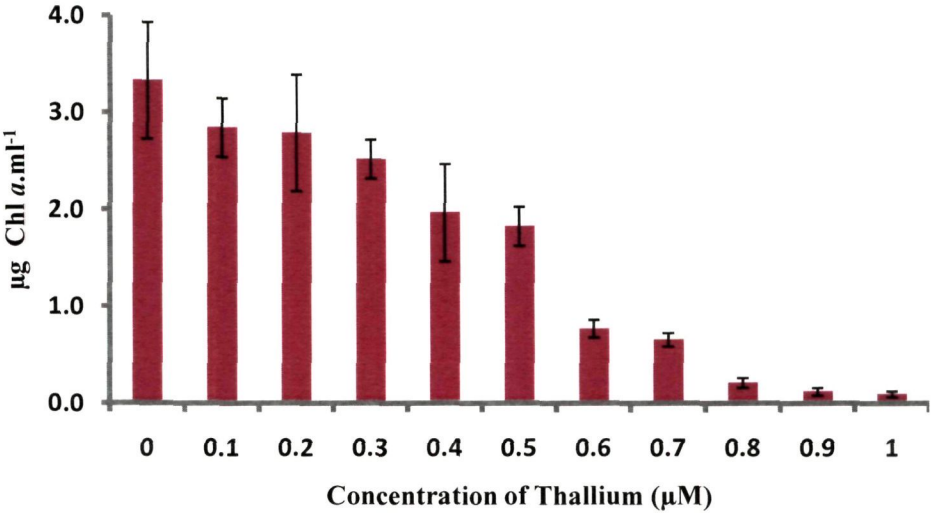


Fig 3.14: Effect of increasing concentrations of thallium sulphate in *Anabaena variabilis* cells grown in 1mM glutamine supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a* .ml^{-1} was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2-medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

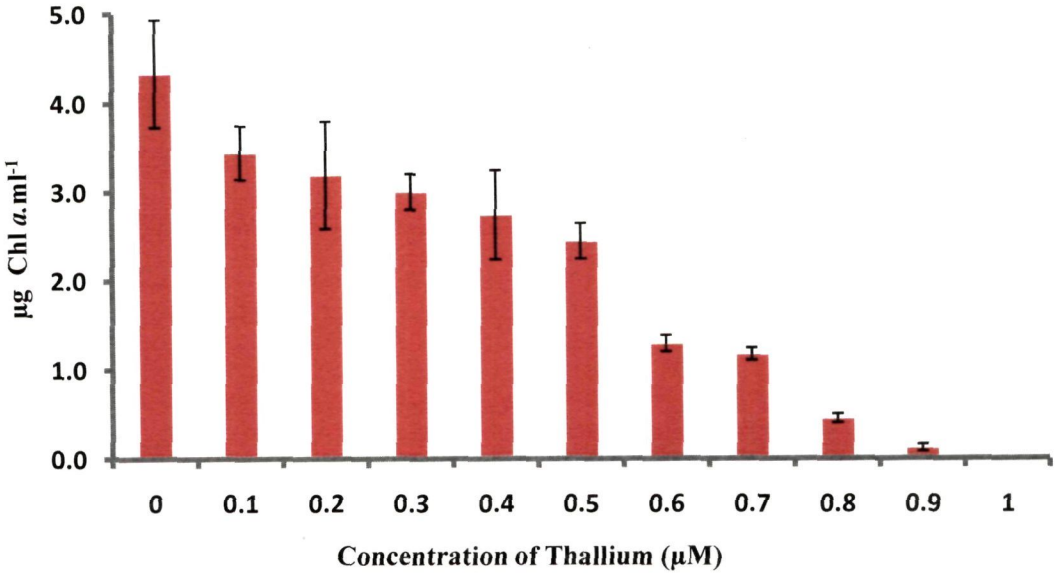


Fig 3.15: Effect of increasing concentrations of thallium sulphate in *Nostoc* ANTH cells grown in 1mM proline supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a* $.ml^{-1}$ was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

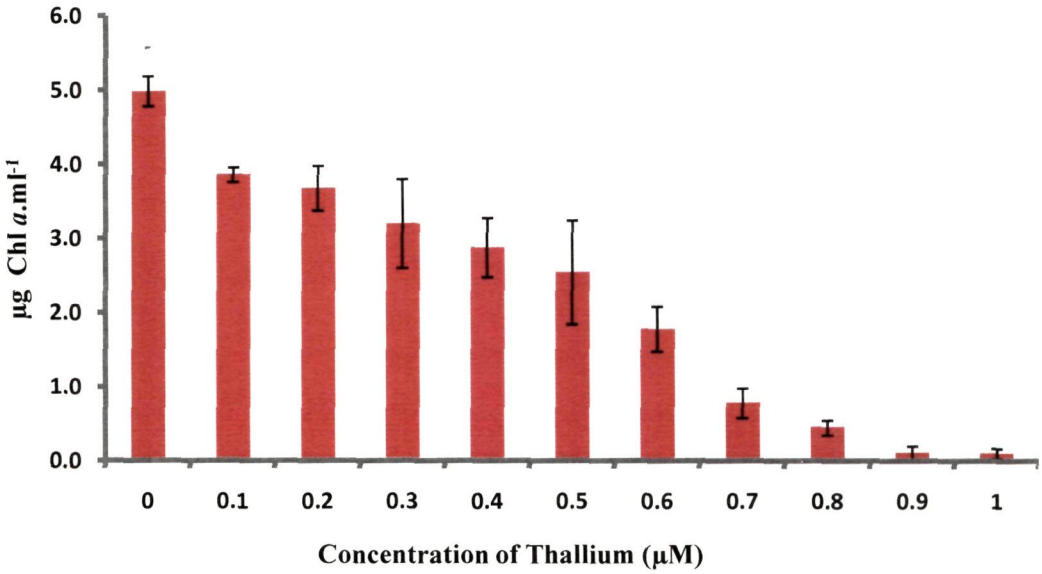


Fig 3.16: Effect of increasing concentrations of thallium sulphate in *Anabaena cycadae* cells grown in 1mM proline supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a* $.ml^{-1}$ was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

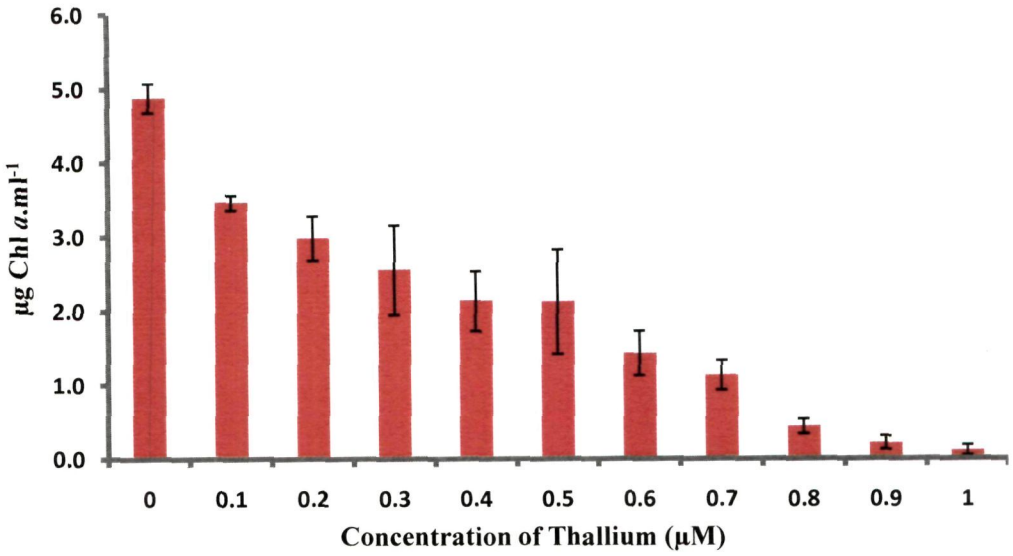


Fig 3.17: Effect of increasing concentrations of thallium sulphate in *Nostoc muscorum* cells grown in 1mM proline supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a* $.ml^{-1}$ was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2-medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

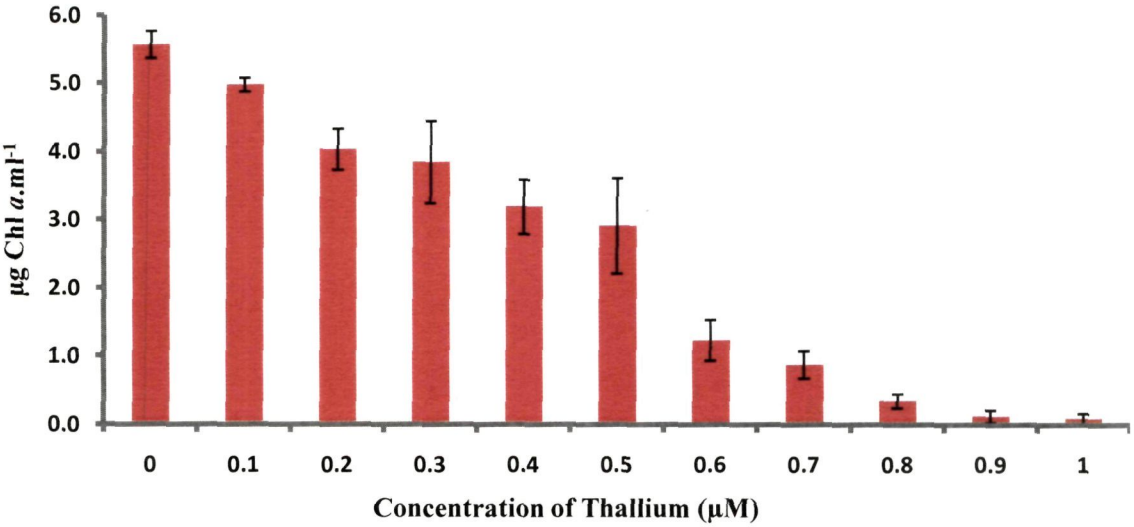


Fig 3.18: Effect of increasing concentrations of thallium sulphate in *Anabaena variabilis* cells grown in 1mM proline supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a* $.ml^{-1}$ was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2-medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

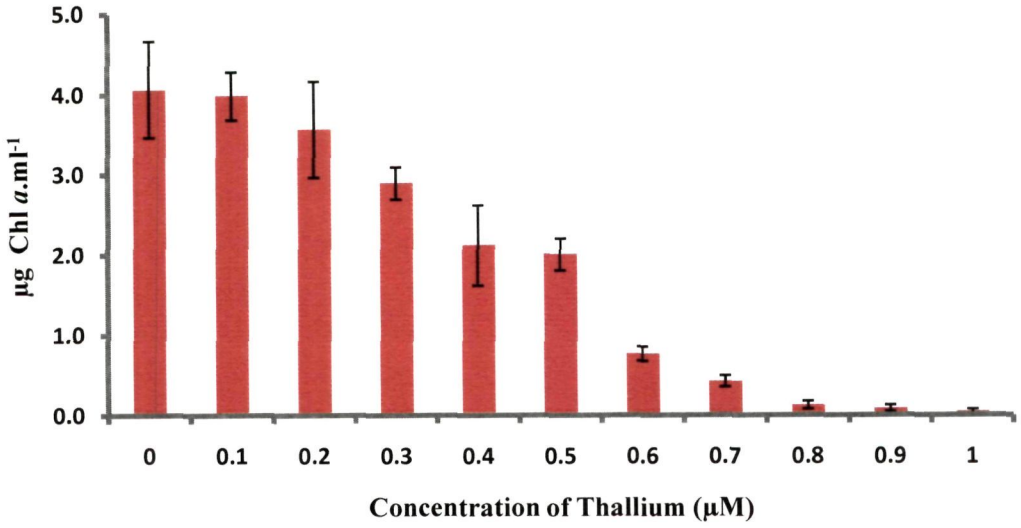


Fig 3.19: Effect of increasing concentrations of thallium sulphate in *Nostoc* ANTH cells grown in 1mM asparagine supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a* $.ml^{-1}$ was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

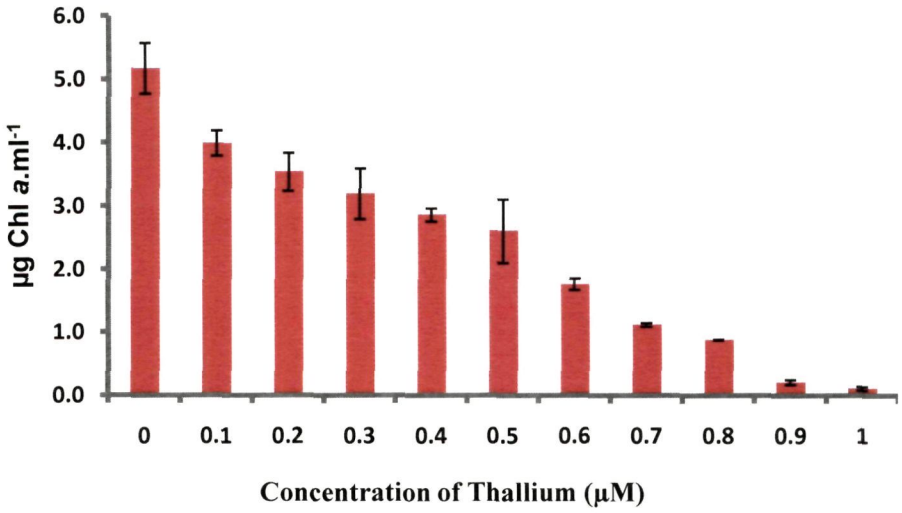


Fig 3.20: Effect of increasing concentrations of thallium sulphate in *Anabaena cycadae* cells grown in 1mM asparagine supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a* $.ml^{-1}$ was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

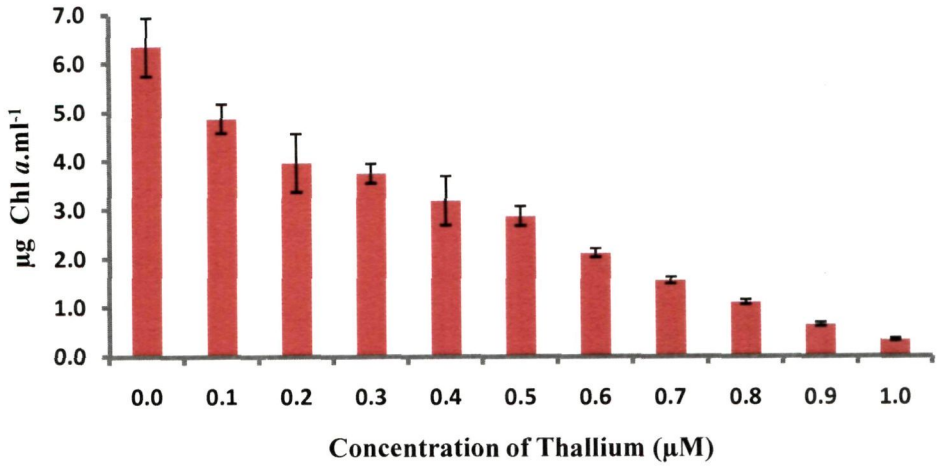


Fig 3.21 Effect of increasing concentrations of thallium sulphate in *Nostoc muscorum* cells grown in 1mM asparagine supplemented BG-11$_0$ medium at 24±2°C. Growth in terms of µg Chl *a*.ml^{-1} was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

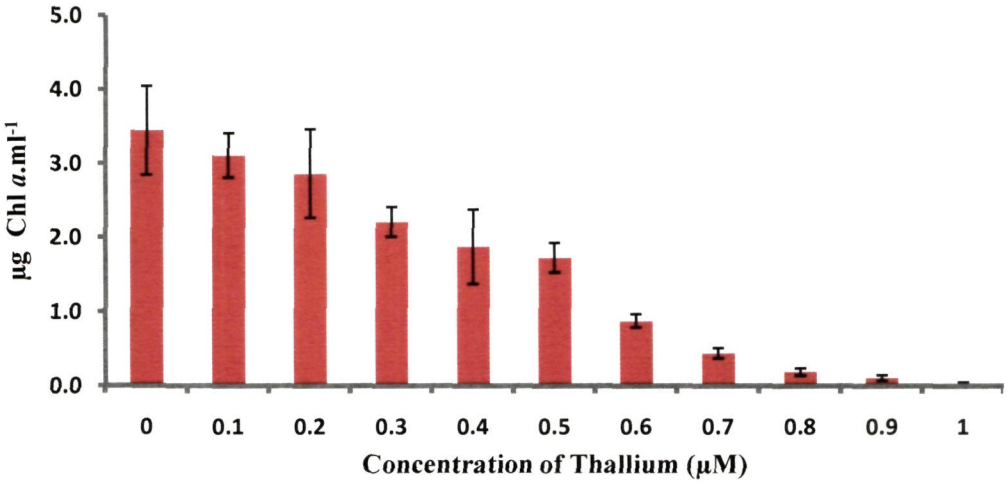


Fig 3.22: Effect of increasing concentrations of thallium sulphate in *Anabaena variabilis* cells grown in 1mM asparagine supplemented BG-11$_0$ medium at 24±2°C. Growth in terms of µg Chl *a*.ml^{-1} was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

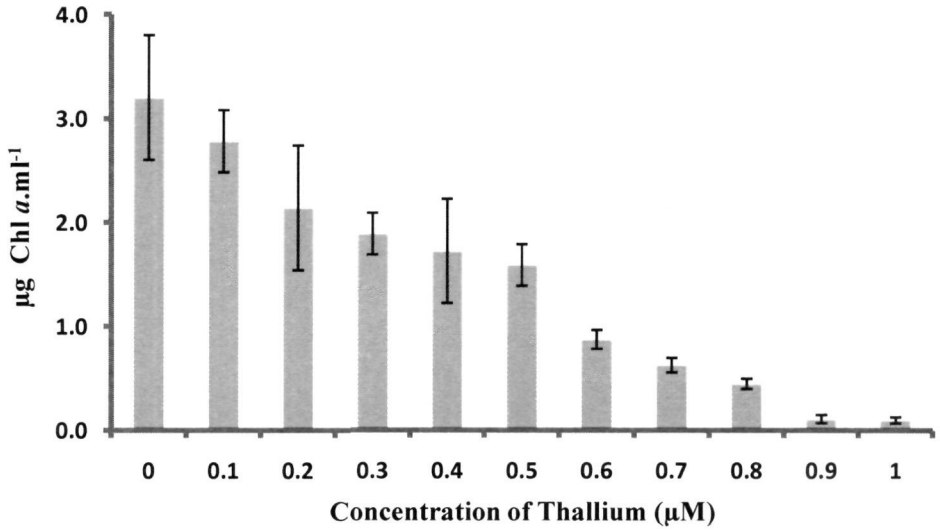


Fig 3.23: Effect of increasing concentrations of thallium sulphate in *Nostoc* ANTH cells grown in 1mM alanine supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a*.ml^{-1} was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

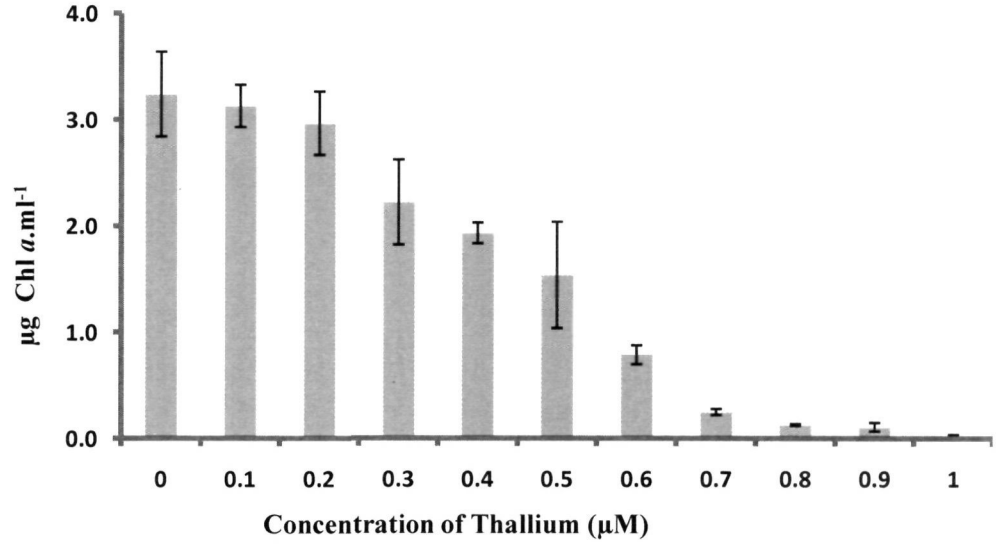


Fig 3.24: Effect of increasing concentrations of thallium sulphate in *Anabaena cycadae* cells grown in 1mM alanine supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a*.ml^{-1} was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

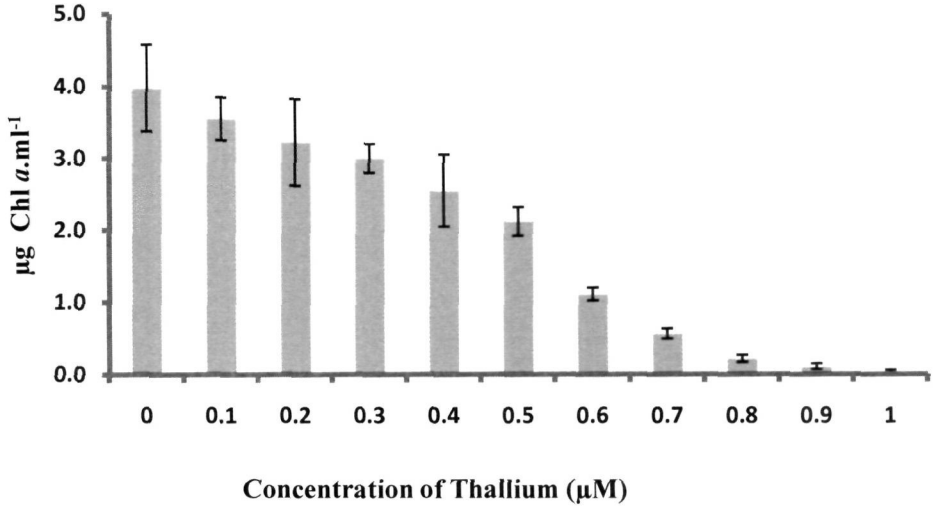


Fig 3.25: Effect of increasing concentrations of thallium sulphate in *Nostoc muscorum* cells grown in 1mM alanine supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a*.ml^{-1} was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

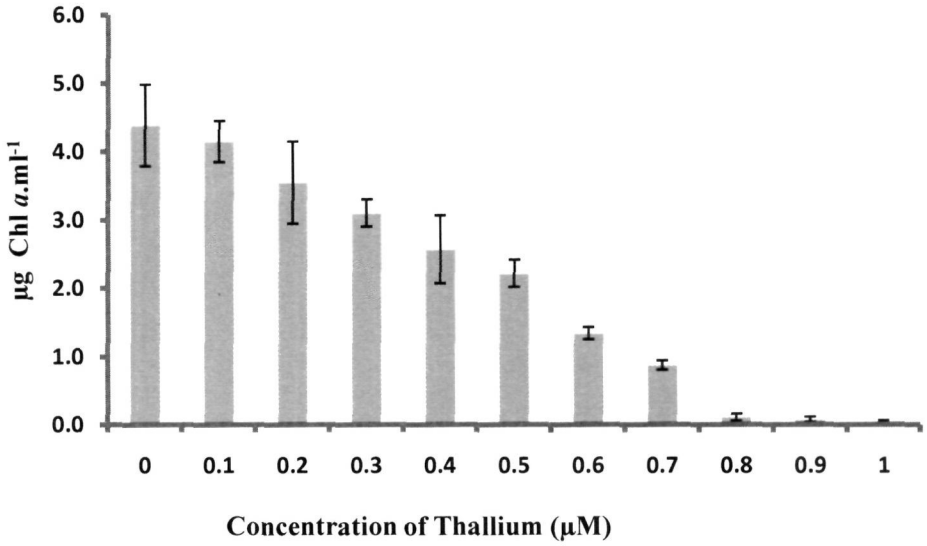


Fig 3.26: Effect of increasing concentrations of thallium sulphate in *Anabaena variabilis* cells grown in 1mM alanine supplemented BG-11_0 medium at 24±2°C. Growth in terms of µg Chl *a*.ml^{-1} was determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

3.4 Effect of thallium on the phycobilisome contents with organic sources.

The effect of thallium on the phycobilisome contents – phycocyanin [PC], allophycocyanin [APC] and phycoerythrin [PE] was determined in cyanobacterial cells of *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Anabaena variabilis* in medium supplemented with amino acids. For this study exponentially grown cells were inoculated at an initial chlorophyll *a* concentration of 0.4 $\mu g.ml^{-1}$ in 100 mL of N_2-medium containing 0.5μM thallium sulphate supplemented with 1mM glutamine, alanine, proline and asparagine respectively. The PC, APC and PE contents were measured 6 days after inoculation and expressed as $\mu g.ml^{-1}$.

Like changes in chlorophyll *a* contents, amino acids showed protection against thallium toxicity to cyanobacteria measured in terms of phycobilisome contents. The percent level of phycobilisome contents in *Nostoc* ANTH (fig 3.27), *Anabaena cycadae* (fig 3.28), *Nostoc muscorum* (fig 3.29) and *Anabaena variabilis* (fig 3.30) in amino acid supplemented media with 0.5μM thallium in comparison to thallium untreated control is given below in table 4.13.

Table: 4.13

Effect of 0.5μM thallium on phycobilisome contents – phycocyanin (PC), Allophycocyanin (APC) and Phycoerythrin (PE) in *Nostoc ANTH, Anabaena cycadae, Nostoc muscorum* and *Anabaena variabilis* cells grown in 1mM alanine, asparagnine, proline and glutamine supplemented BG-11_0 medium at 24 ± 2°C.

Cultures Used	Amino acids	% Protection		
		[PC] 0.5μM Tl^{2+}	[APC] 0.5μM Tl^{2+}	[PE] 0.5μM Tl^{2+}
Nostoc ANTH	Alanine	62	59	51
	Asparagnine	74	65	61
	Proline	58	56	54
	Glutamine	73	63	56
Anabaena cycadae	Alanine	77	67	59
	Asparagnine	62	58	56
	Proline	70	69	66
	Glutamine	62	58	53
Nostoc muscorum	Alanine	74	66	60
	Asparagnine	70	65	54
	Proline	66	52	50
	Glutamine	65	56	51
Anabaena variabilis	Alanine	60	52	54
	Asparagnine	58	54	52
	Proline	68	56	52
	Glutamine	70	67	61

The PC, APC and PE contents in $\mu g.ml^{-1}$ were determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2-medium. The results shown were in terms of percent protection as compared with the respective controls cultures grown in thallium deficient medium. The values represented are average measurement of two replicates each of two independent experiments.

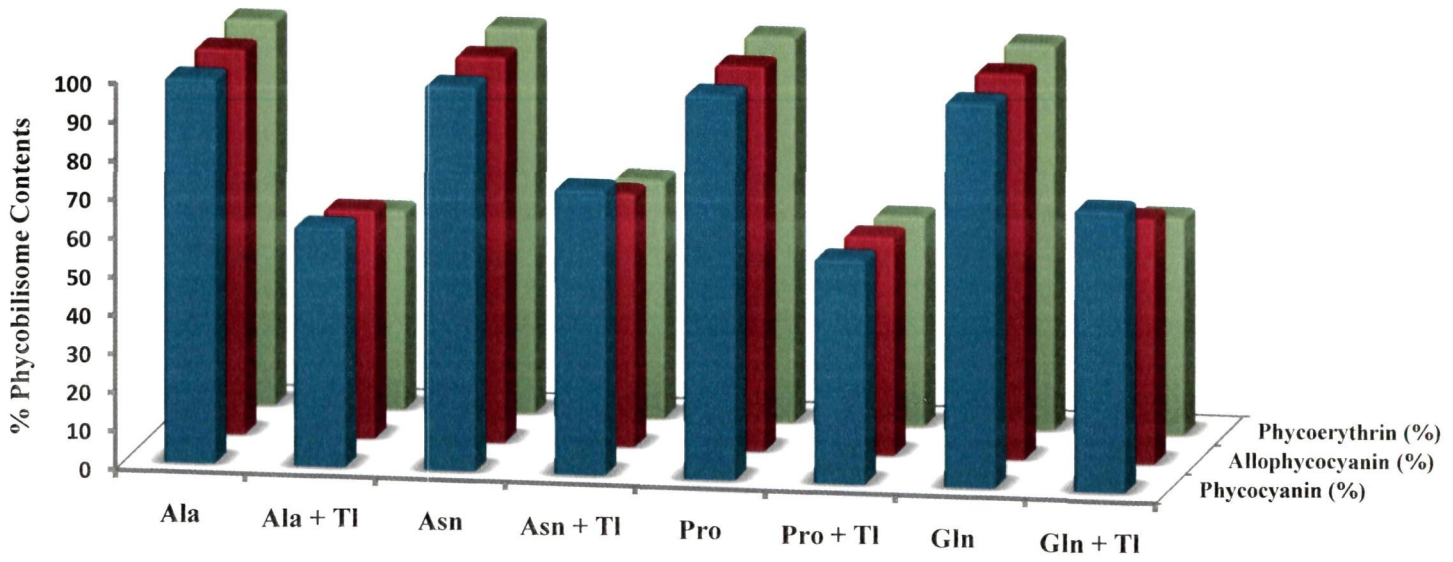


Fig 3.27: Effect of 0.5µM thallium sulphate on phycobilisome contents (phycocyanin, allophycocyanin & phycoerythrin) in *Nostoc* ANTH cells grown in 1mM alanine (ala), asparagine (asp), proline (pro) or glutamine (gln) supplemented BG-11_0 medium at 24±2°C. The PC, APC and PE contents in $\mu g.ml^{-1}$ were determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2-medium. The values were plotted in percent protection taking the control culture values as 100%. The values represented are average measurement of two replicates each of two independent experiments.

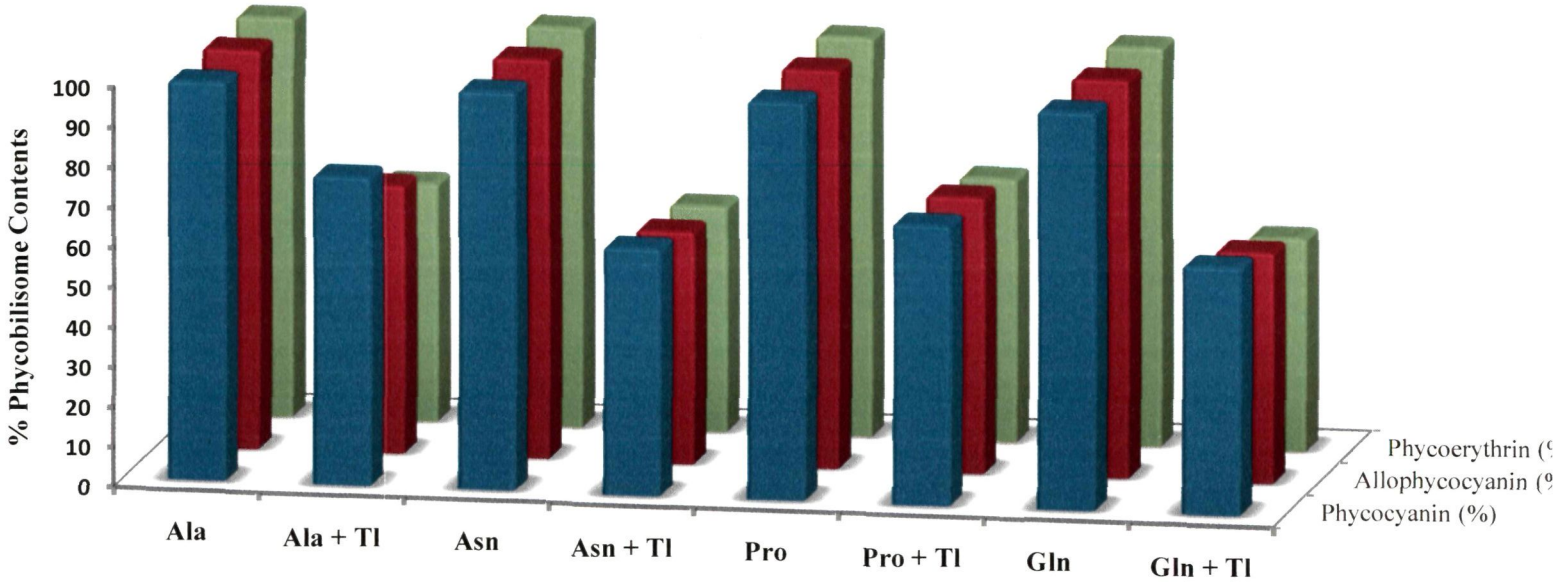


Fig 3.28: Effect of 0.5µM thallium sulphate on phycobilisome contents (phycocyanin, allophycocyanin & phycoerythrin) in *Anabaena cycadae* cells grown in 1mM alanine (ala), asparagine (asp), proline (pro) or glutamine (gln) supplemented BG-11_0 medium at 24±2°C. The PC, APC and PE contents in $\mu g.ml^{-1}$ were determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values were plotted in percent protection taking the control cultures values as 100% The values represent average measurement of two replicates each of two independent experiments.

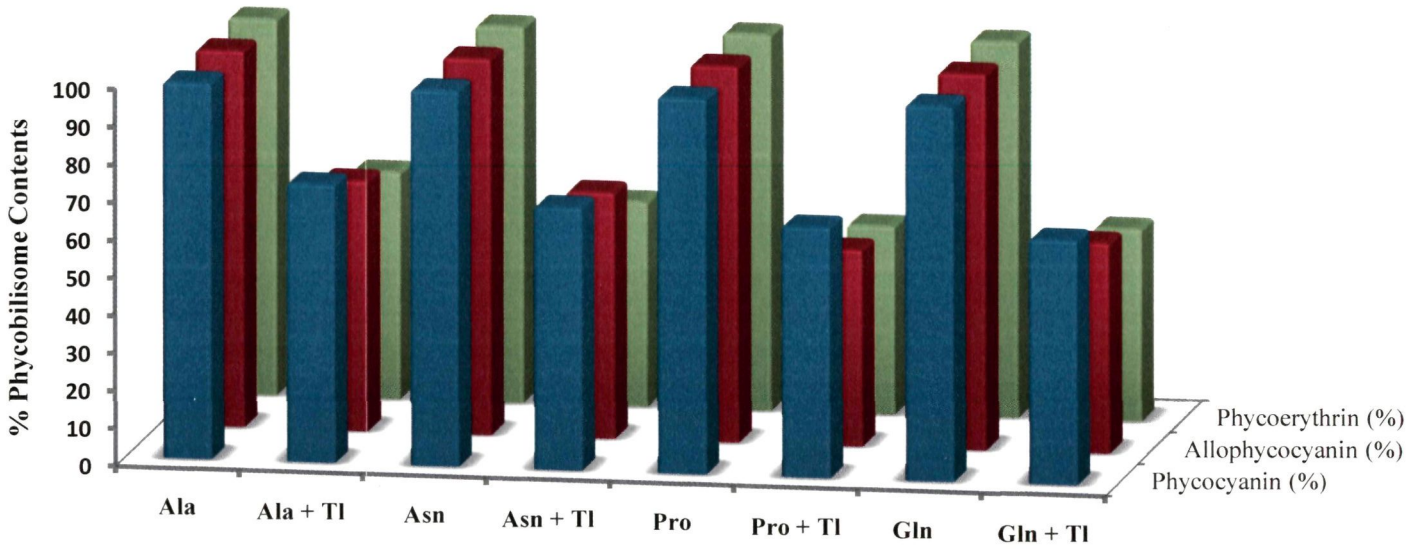


Fig 3.29: Effect of 0.5µM thallium sulphate on phycobilisome contents (phycocyanin, allophycocyanin & phycoerythrin) in *Nostoc muscorum* cells grown in 1mM alanine (ala), asparagine (asp), proline (pro) or glutamine (gln) supplemented BG-11_0 medium at 24±2°C. The PC, APC and PE contents in $\mu g.ml^{-1}$ were determined 6 days after inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values were plotted in percent protection taking the control cultures values as 100%. The values represent average measurement of two replicates each of two independent experiments.

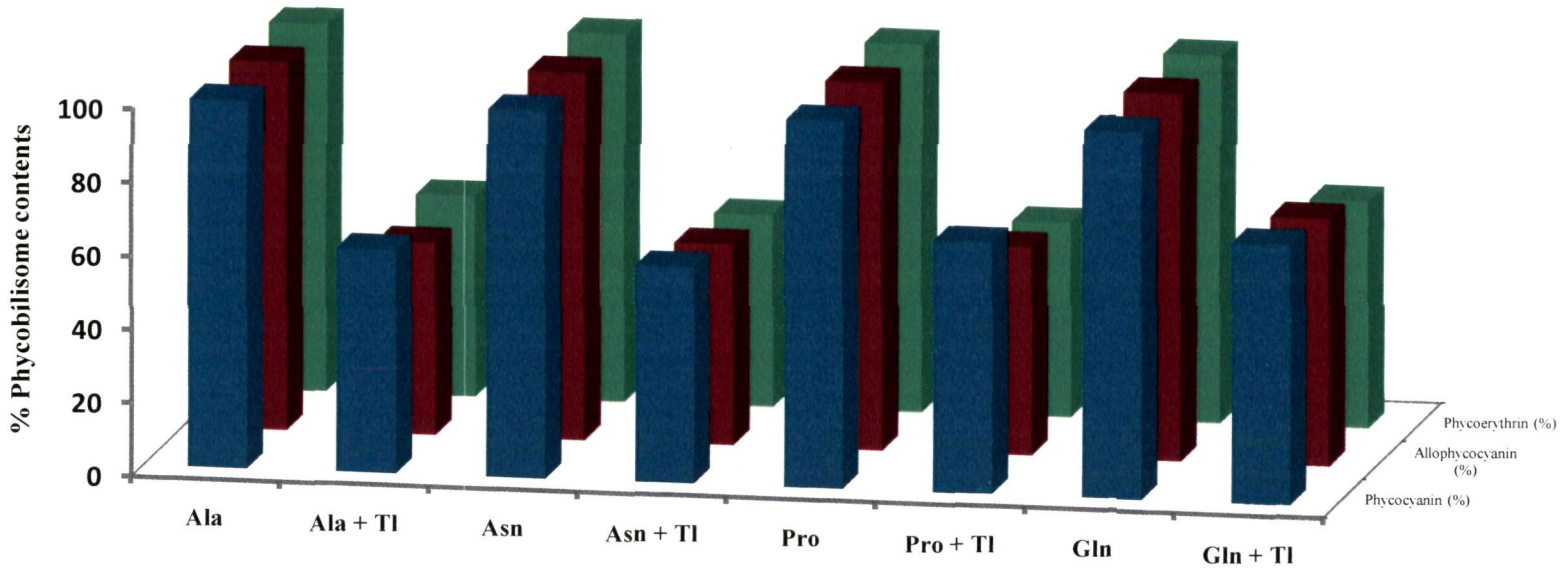


Fig 3.30: Effect of 0.5µM thallium sulphate on phycobilisome contents (phycocyanin, allophycocyanin & phycoerythrin) in *Anaba variabilis* cells grown in 1mM alanine (ala), asparagine (asp), proline (pro) or glutamine (gln) supplemented BG-11_0 medium at 24±2 The PC, APC and PE contents in $\mu g.ml^{-1}$ were determined 6 days after inoculation. Experimental samples were inoculated at an initial Cl concentration of 0.4 $\mu g.ml^{-1}$ by using exponentially growing cyanobacterial cells in N_2 medium. The values were plotted in perc protection taking the control cultures values as 100%. The values represent average measurement of two replicates each of two independ experiments

3.5 Thallium uptake activities:

The thallium uptake activity in cyanobacterial strains *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Mastigocladus sp.* grown in N_2, nitrate, ammonium or amino acids namely glutamine, proline, asparagine and alanine supplemented media was estimated. Exponentially grown cyanobacterial cells were harvested by centrifugation, washed twice with 10 mM HEPES – NaOH buffer (pH 7.0), resuspended in the same and equilibrated for 30 mins at 24±2°C under photoautotrophic growth conditions. $^{204}Tl^{2+}$ labelled thallium was then added to the cell suspension to a final concentration of 20 µM (specific activity of 194.1 Bq μmol^{-1}). Samples were taken at regular intervals and radioactivity was counted as described in materials and methods. The intracellular ^{204}Tl accumulation was expressed as Bq.μg^{-1} Chl *a*.

In N_2 medium, overall pattern of $^{204}Tl^{2+}$ uptake showed a biphasic pattern. Such a $^{204}Tl^{2+}$uptake pattern was marked by an initial rapid phase lasting for about 10 mins followed by subsequent slower second phase showing very little increase in intracellular radioactivity. Subsequent decrease in the intracellular level of thallium after addition of 2mM K^+ was also observed. This suggests that K^+ is an antagonist to thallium in the cyanobacterium. The uptake kinetics of thallium was similar for all four cyanobacterial strains. The first rapid phase which lasted for 10 mins showed uptake rate of 2.6 Bq μg^{-1} Chl *a*.min^{-1} in *Nostoc* ANTH (fig 3.31a) followed by 4.64 Bq μg^{-1} Chl *a*.min^{-1} in *Anabaena cycadae* (fig 3.31b), 4.8 Bq μg^{-1} Chl *a*.min^{-1} in *Nostoc muscorum* (fig 3.32a) and 1.96 Bq μg^{-1} Chl *a*.min^{-1} in *Mastigocladus* sp. (fig 3.32b) respectively.

The thallium uptake pattern was found to be similar in cyanobacterial cells grown in nitrate, ammonium or amino acid supplemented medium. The uptake rate were 2.9 Bq μg^{-1} Chl *a*.min^{-1} in *Nostoc* ANTH (fig 3.33a) followed by

2.8 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Anabaena cycadae* (fig 3.33a), 1.5 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Nostoc muscorum* (fig 3.34a) and 1.1 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Mastigocladus* sp. (fig 3.34b) in nitrate grown cells respectively. In ammonium grown cells the uptake rate was found to be 4.4 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Nostoc* ANTH (fig 3.35a) followed by 5.1 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Anabaena cycadae* (fig 3.35b), 6.1 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Nostoc muscorum* (fig 3.36a) and 1.1 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Mastigocladus* sp. (fig 3.36b).

The rate of rapid phase of $^{204}Tl^{2+}$ uptake activity in amino acid supplemented medium was as follows: The uptake rate in proline grown cells was 2.7 Bq μg $^{-1}$ Chl *a* .min^{-1} in *Nostoc* ANTH (fig 3.37) followed by 4.7 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Anabaena cycadae* (fig 3.38) and 3.9 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Nostoc muscorum* (fig 3.39). In glutamine grown cells the uptake rate was found to be 2.9 Bq μg $^{-1}$ Chl *a*.min^{-1} , 4.6 Bq μg $^{-1}$ Chl *a*.min^{-1} and 4.1 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Nostoc* ANTH (fig 3.37), *Anabaena cycadae* (fig 3.38) and *Nostoc muscorum* (fig 3.39) cells respectively.

In asparagnine supplemented growth medium thallium uptake rate was found to be 6.2 Bq μg $^{-1}$ Chl *a* .min^{-1} in *Nostoc* ANTH (fig 3.37) followed by 5.2 Bq μg $^{-1}$ Chl *a* .min^{-1} in *Anabaena cycadae* (fig 3.38) and 4.1 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Nostoc muscorum* (fig 3.39). In alanine supplemented growth medium the uptake rate was 3.6 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Nostoc* ANTH (fig 3.37), 3.4 Bq μg $^{-1}$ Chl *a*.min^{-1} in *Anabaena cycadae* (fig 3.38) and 3.2 Bq μg $^{-1}$ Chl *a* .min^{-1} in *Nostoc muscorum* (fig 3.39).

The uptake pattern in nitrate, ammonium, proline, glutamine, asparagnine grown cells after rapid phase lasting for 10 mins showed a slower second phase with moderate uptake rate suggesting for inability of cells for further assimilation of intracellular thallium. Interestingly intracellular thallium was chased out from into the external medium by subsequent addition of 2mM potassium. These results suggest that

intracellular thallium after intake remains in free form and acts as chemical analogue of potassium. All cyanobacterial strains produced similar pattern of thallium uptake activity except for *Mastigocladus* sp. which showed 50% reduction in activity.

Since addition of K^+ to thallium exposed cells led to extrusion of intracellular thallium, the toxicity thallium at 0.5μM concentration was assessed in the presence of increasing K^+ concentration in the cyanobacterial strains *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Anabaena variablis*. The cultures were inoculated with an initial Chl *a* concentration of 0.4 $\mu g.ml^{-1}$ having increasing concentration of potassium from 0mM to 25 mM concentrations in N_2-medium containing 0.5μM thallium. The effect of potassium concentrations on growth of cyanobacterial strains was determined by measuring changes in chlorophyll *a* content 6 days after inoculation. A K^+ concentration of 25mM was required to spare the cyanobacteria from thallium toxic effects. At the maximum concentration of 25 mM K^+ concentration, the Chl *a* content was found to be 4.25 $\mu g.ml^{-1}$ in *Nostoc* ANTH (fig 3.40) followed by 3.16 $\mu g.ml^{-1}$ in *Anabaena cycadae* (fig 3.41), 2.65 $\mu g.ml^{-1}$ in *Nostoc muscorum* (fig 3.42) and 2.54 $\mu g.ml^{-1}$ in *Anabaena variabilis* (fig 3.43). However, growth in K^+ deficient thallium containing medium was found to be 0.65 $\mu g.ml^{-1}$ in *Nostoc* ANTH followed by 0.32 $\mu g.ml^{-1}$ in *Anabaena cycadae*, 0.24 $\mu g.ml^{-1}$ in *Nostoc muscorum* and 0.29 $\mu g.ml^{-1}$ in *Anabaena variabilis* respectively. These results suggest that toxicity of thallium results due to its interference with K^+ dependent activities in the cells. At the same time since a very high K^+ concentration was needed to protect cyanobacterial cells from thallium toxicity, thallium might be inhibiting other intracellular activities in addition to K^+ dependent processes as well. Additionally affinity of thallium to potassium dependent processes might be more than potassium. However thallium

inability of thallium to substitute for potassium physiologically may be the main cause of its toxic effect(s) in cyanobacteria.

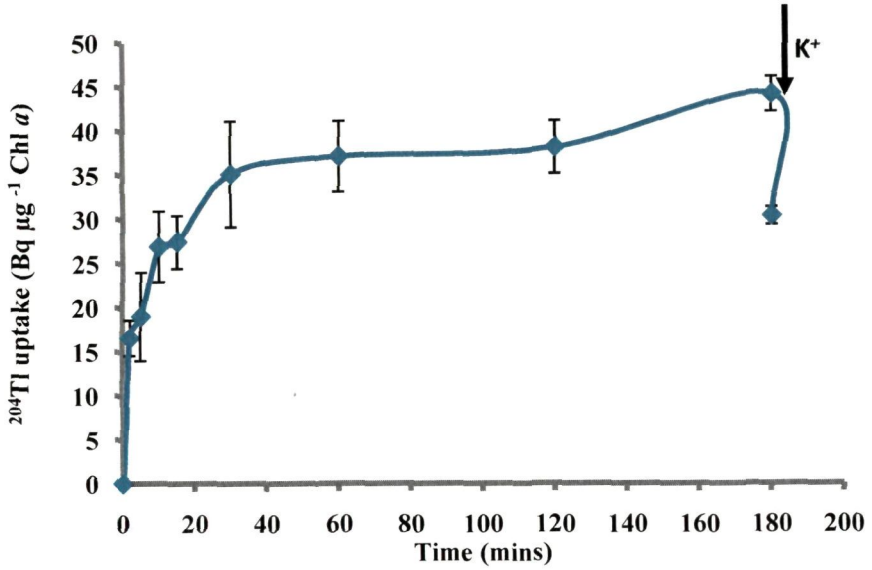


Fig 3.31a

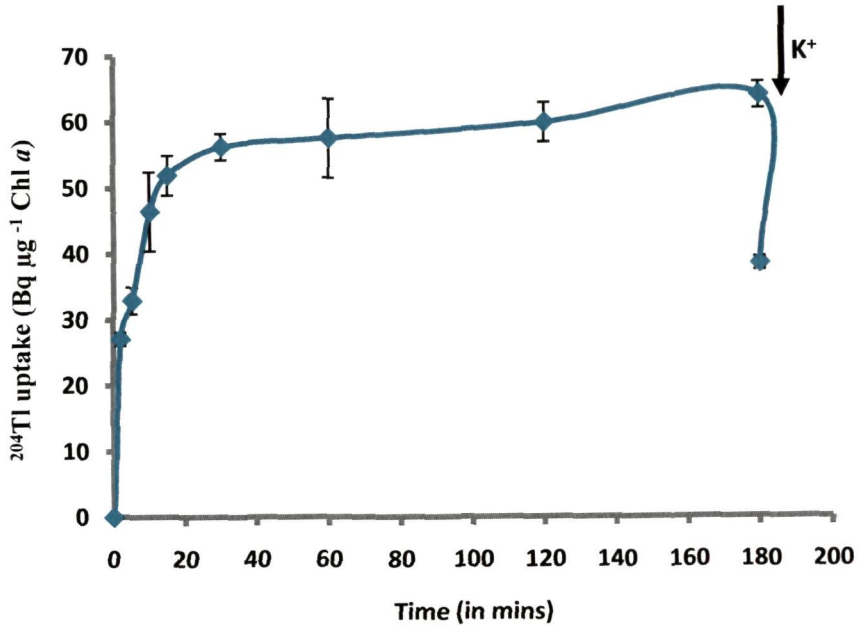


Fig 3.31b

Fig 3.31: Uptake of ^{204}Tl by cells of ***Nostoc* ANTH** (Fig 3.31a) **and *Anabaena cycadae*** (Fig 3.31b) grown in N_2-medium at 24±2°C. Cells were suspended in 10 mM HEPES-NaOH buffer, pH 7.0. Uptake reaction was started by adding ^{204}Tl to a final concentration of 20 µM (specific activity of 194.1 Bq.µmol^{-1}). At different time intervals cells were separated from their bathing medium and intracellular ^{204}Tl label was determined. K^+ was added after three hours of thallium uptake as indicated by (↓). K^+ refers to 2mM KCl added to bathing medium. Values are plotted after subtracting radioactivity incorporated in toluene-treated cells and represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

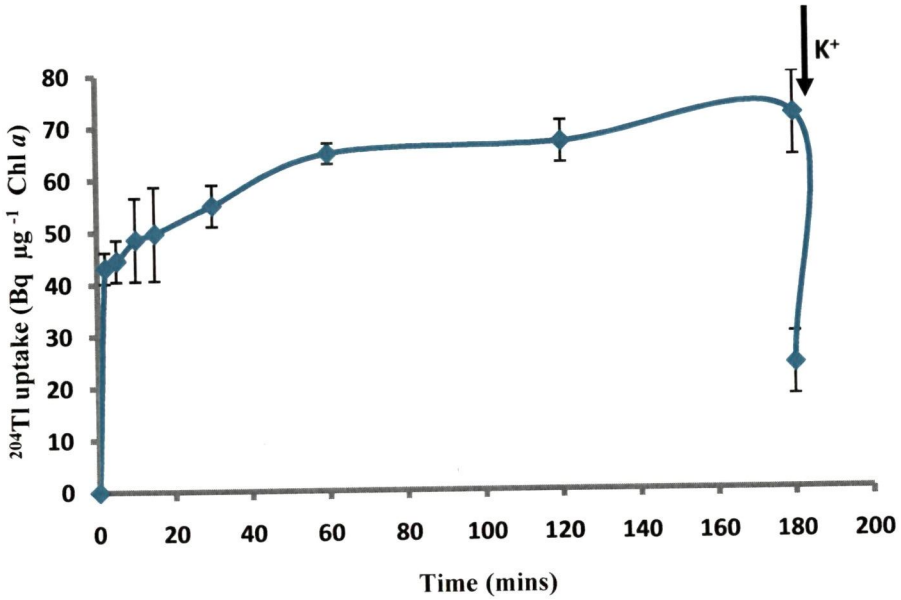


Fig 3.32a

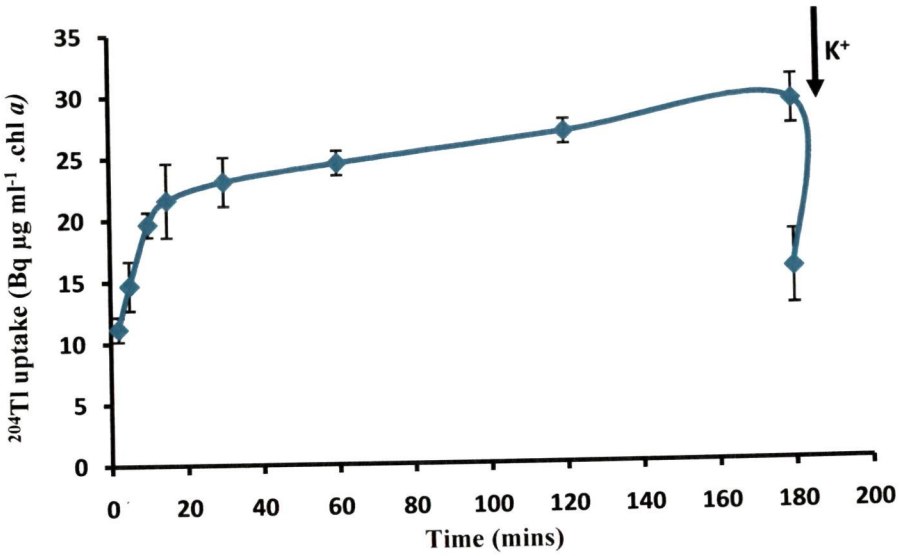


Fig 3.32b

Fig 3.32: Uptake of ^{204}Tl by cells of ***Nostoc muscorum*** (Fig 3.32a) grown in N_2-medium **and *Mastigocladus* sp.** (Fig 3.32b) grown in D-medium at 24±2°C. Cells were suspended in 10 mM HEPES-NaOH buffer, pH 7.0. Uptake reaction was started by adding ^{204}Tl to a final concentration of 20 µM (specific activity of 194.1 Bq.µmol^{-1}). At different time intervals cells were separated from their bathing medium and intracellular ^{204}Tl label was determined. K^+ was added after three hours of thallium uptake as indicated by (↓). K^+ refers to 2mM KCl added to bathing medium. Values are plotted after subtracting radioactivity incorporated in toluene-treated cells and represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

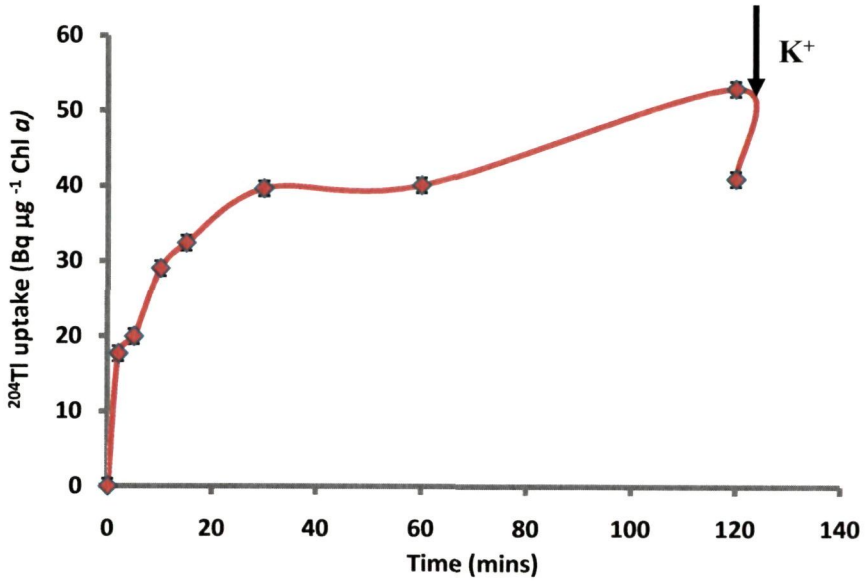


Fig 3.33a

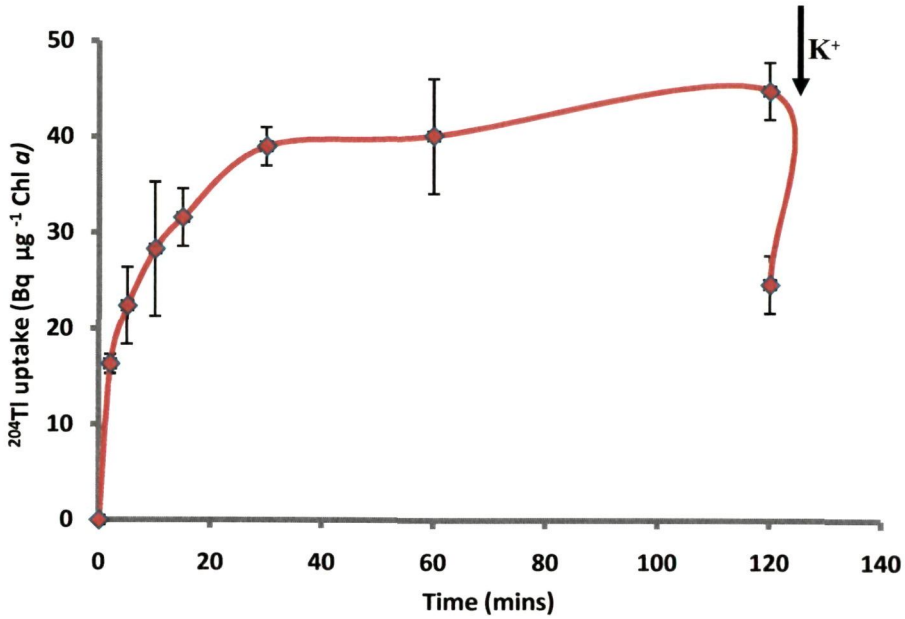


Fig 3.33b

Fig 3.33: Uptake of ^{204}Tl by cells of *Nostoc* ANTH (Fig 3.33a) and *Anabaena cycadae* (Fig 3.33b) grown in NO_3^- medium at 24±2°C. Cells were suspended in 10 mM HEPES-NaOH buffer, pH 7.0. Uptake reaction was started by adding ^{204}Tl to a final concentration of 20 µM (specific activity of 194.1 Bq.µmol^{-1}). At different time intervals cells were separated from their bathing medium and intracellular ^{204}Tl label was determined. K^+ was added after two hours of thallium uptake as indicated by (↓). NO_3^- refers to 5mM $NaNO_3$ added to N_2-medium; K^+ refers to 2mM KCl added to bathing medium. Values are plotted after subtracting radioactivity incorporated in toluene-treated cells and represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

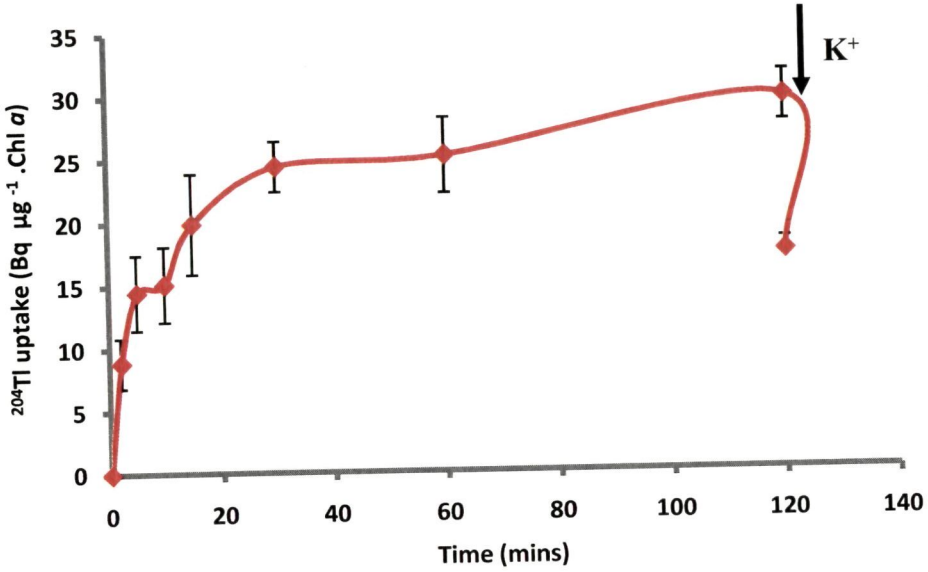


Fig 3.34a

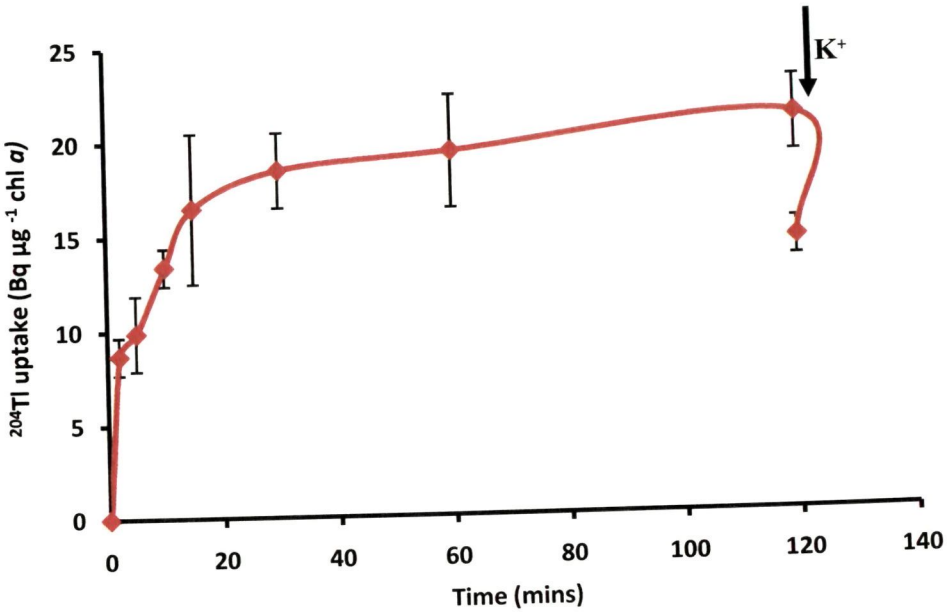


Fig 3.34b

Fig 3.34: Uptake of ^{204}Tl by cells of *Nostoc muscorum* (Fig 3.34a) and *Mastigocladus* sp. (Fig 3.34b) grown in NO_3^- medium at 24±2°C. Cells were suspended in 10 mM HEPES-NaOH buffer, pH 7.0. Uptake reaction was started by adding ^{204}Tl to a final concentration of 20 µM (specific activity of 194.1 Bq.µmol^{-1}). At different time intervals cells were separated from their bathing medium and intracellular ^{204}Tl label was determined. K^+ was added after two hours of thallium uptake as indicated by (⬇). NO_3^- refers to 5mM $NaNO_3$ added to N_2-medium and D-medium; K^+ refers to 2mM KCl added to bathing medium. Values are plotted after subtracting radioactivity incorporated in toluene-treated cells and represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

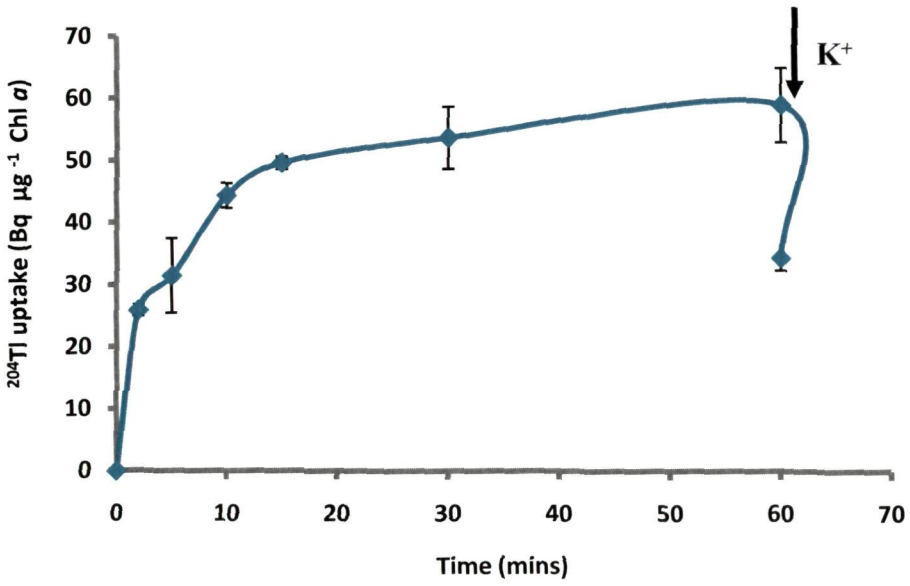


Fig 3.35a

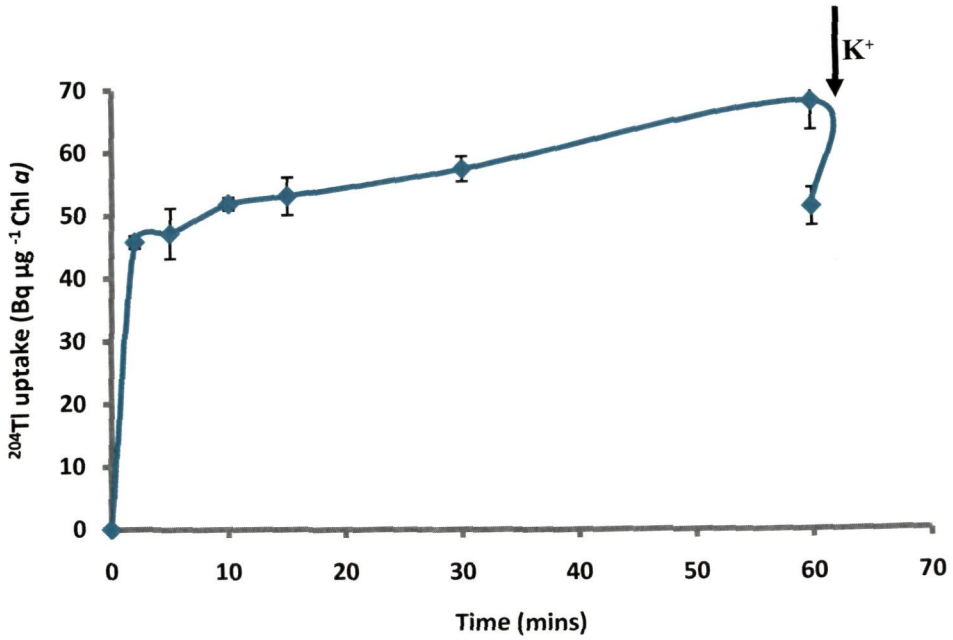


Fig 3.35b

Fig 3.35: Uptake of ^{204}Tl by cells of *Nostoc* ANTH (Fig 3.35a) and *Anabaena cycadae* (Fig 3.35b) grown in NH_4^+ medium at 24±2°C. Cells were suspended in 10 mM HEPES-NaOH buffer, pH 7.0. Uptake reaction was started by adding ^{204}Tl to a final concentration of 20 µM (specific activity of 194.1 Bq.µmol^{-1}). At different time intervals cells were separated from their bathing medium and intracellular ^{204}Tl label was determined. K^+ was added after one hour of thallium uptake as indicated by (↓). NH_4^+ refers to 2mM NH_4Cl added to N_2-medium; K^+ refers to 2mM KCl added to bathing medium. Values are plotted after subtracting radioactivity incorporated in toluene-treated cells and represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

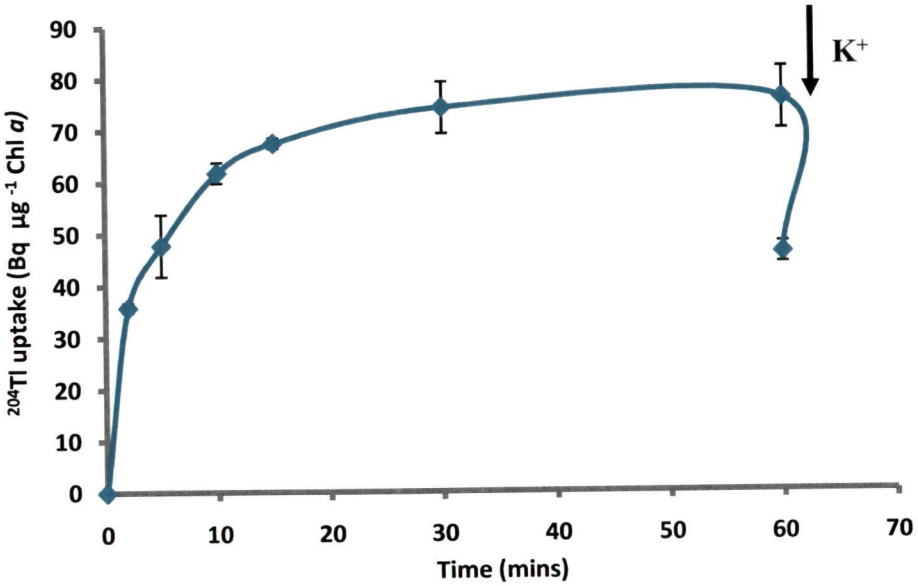


Fig 3.36a

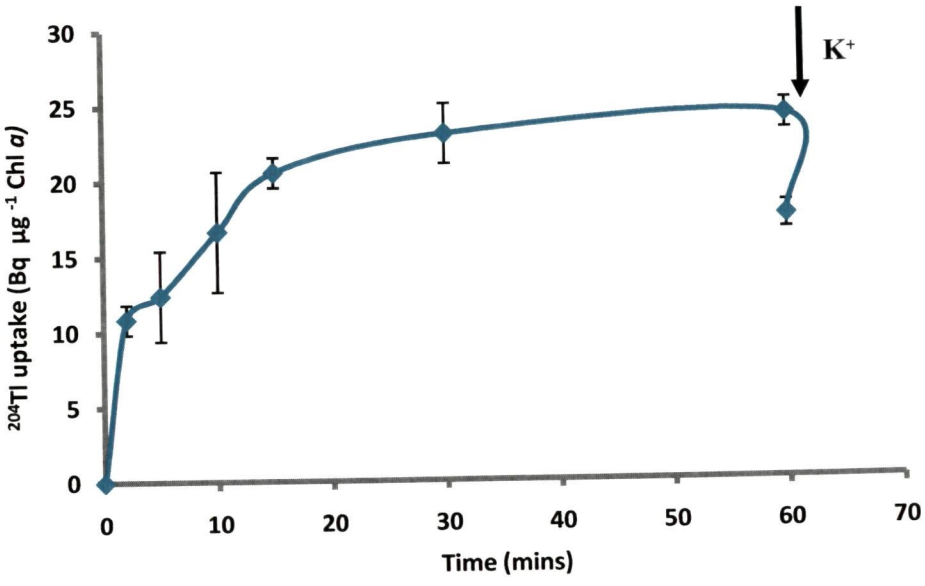


Fig 3.36b

Fig 3.36: Uptake of ^{204}Tl by cells of *Nostoc muscorum* (Fig 3.36a) and *Mastigocladus* sp. (Fig 3.36b) grown in NH_4^+ medium at 24±2°C. Cells were suspended in 10 mM HEPES-NaOH buffer, pH 7.0. Uptake reaction was started by adding ^{204}Tl to a final concentration of 20 µM (specific activity of 194.1 Bq.µmol^{-1}). At different time intervals cells were separated from their bathing medium and intracellular ^{204}Tl label was determined. K^+ was added after one hour of thallium uptake as indicated by (⬇). NH_4^+ refers to 2mM NH_4Cl added to N_2-medium and D-medium; K^+ refers to 2mM KCl added to bathing medium. Values are plotted after subtracting radioactivity incorporated in toluene-treated cells and represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

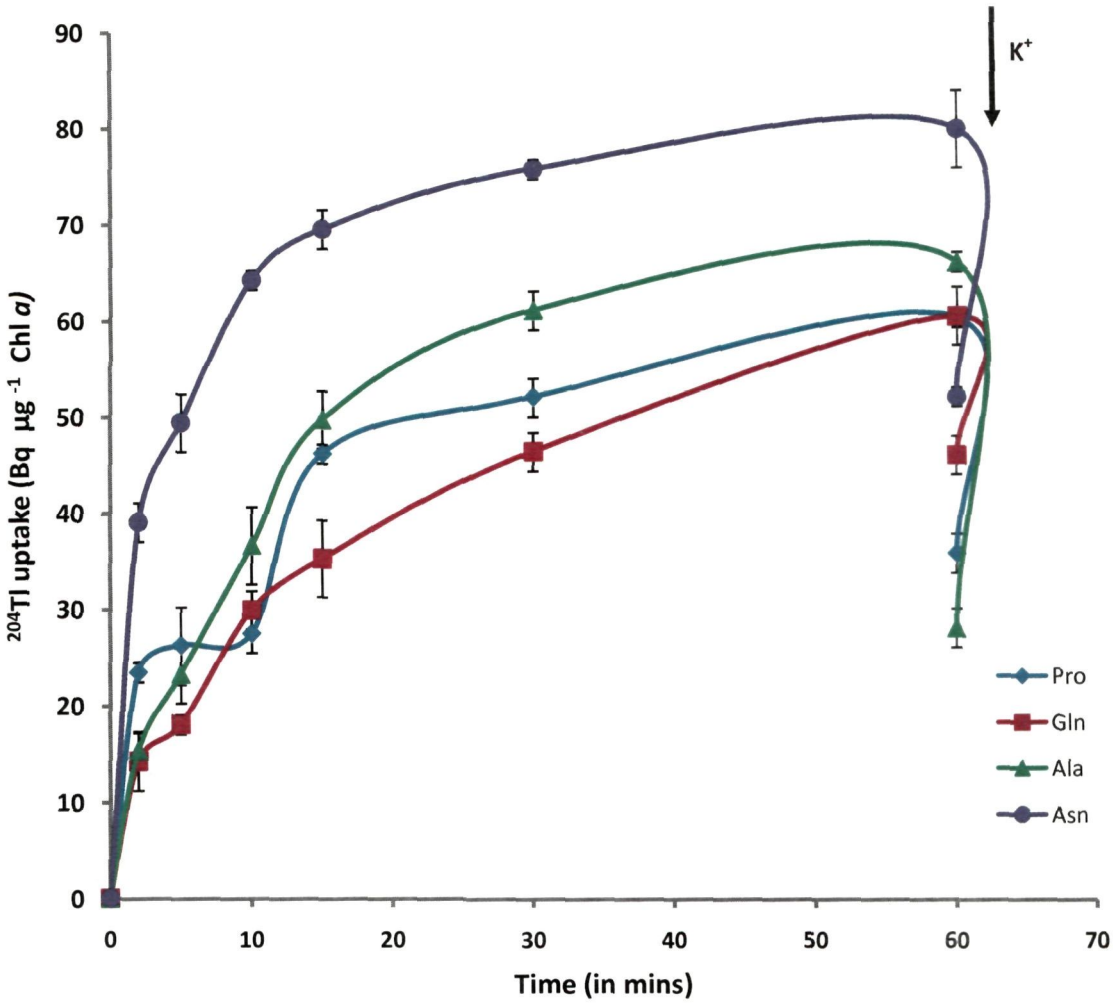


Fig 3.37: Uptake of ^{204}Tl by cells of ***Nostoc* ANTH** grown in N_2- medium supplemented with 1mM proline (♦), alanine (▲), asparagine (●) and glutamine (■) at 24±2°C. Cells were suspended in 10 mM HEPES-NaOH buffer, pH 7.0. Uptake reaction was started by adding ^{204}Tl to a final concentration of 20 µM (specific activity of 194.1 Bq.µmol^{-1}). At different time intervals cells were separated from their bathing medium and intracellular ^{204}Tl label was determined. K^+ was added after one hour of thallium uptake as indicated by (↓). K^+ refers to 2mM KCl added to bathing medium. Values are plotted after subtracting radioactivity incorporated in toluene-treated cells and represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

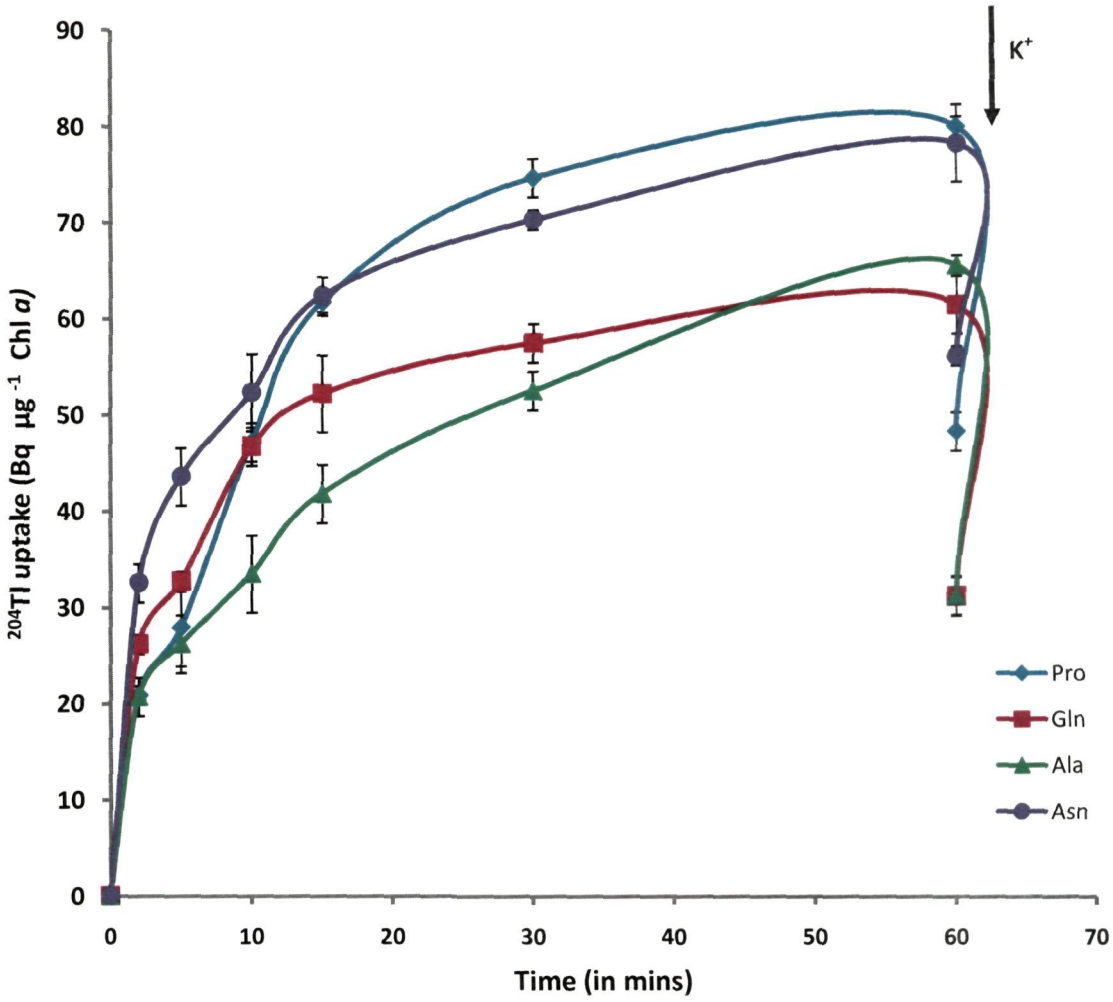


Fig 3.38: Uptake of ^{204}Tl by cells of ***Anabaena cycadae*** grown in N_2- medium supplemented with 1mM proline (♦), alanine (▲), asparagine (●) and glutamine (■) at 24±2°C. Cells were suspended in 10 mM HEPES-NaOH buffer, pH 7.0. Uptake reaction was started by adding ^{204}Tl to a final concentration of 20 µM (specific activity of 194.1 Bq.µmol^{-1}). At different time intervals cells were separated from their bathing medium and intracellular ^{204}Tl label was determined. K^+ was added after one hour of thallium uptake as indicated by (↓). K^+ refers to 2mM KCl added to bathing medium. Values are plotted after subtracting radioactivity incorporated in toluene-treated cells and represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

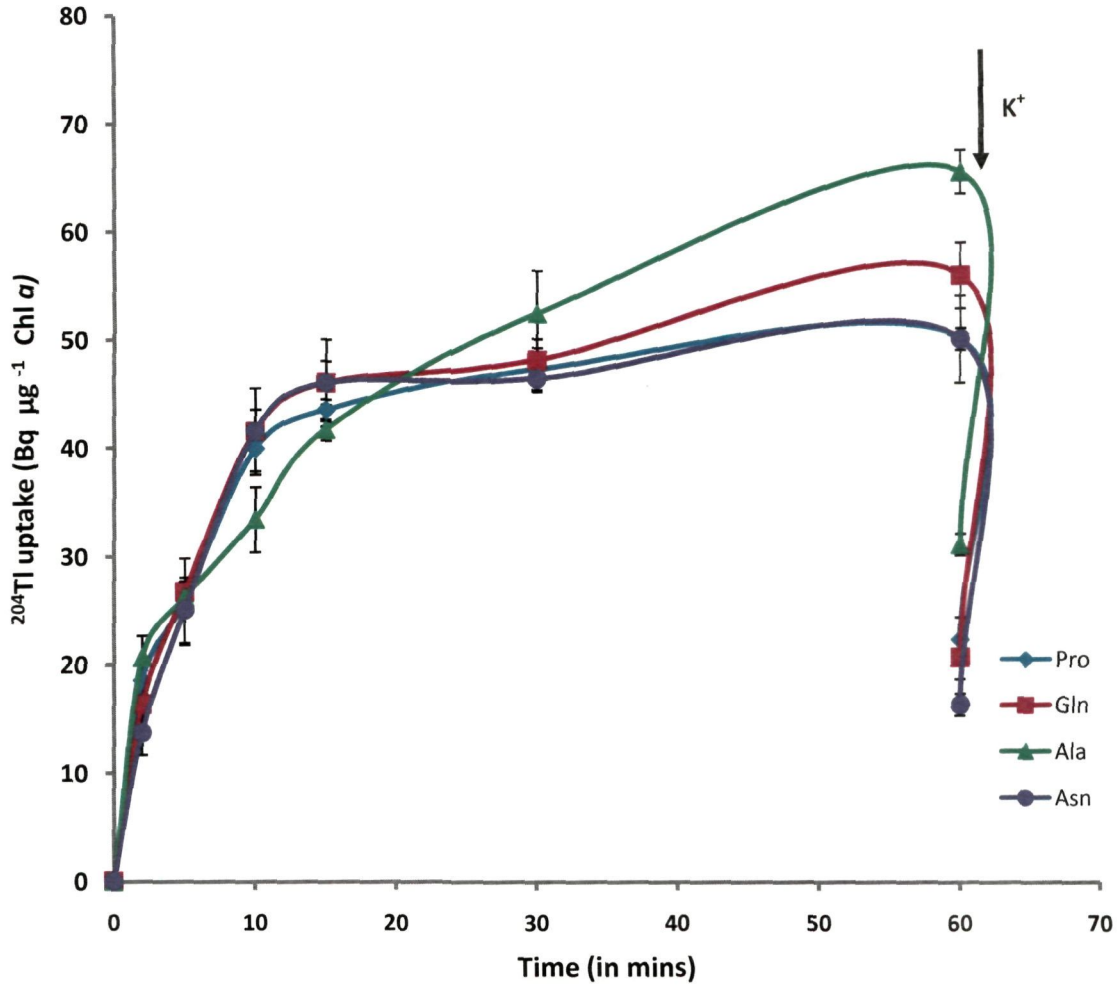


Fig 3.39: Uptake of ^{204}Tl by cells of ***Nostoc muscorum*** grown in N_2- medium supplemented with 1mM proline (♦), alanine (▲), asparagine (●) and glutamine (■) at 24±2°C. Cells were suspended in 10 mM HEPES-NaOH buffer, pH 7.0. Uptake reaction was started by adding ^{204}Tl to a final concentration of 20 µM (specific activity of 194.1 Bq.µmol^{-1}). At different time intervals cells were separated from their bathing medium and intracellular ^{204}Tl label was determined. K^+ was added after one hour of thallium uptake as indicated by (↓). K^+ refers to 2mM KCl added to bathing medium. Values are plotted after subtracting radioactivity incorporated in toluene-treated cells and represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

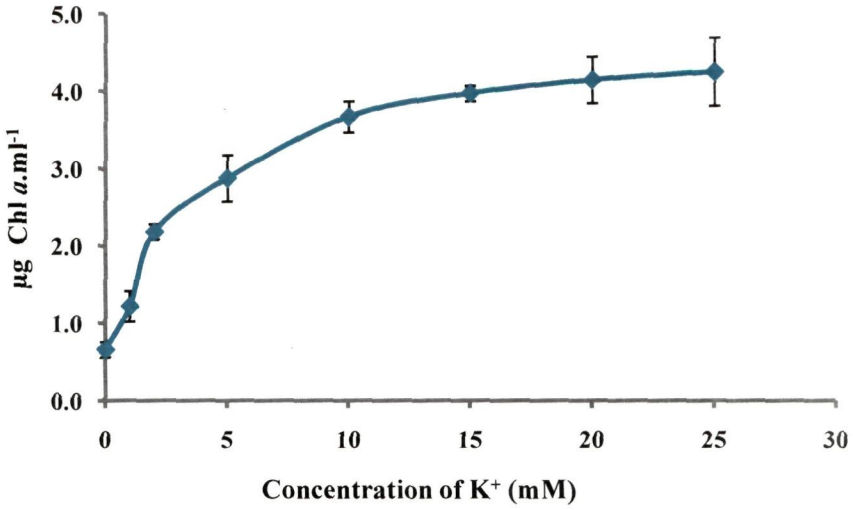


Fig 3.40: Effect of increasing concentration of potassium (K^+) in *Nostoc* ANTH cells grown in N_2-medium containing 0.5µM thallium sulphate at 25±2°C. Growth in terms of µg Chl *a*.ml^{-1} was determined 6 days inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2-medium. K^+ refers to KCl added to N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

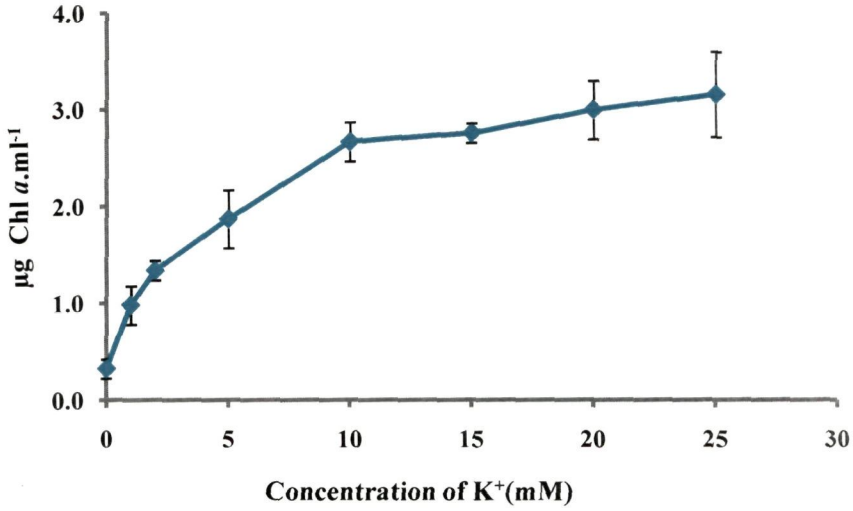


Fig 3.41: Effect of increasing concentration of potassium (K^+) in *Anabaena cycadae* grown in N_2-medium containing 0.5µM thallium sulphate at 25±2°C. Growth in terms of µg Chl *a*.ml^{-1} was determined 6 days inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2-medium. K^+ refers to KCl added to N_2-medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

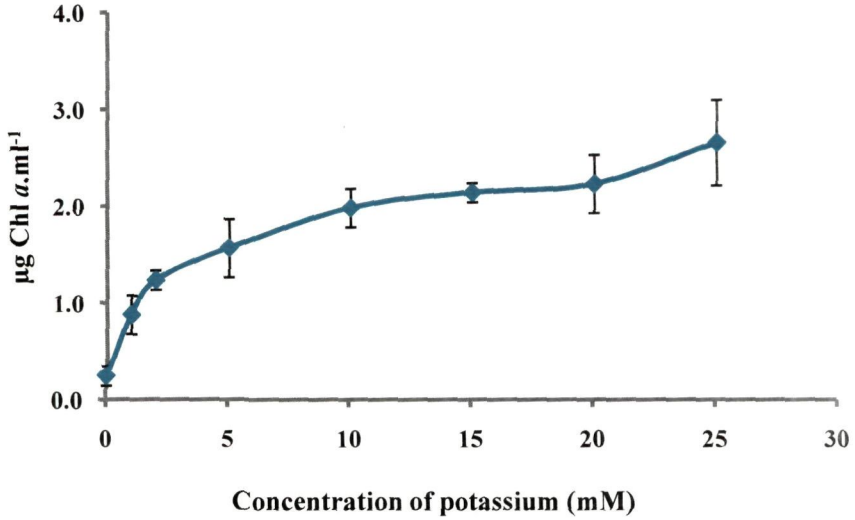


Fig 3.42: Effect of increasing concentration of potassium (K^+) in *Nostoc muscorum* cells grown in N_2-medium containing 0.5µM thallium sulphate at 25±2°C. Growth in terms of µg Chl *a*.ml^{-1} was determined 6 days inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2-medium. K^+ refers to KCl added to N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

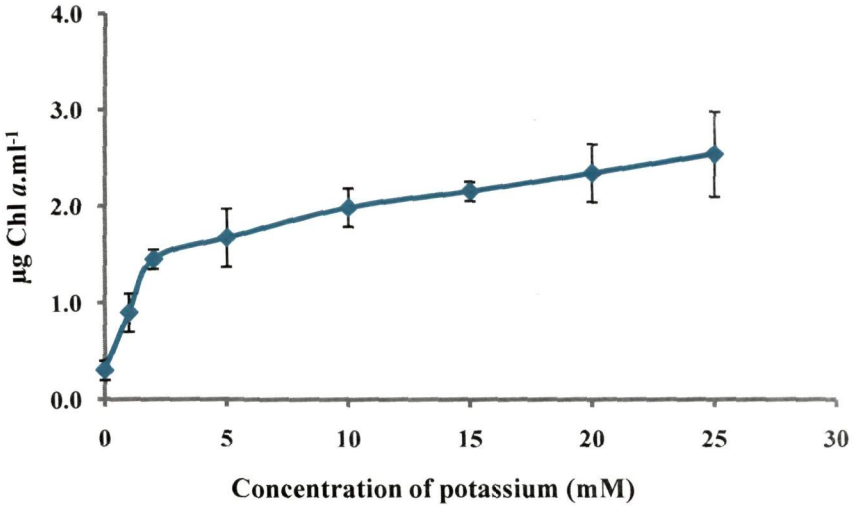


Fig 3.43: Effect of increasing concentration of potassium (K^+) in *Anabaena variabilis* cells grown in N_2-medium containing 0.5µM thallium sulphate at 25±2°C. Growth in terms of µg Chl *a*.ml^{-1} was determined 6 days inoculation. Experimental samples were inoculated at an initial Chl *a* concentration of 0.4 µg.ml^{-1} by using exponentially growing cyanobacterial cells in N_2-medium. K^+ refers to KCl added to N_2- medium. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

3.6 Characterization of thallium resistant mutant strain of *Anabaena cycadae* (Tl-R)

A thallium resistant (Tl-R) mutant strain of *Anabaena cycadae* was isolated by growing cyanobacterial cell in 1.5% agar supplemented N_2–media containing 0.8μM thallium sulphate. After an incubation period of two weeks, pin head colonies were picked up with tooth picks and inoculated in 0.8μM thallium containing liquid N_2-medium. The resistant phenotype of mutant cells was checked and ascertained by growing *Anabaena cycadae* mutant cells in 0.8 μM thallium containing medium after seven generation of growth in thallium deficient media (fig 3.44). The mutant strain was always maintained in N_2-medium containing 0.8μM thallium sulphate. The growth behaviour, heterocyst frequency, GS activity, NR activity, NiR activity, O_2 consumption and evolution activity of mutant strain was determined and compared with wild *Anabaena cycadae*.

The growth behaviour of Tl-R mutant and wild type *Anabaena cycadae* cells in the presence of 0.5μM thallium is shown in fig 3.45 Tl-R mutant exhibited maximum growth in the nitrate medium showing a Chl *a* concentration of 2.86 $\mu g.ml^{-1}$ as compared with the wild type showing Chl *a* content of 0.39 $\mu g.ml^{-1}$. The next maximum growth was observed in mutant cells grown in N_2-medium. The Chl *a* content in mutants was found to be 2.11 $\mu g.ml^{-1}$ and wild type exhibited Chl *a* concentration of 0.33 $\mu g.ml^{-1}$. In ammonium supplemented medium Chl *a* was found to be 1.3 $\mu g.ml^{-1}$ in Tl-R mutants and 0.278 $\mu g.ml^{-1}$ in wild type strains. The growth behaviour studies suggest that the Tl-R strains exhibited better growth as compared with the wild type strains when grown in the presence of 0.5μM thallium.

Heterocyst frequency (%) of mutant strain in N_2- medium was 8±0.5 (table 3.14) in 0.5μM thallium containing media whereas in the medium without thallium heterocyst

frequency was found to be 9±1. Thallium resistant strain like wild type cyanobacterial strains did not produce heterocyst when grown in combined nitrogen source supplemented media (table 4.15 & 4.16).

GS activity of Tl – R mutants with 0.5μM thallium concentration in N_2-medium, nitrate and ammonium supplemented medium was found to be 830.5±19 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein, 916.71±12 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein and 475.21±21 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein. A slight difference in GS activity was observed when compared with mutant cells grown in thallium deficient medium with GS activity level of 965.2±37 nmol - γ - glutamylhydroxamate formed .min^{-1}.mg^{-1}.protein in N_2 medium, 953.4±30 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein in nitrate and 516.89±18 nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein in ammonium medium. The difference was found to be 14% (table 4.14), 3% (table 3.15) and 7.9% (table 3.16).

NR activity of the Tl-R mutant in N_2, nitrate and ammonium medium at 0.5 μM thallium concentrations was found to be 1.65±0.19 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein, 4.92±0.35 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein and 0.31±0.06 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein respectively. NR activity in mutant strain grown in thallium deficient medium was found to be 1.74±0.14 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in N_2 medium, 5.1±0.16 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in nitrate medium and 0.41 ±0.09 nmol NO_2^- formed.min^{-1}.mg^{-1}.protein in ammonium medium which was not significantly different from values obtained in thallium grown cells. The difference was found to be 5% (table 4.14) in N_2-medium, 4% (table 4.15) in nitrate and 24% (table 4.16) in ammonium medium respectively.

NiR activity of the Tl-R mutant in N_2, nitrate and ammonium medium at 0.5 μM thallium concentrations varied by 6% (table 4.14) in N_2-medium, 22% (table 4.15) in nitrate medium and 23% (table 4.16) in ammonium when compared with the mutant cells grown in thallium deficient medium. The NiR activity of mutant strain in thallium deficient medium was 526.5±28 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein in N_2-medium, 598.8±23 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein in NO_3^- medium and 270.4±22 nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein in NH_4^+ medium. These results indicate the capacity of mutant cells to tolerate toxic effect of thallium by maintaining growth, physiological and biochemical activities in thallium treated conditions.

O_2 evolution activities of the Tl-R mutants in response to 0.5μM thallium concentration in N_2, nitrate and ammonium medium was 127.4±21 nmoles O_2 evolved μg^{-1}Chl *a*.hr^{-1}, 199±23 nmoles O_2 evolved μg^{-1}Chl *a*.$hr^{-1}$and 112±20 nmoles O_2 evolved μg^{-1}Chl *a*.hr^{-1}. The mutant cells grown in thallium deficient medium when compared with the Tl-R mutants at 0.5μM thallium concentration showed slight decrease in O_2 evolution rate. The activity in thallium deficient medium was found to be 125.3±10 nmoles O_2 evolved μg^{-1}Chl *a*.hr^{-1}, 188.3±27 nmoles O_2 evolved μg^{-1}Chl *a*.hr^{-1}, 109.3±15 nmoles O_2 evolved μg^{-1}Chl *a*.hr^{-1}in N_2, nitrate and ammonium supplemented medium. The difference in activity was found to be 2% in N_2 (table 4.14) and ammonium medium (table 4.16) and 6% in nitrate medium (table 4.15). Similarly O_2 consumption activities of Tl-R mutants in presence of 0.5μM thallium showed only slight difference of 4% (table 4.14), 5% (table 4.15) and 7% (table 4.16) when compared with the mutant cells grown in thallium deficient medium. The O_2 consumption activity in cells grown in thallium deficient medium was found to be 66.7±7 nmoles O_2 consumed μg^{-1}.Chl *a*.ml^{-1}.hr^{-1}, 67.8±9 nmoles O_2 consumed μg^{-1}.Chl *a*.ml^{-1}.$hr^{-1}$and 50.1±5 nmoles O_2

consumed μg^{-1}.Chl *a*.ml^{-1}.hr^{-1} in N_2, nitrate and ammonium supplemented medium respectively.

The thallium uptake activity of Tl-R mutants grown in N_2-medium was also estimated. Exponentially grown mutant cells were harvested by centrifugation, washed twice with 10 mM HEPES – NaOH buffer (pH 7.0), resuspended in the same and equilibrated for 30 mins at 24±2°C under photoautotrophic growth conditions. $^{204}Tl^{2+}$ labelled thallium sulphate was then added to the cell suspension to a final concentration of 20 μM (specific activity of 194.1 Bq μmol^{-1}). Samples were taken at regular intervals and intracellular ^{204}Tl accumulation was determined and expressed in terms of Bq μg^{-1} Chl *a*.

As shown in fig 3.46, overall pattern of $^{204}Tl^{2+}$ uptake activity in mutant strain showed a biphasic pattern with an initial rapid phase lasting for 10 mins and showing uptake rate of 4.6 Bq μg^{-1}Chl *a*.min^{-1}. The initial rapid phase was followed by a second slower phase which exhibited very slow increase in intracellular thallium level. Subsequent decrease in the intracellular level of thallium after addition of 2mM K^+ was also observed. This suggests again that Tl is a sufficiently similar chemical analogue of K^+ and in mutant phenotype probable mutations of thallium target sites/structures might be the reason for development of thallium resistant phenotype of cyanobacterium *Anabaena cycadae*. Such strains able to grow in presence of thallium and accumulate thallium intracellularly could be an appropriate candidate for bioremediation of thallium from thallium polluted areas in the environment.

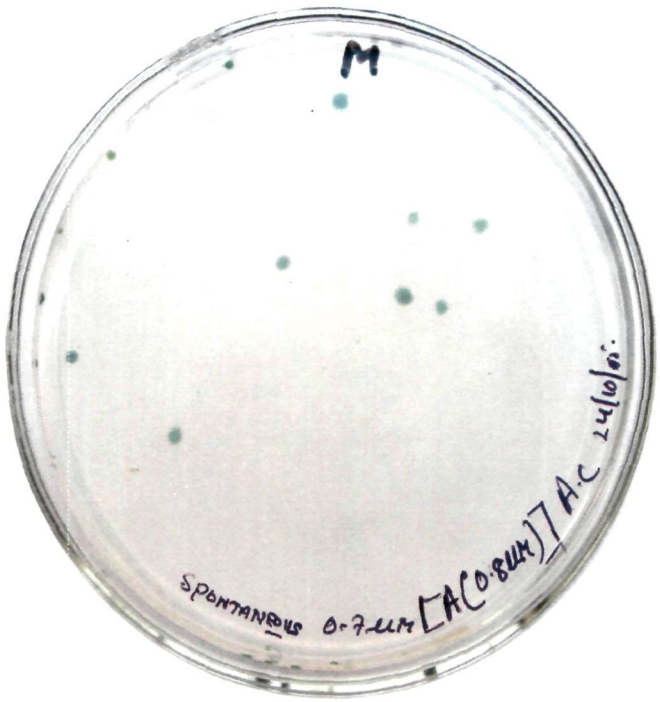


Fig 3.44: Mutant colonies of *Anabaena cycadae* developed in N_2-medium containing 0.8μM thallium with 1.5% agar. The inoculated plates were incubated for 2 weeks in the growth chamber at 24±2°C and a photon fluence rate of 50μmol m^{-2} s^{-1}. The plates were examined for the number of surviving colonies growing on each plate. The pin head colonies were picked up with tooth picks and inoculated in 0.8μM thallium containing liquid N_2- medium. The percentage survival was determined by treating the total number of surviving colonies on control plates as 100%. The resistant phenotype of mutant cells was checked and ascertained by growing *Anabaena cycadae* mutant cells in 0.8μM thallium containing medium after seven generation of growth in thallium deficient media The mutant strain was always maintained in N_2-medium containing 0.8μM thallium sulphate.

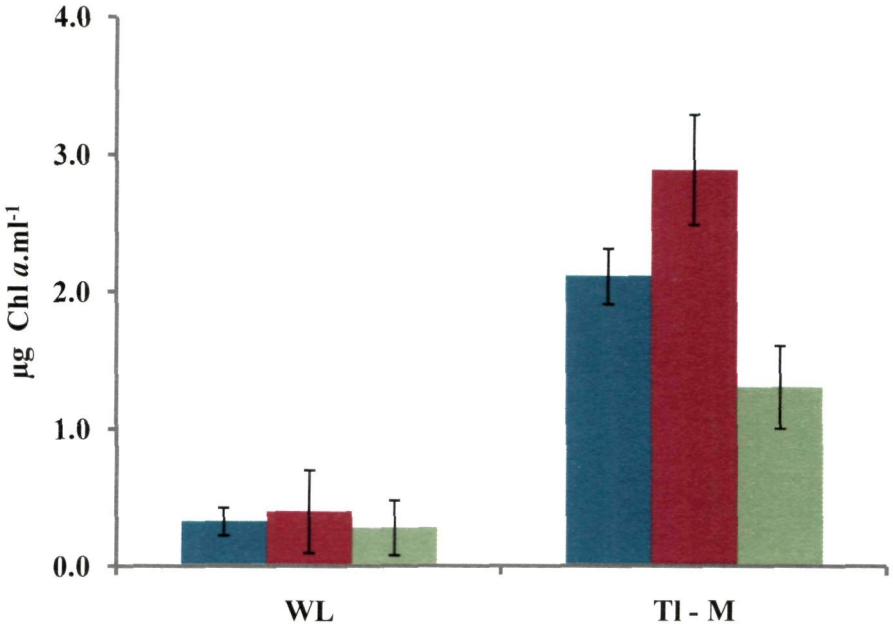


Fig 3.45: Effect of thallium on growth of Tl-R mutant and its wild type *Anabaena cycadae* grown in N_2 (■), NO_3^- (■) and $NH4^+$ (■) medium containing 0.5µM thallium. Growth was determined 6 days after inoculation. N_2 refers to nitrogen free medium, NO_3^- to medium N_2 with 5mM $NaNO_3$, NH_4^+ to medium N_2 with 2mM NH_4Cl as N source. The values represents mean ± standard deviation (SD) from two independent experiments with two replicates each.

Table 4.14

The frequency of heterocyst (per 100 vegetative cells), Glutamine synthetase activity (transferase) (nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}) in of Tl – R mutants of *Anabaena cycadae* cells grown in N_2-medium at 24±2°C and determined 6 days after inoculation.

Characteristics	N_2 medium		
	Tl –R without Tl	**Tl –R with 0.5μM Tl**	*p*
Heterocyst frequency (%)	9 ± 01	8 ± 0.5	< 0.001
Glutamine synthetase (transferase)	965.2 ± 37	830.5 ± 19	< 0.001
Nitrate reductase	1.74 ± 0.14	1.65 ± 0.19	NS
Nitrite reductase	526.5 ± 28	492.7 ± 15	< 0.001
O_2 evolution	125.3 ± 10	127.4 ± 21	< 0.001
O_2 Consumption	66.7 ± 07	69.11 ± 03	NS

The values presented are mean ± standard deviation (SD) from two independent experiments with two replicates each. N_2 refers to nitrogen free medium; '*p*' indicates level of significance, the values (p <0.001) indicates statistically significant and NS not significant values.

Table 4.15

The frequency of heterocyst (per 100 vegetative cells), Glutamine synthetase activity (transferase) (nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}) in Tl – R mutants of *Anabaena cycadae* cells grown in NO_3^- medium at 24±2°C and determined 6 days after inoculation.

Characteristics	NO_3^- medium		
	Tl –R without Tl	**Tl –R with 0.5μM Tl**	***p***
Heterocyst frequency(%)	0.0	0.0	< 0.001
Glutamine synthetase (transferase)	953.4 ± 30	916.71 ± 12	< 0.001
Nitrate reductase	5.1 ± 0.16	4.92 ± 0.35	NS
Nitrite reductase	598.8 ± 23	464.1 ± 09	< 0.001
O_2 evolution	188.3 ± 27	199 ± 23	< 0.001
O_2 Consumption	67.8 ± 09	71 ± 8.9	NS

The values presented are mean ± standard deviation (SD) from two independent experiments with two replicates each. NO_3^- refers to N_2 medium supplemented with 5mM $NaNO_3$; '*p*' indicates level of significance, the values (p <0.001) indicates statistically significant and NS not significant values.

Table 4.16

The frequency of heterocyst (per 100 vegetative cells), Glutamine synthetase activity (transferase) (nmol - γ – glutamylhydroxamate formed.min^{-1}.mg^{-1}.protein), Nitrate reductase activity (nmol NO_2^- formed.min^{-1}.mg^{-1}.protein), Nitrite reductase activity (nmol of NO_2^- consumed.min^{-1}.mg^{-1}.protein), O_2 evolution (nmoles O_2 evolved μg^{-1} Chl *a*.hr^{-1}), O_2 consumption (nmoles O_2 consumed μg^{-1} Chl *a*.hr^{-1}) in Tl – R mutants of *Anabaena cycadae* cells grown in NH_4^+ medium at 24±2°C and determined 6 days after inoculation

Characteristics	**NH_4^+ medium**		
	Tl –R without Tl	**Tl –R with 0.5μM Tl**	***p***
Heterocyst frequency (%)	0.0	0.0	< 0.001
Glutamine synthetase (transferase)	516.89 ± 18	475 ± 21	< 0.001
Nitrate reductase	0.41 ± 0.09	0.31 ± 0.06	NS
Nitrite reductase	270.4 ± 22	208.5 ± 16	< 0.001
O_2 evolution	109.8 ± 15	112 ± 20	< 0.001
O_2 Consumption	50.1 ± 05	53.6 ± 06	NS

The values presented are mean ± standard deviation (SD) from two independent experiments with two replicates each. NH_4^+ refers to N_2 medium supplemented with 2mM NH_4Cl; '*p*' indicates level of significance, the values (*p* <0.001) indicates statistically significant and NS not significant values.

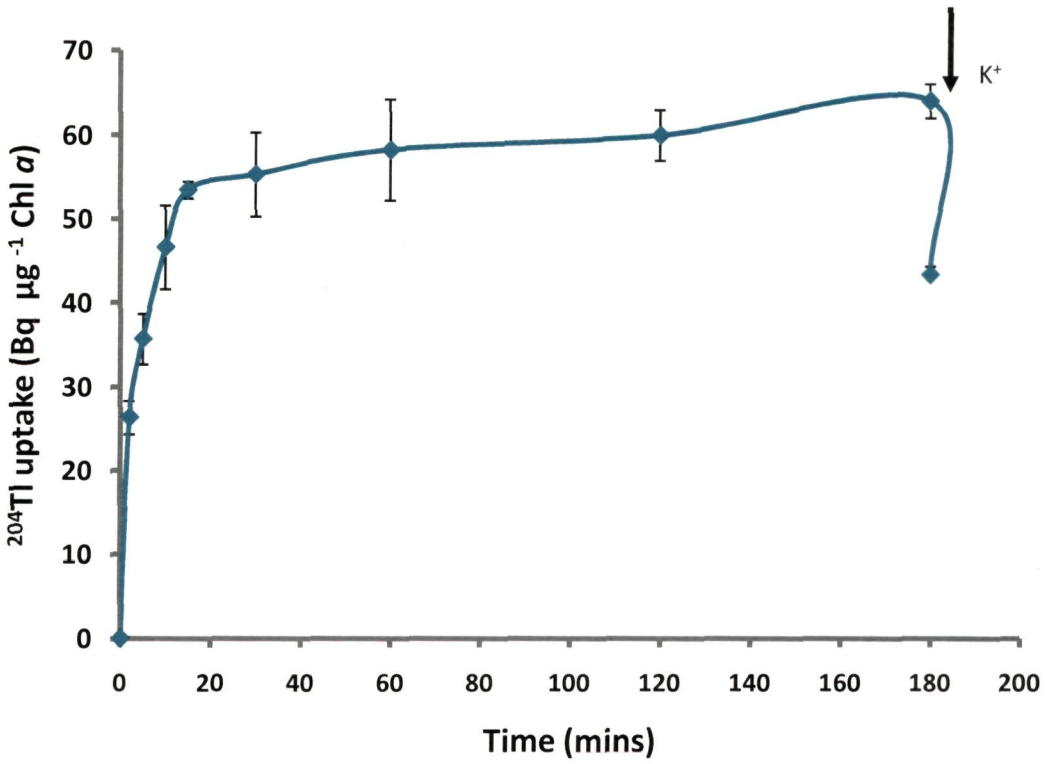


Fig 3.46: Uptake of ^{204}Tl by Tl-R mutant cells of *Anabaena cycadae* grown in N_2 medium at 24±2°C. Cells were suspended in 10 mM HEPES-NaOH buffer, pH 7.0. Uptake reaction was started by adding ^{204}Tl to a final concentration of 20 µM (specific activity of 194.1 Bq.µmol^{-1}). At different time intervals cells were separated from their bathing medium and intracellular ^{204}Tl label was determined. K^+ was added after one hour of thallium uptake as indicated by (↓). K^+ refers to 2mM KCl added to bathing medium. Values are plotted after subtracting radioactivity incorporated in toluene-treated cells and represent mean ± standard deviation (SD) from two independent experiments with two replicates each.

4. DISCUSSION

CHAPTER - 4

DISCUSSION

Removal of heavy metals contaminants from environment has now become established technique in the bioremediation of industrial effluents. Selection of the requisite organisms depends on the metal accumulation potential, metal sensitivity, stability under harsh environmental conditions and simpler nutritional requirements (Davis *et al.*, 2000). Cyanobacterial cells have a remarkable ability to take up and accumulate heavy metals from their external environment (De Filippis & Pallaghy, 1994). In the present study cyanobacteria has been chosen as a suitable group for testing their potential to accumulate toxic metal cations because of their autotrophic mode of nutrition and ability to adjust in changing environment over the other group of microbes (Fogg, 1982). The various cyanobacterial strains *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum* and *Mastigocladus* sp. were tested for their capacity to grow in thallium containing environment. Thallium was found to be equally and extremely toxic to all five cyanobacterial strains with 0.5μM being lethal dose concentration. Results of lethal dose concentration in terms of decrease in chlorophyll *a* content are shown in Fig 3.1, 3.2, 3.3, 3.4 & 3.5. Toxicity of heavy metal to cyanobacteria has been very well researched. Asthana *et al* (1999) have reported findings on the Ni^{2+} toxicity to growth behaviour in *Nostoc muscorum*. Similar reports are well established on toxicities studies of Cu^{2+} in *Anabaena* sp. (Laube *et al.*, 1980), Cd^{2+} in *Nostoc muscorum* (Singh & Pandey, 1981), Ni^{2+} & Zn^{2+} toxicity in *Nostoc muscorum* (Rai *et al.*, 1990), metal induced inhibition of photosynthesis & cell division (Conway, 1978), interaction of metals with nucleic

acids, competition for enzyme binding site (Singh & Pandey, 1981), and N_2 fixation (Rai & Raizada, 1986).

Combined nitrogen sources such as nitrate and ammonium supports better growth of cyanobacteria (Ripikka *et al.*, 1979). The toxicity of heavy metals is an independent phenomenon and not regulated by combined nitrogen source however in context to present study the growth was inhibited in the combined nitrogen sources medium. However, in contrast one such study by Singh *et al* (1994) on cesium toxicity was found to be regulated by nitrogen sources in the media and presence of nitrate or ammonium in the medium protected *Nostoc muscorum* from toxic effect of monovalent alkali cation i.e. Cs^+.

Utilization of amino acids as nitrogen source by an organism depends on its ability to transport and degrade them by specific pathways to generate ammonia for assimilation as nitrogen source. This aspect of nitrogen regulation has been studied in enteric bacteria in some detail. Some cyanobacteria have been shown to be able to take up some amino acids (Flores & Herrero, 1994) for their growth. Amino acids like alanine, asparagnine and glutamine could be used as a sources of nitrogen by a number of cyanobacteria (Flores & Herrero, 1994), while arginine has been described to serve as a source of carbon in *Synechocystis* strain PCC 6308 (Weathers, 1978). However, the role of amino acids in regulation of heavy metal toxicity has not been studied extensively. Amino acids are very selective in supporting cyanobacterial growth. A particular amino acid can be toxic to one cyanobacterium while supporting growth of other strains (Flores, 1983; Flores & Herrero, 1994). Several reports depict that the sulphur containing amino acids decrease the heavy metal toxicity (Singh & Pandey, 1981; Rai, 1990). The present

study reveals growth of cyanobacteria in amino acid supplemented medium containing lethal dose concentration of thallium. Majority of amino acids were found growth inhibitory whereas alanine, glutamine, asparagine and proline were found to be growth stimulatory and reduced the toxic effects of thallium to cyanobacteria. Aromatic amino acids tyrosine, tryptophan & phenyl alanine could reduce thallium toxicity slightly in *Nostoc* ANTH, *Anabaena* cycadae, *Nostoc muscorum* and *Anabaena variabilis*. Protection of cyanobacteria from metal stress in the presence of alanine, glutamine, asparagine and proline could be used to generate amino acid mutant overproducing strains for their use in bioremediation of metal cations.

The effect of heavy metals on light harvesting complex (LHC) *i.e.* phycobillisome contents - Phycocyanin (PC), Allophycocyanin (APC) and Phycoerythrin (PE) of cyanobacteria has been reported and are composed of chromophoric and non-chromophoric proteins arranged in a definite order to maximize energy trapping and eventually channelling it to PSII reaction centre (Patterson, 1995). The phycobilisomes pigments absorb light in the wavelength range of 500-650 nm and transfer energy to the Chl *a* for photosynthesis. Energy transfer with the phycobilisome typically occurs in 100-200 ps efficiently over an array of several hundred chromophores. The light is harvested by the phycobilisome pigments and transferred to the reaction center (RC) in the following sequence: PC→APC →PE→ Chl *a* (Bald *et al.*, 1996). The energy transfer within the phycobilisome pigments could be influenced by several environmental factors like low temperature, high temperature (Li *et al.*, 2001; Li *et al.*, 2004; Murthy *et al.*, 2004; Wen *et al.*, 2005), heavy metals salt stress (Sudhir *et al.*, 2005) and state transition (Li *et al.*, 2004). Metals are known to interfere with the light trapping molecules present in the thylakoids and affect the production of phycobilisome

content (Yamanka *et al.*, 1980). Present study reveals the inhibitory effect of heavy metal thallium in production of phycobilisome contents with a decrease from 71% to 95% when compared to control cultures (Fig 3.6, 3.7, 3.8, 3.9 and 3.10). These finding are consistent with similar reports involving effect of thallium to phycobilisome in *Synechocystis* sp PCC6803 (Aoki *et al.*, 2008) and *Anacystis nidulans* (Gupta & Singhal, 1996) where reduction in the PC, APC and PE content in response to thallium exposure occurred and in a range of 71% to 79%. Cyanobacterial strains in amino acids glutamine, alanine, proline and asparagine supplemented medium also showed reduction in thallium toxicity at 0.5μM concentration measured as changes in phycobilisome contents.

In the absence of combined nitrogen source, free living cyanobacteria develop regularly spaced heterocyst which represents 5-10% of the total cell population in the cyanobacterial filament. Heterocysts are the sites of nitrogen fixation and provide oxygen protection to nitrogenase (Gallon, 1992). The N_2 fixation in heterocyst is mediated by the nitrogenase enzyme (Bergman, 1981) and therefore requires a great deal of chemical energy, released from the hydrolysis of ATP and many redundant pools. The effect of heavy metals on heterocyst frequency has been reported earlier on toxic effect of Cu^2 in *Anabaena* sp. (Laube *et al.*, 1980), Cd^{2+} and Cu^{2+} in *Ankistrodesmus braunii* and *Anabaena* 7120 (Massalski *et al.*, 1981), Cd^{2+}, Ni^{2+}, Hg^{2+} and Cr^{2+} in *Nostoc muscorum* (Rai & Raizada, 1986), Ni^{2+} and Zn^{2+} in *Nostoc muscorum* (Rai *et al.*,1990). In the present study heterocyst frequency in cyanobacterial filaments decreased ranging from 67% to 7 % in the N_2 medium and no heterocyst formation was observed in the inorganic combined nitrogen sources medium. The effect of thallium was not only observed in heterocyst frequency but also in nitrogenase enzyme activity. Nitrogenase is one of the most sensitive

enzymes which respond quickly to changes in environment e.g. to metal pollution by inhibiting it drastically Heavy metals cause nitrogenase inhibition mainly by depleting cellular ATP level and reductant pools available to nitrogenase. Heavy metals which act in this way are Hg^{2+} (Strattion & Huber, 1979) and Cu^{2+} (Wurtsbaugh & Horne, 1982). The sources of ATP and reducing equivalents for nitrogenase are provided by photosynthetic activity of the phototrophic organisms (Bergman, 1981). Similar results were also reported on effect of Ni^{2+} toxicity to nitrogenase activity in *Nostoc muscorum* (Asthana *et al.*,1990), heavy metals toxicity on *Anabaena doliolum* (Mallick & Rai, 2006)

Microorganisms assimilate diazotrophically generated NH_4^+ through GS-GOGAT pathway. In addition glutamate dehydrogenase also takes part in assimilation of ammonia using α-ketoglutarate as amino group acceptor. GOGAT pathway is the only route of ammonia assimilation resulting from N_2 fixation, nitrate assimilation or exogenous supply in heterocystous cyanobacteria (Stewart & Rowell, 1975), in *Azotobacter vinelandii* (Kleiner, 1975), in *Rhodobacter capsulatus* (Johansson & Gest, 1976). In heterocystous cyanobacteria growing diazotrophically heterocyst is the site of aerobic N_2 fixation and glutamine production whereas vegetative cells are the site of glutamate production (Thomas *et al.*, 1977). Presence of glutamate synthetase activity in vegetative cells but not in heterocyst has been found to be reason for localization of glutamate production in vegetative cells during cyanobacterial diazotrophic growth (Verma *et al.*, 1990). Presence investigation revealed drastic inhibition of GS activity in thallium grown cyanobacterial cells. It is worth mentioning an observation of IP *et al.*, (1983) showing inhibition of GS in cultures exposed to Hg^{2+} and Cd^{2+} in the medium. Similar reports were also presented on Cu^{2+} toxicity in *Anabaena* sp. (Laube *et al.*, 1980), Ni^{2+} & Zn^{2+} toxicity

in *Nostoc muscorum* (Rai *et al.*,1990), effect of heavy metals on nitrogen assimilation of *Anabaena doliolum* (Mallick & Rai, 2006) which correlates the present study with thallium toxicity.

Assimilatory N reduction, performed by bacterial and eukaryotic organisms (Solomonson & Barber, 1990; Zvyagilskaya *et al.*, 1996) includes the reduction of nitrate and nitrite compounds, often mediated by nitrate or nitrite reductase enzymes, for synthesis of amino acids. NR, a very crucial enzyme catalyzes the reduction of nitrate to nitrite, the first step in the assimilatory reduction of nitrate (Campbell & Campbell, 1998). Ammonium (NH_3), the end product of the nitrate-reducing pathway, behaves as a very effective antagonist of nitrate assimilation (Losada & Guererro 1979, Manzano *et al.,* 1976). In addition, NiR enzyme catalyzes the six-electron reduction of nitrite form by NR enzyme to ammonia, using reduced ferredoxin (Fd) as the electron donor. The Fd-dependent NiRs of plants, algae and cyanobacteria are monomeric proteins (Dose *et al*, 1997). Heavy metals stress or high salinity interferes with the metabolism of inorganic and organic compounds. In combination NR and NiR enzyme facilitate conversion of oxidized form of nitrogen into its most reduced state i.e. NH_3 which is incorporated in amino acids. These pathways are severely affected under stress; however inhibition level may vary from organism to organism (Incharoesankdi, 2006). Tripathy *et al* (2004) have reported that at varying concentration of heavy metals e.g. Ni^{2+}, Zn^{2+}, Cd^{2+}, ammonia could not be assimilated via nitrate reductase enzyme due to NR & NiR inhibition. Thallium was also found to inhibit both NR and NiR activity in the cyanobacteria used in the present investigation. The inhibition in NR activity ranged from 50% to 76% and NiR from 58% to 79% in various combined nitrogen sources. Similar results have been reported involving NR and NiR activity inhibition in response to

Cu^{2+} toxicity in *Anabaena* sp. (Laube *et al.*, 1980), Ni^{2+} and Zn^{2+} toxicity in *Nostoc muscorum* (Rai *et al.*,1990), Cu^{2+} and Ni^{2+} toxicity *Chorella vulgaris* (Mallick *et al.*, 1994), Cr^{2+} toxicity in *Aulosira fertilissima*g (Bannerjee *et al.*, 2004), heavy metals effect on NR & NIR in *Anacystis nidulans* & *Chlorella vulgaris* (Awasthi, 2005) and heavy metals toxicity in *Anabaena doliolum* (Mallick & Rai, 2006)

The photosynthetic electron transport chain of oxygenic organisms has been reviewed extensively (Hippler *et al.*, 1998; Whitmarsh, 1998). Photosystem II (PS II) uses light energy to split water and to reduce the PQ (plastoquinone) pool. Electrons are transported from the PQ pool to the cytochrome b_6f complex and from there to a soluble electron carrier on the luminal side of the thylakoids membrane. In cyanobacteria this soluble carrier may be plastocyanin or cytochrome c_{553}, depending on the species. Either of these soluble one-electron carriers can reduce the oxidized PS I reaction centre chlorophyll, P_{700} centre. The reduced PSI transfers electron to ferredoxin (Fd) and eventually to NADP. The NADP serves as reducing equivalent supporting the dark reaction of CO_2 fixation. Environmental factors such as temperature, UV-light, irradiance, drought, salinity and heavy metals are known to affect photosynthesis in both cyanobacteria and plants. In cyanobacteria, several studies have been conducted on photosynthetic electron transport activities under various stress conditions in whole cells as well as thylakoids membranes (Pucket, 1976; Barman, 2000 and Gambrell, 2004). O_2 evolution and consumption activities in the present study exhibited drastic decrease under thallium treated conditions. The inhibition of O_2 evolution & consumption are also reported in Cr^{2+} toxicity in presence of carbon sources on PS II in selected cyanobacterium (Rai & Dubey, 1988), Ni^{2+} toxicity to O_2 evolution and consumption in *Nostoc muscorum* (Asthana *et al.*, 1990), Ni^{2+} and Zn^{2+} toxicity in *Nostoc muscorum* (Rai *et al.*,1990),

interactive effects of Ca^{2+} & Cd^{2+} on *Nostoc* UAM208 (Fernandez, 1995), Zn^{2+}, Pb^{2+} and Cd^{2+} toxicity on various enzymatic activities of PSI, PSII in *Phragmites australis* (Ye *et al.*, 1997), Cr^{2+} toxicity in *Aulosira fertilissima* (Bannerjee *et al.*, 2004), heavy metals in *Anabaena doliolum* (Mallick & Rai, 2006), Na^{+} stress in *spirulina platensis* (Sudhir *et al.*, 2005), heavy metals effect on phosynthetic pigments in *Anacystis nidulans* and *Chlorella vulgaris* (Awasthi, 2005), ,

Amino acid proline acts as intracellular osmoprotectant under stress conditions e.g. heavy metal stress, osmotic stress, and temperature stress in prokaryotic and eukaryotic organisms by quenching ROS-reactive oxygen species (Farago & Mullen, 1979). A general increase in the level of heavy metals to microbial cultures poses a pervasive threat resulting in production of ROS. The toxic effects of ROS thus produced are prevented by the production of proline that accumulates in eukaryotes, prokaryotes including several cyanobacteria under stress, providing the cells protection against oxidative damages (Ahmad & Hellebust, 1988). Accumulation of proline under stress conditions has been reported profusely (Smirnoff & Cumbes, 1989; Chang, 1991; Bassi & Sharma, 1993; Costa & Morel, 1994; Kavi Kishor *et al.*, 1995; Singh *et al.*, 1996; Schat *et al.*, 1997; Wu *et al.*, 1998).

In other instance many researchers believe that proline accumulation is a symptom of injury which does not confer tolerance against metal or other stresses (Bhaskaran *et al.*, 1985; Lutts *et al.*, 1996). Schat *et al.* (1997) and observed that metal induced proline accumulation did not occur until the damage had been caused and consequently it did not apparently prevent metal toxicity. Present study indicated increase in intracellular proline level in response to thallium treatment. However,

accumulation of proline was not similar in different cyanobacteria and also increase in proline level does emphasize role of proline under heavy metal stress. The maximum increase in proline accumulation was observed in *Nostoc* ANTH with 45 % increase in N_2 medium, 21 % increase in ammonium medium and 14 % increase in nitrate medium. The other organism also showed increases though to a lesser level ranging from 9.1 % to 12.5 % in N_2, nitrate and ammonium medium. Results of present work are in conformation with earlier findings reporting proline accumulation in *Nostoc muscorum* (Rai *et al.*, 1990) under Ni^{2+} and Zn^{2+} treatments. The observation in the present investigation supports the role of intracellular proline to mitigate the toxic effects of ROS. Proline that accumulates heavily in several cyanobacteria under stress, provide protection against damage by ROS in *Spirulina platensis* (Ahmad & Hellebust, 1988). Production of proline in response to heavy metal stress has been studied thoroughly in *Chlorella vulgaris* showing large increase in intracellular proline level when exposed to Cu^{2+}, Cr^{2+}, Ni^{2+} and Zn^{2+} (Mehta & Gaur, 1999). Other reports include increased proline levels under heavy metal stress of Cd^{2+}, Mn^{2+} and Ni^{2+} in *Scenedesmus armatus* (Enany & Issa, 2001), free proline positive correlation with copper tolerance in the chlorophyta *Trebouxia erici* (Backor *et al.*, 2009), increased proline accumulation in Cd^{2+} and Pb^{2+} toxicity under stress in *Westiellopsis prolific* (Fatma *et al.*, 2007), protective role of proline accumulation in response to toxic copper in *chlorella* sp. (Tzong Wu, 1998).

An organism to be used for bioremediation purposes should be able to survive and accumulate toxic compound in polluted ecosystems. In this respect understanding of kinetics of heavy metal uptake activity will provide much needed information for developing any meaningful strategy of bioremediation. Therefore ability to bio-accumulate heavy metal should be treated as an important criteria of

strains for bioremediation purposes (Backor *et al.*, 2009; Ghosh *et al.*, 2008). Although it is difficult to relate data obtained in the laboratory to what is actually occurring under field conditions (Hornstorm *et al*, 1984) but it is obvious from this study that cyanobacteria could well be used as a tool for removing metal cations from thallium contaminated areas. North East region of India which harbours many cement plant and coal mining areas suffers from thallium pollution. Thallium is released mainly due to cement smelting and coal burning operations requires a suitable environmentally friendly technology for bioremediation of thallium released in the open. Knowledge of thallium transport activity in cyanobacteria will provide clue for thallium immobilization in cyanobacterial cells. This study presents data on thallium transport activity in cyanobacteria which was most important objective of the present work.

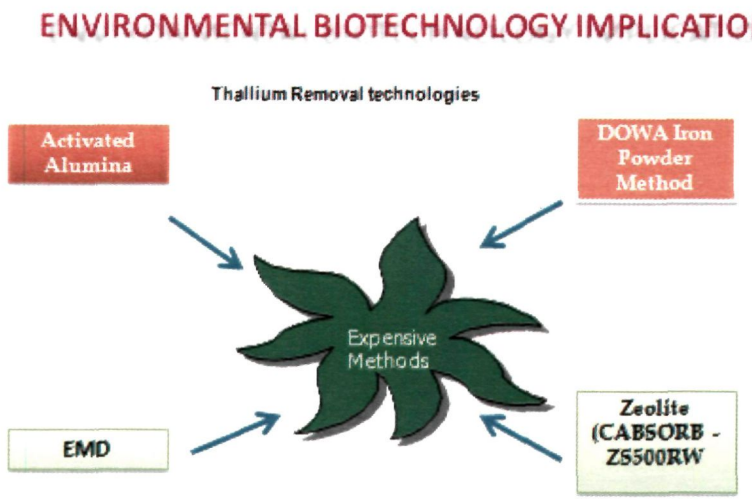
The present study on thallium uptake showed a biphasic pattern in uptake activity which was inhibited by potassium. Addition of potassium in thallium pre treated culture caused extrusion of intracellular thallium. This observation suggests for a competition between Tl^{2+} and K^+ acting on the same intracellular site. Inhibition of thallium toxicity in 25mM K^+ supplemented medium also confirms the same fact. Nitrogen sources did not affect thallium uptake activity. This indicates that even in combined nitrogen source supplemented condition thallium uptake should proceed. Further support of growth by nitrogen compounds would allow more accumulation of thallium. Further since K^+ exerts inhibitory effect on Tl^{2+} uptake activity, K^+ concentration should be maintained a low level only for realising better removal of thallium pollutants. Nitrogen source dependent regulation of cation toxicity and uptake by microalgal and cyanobacterial clay mixtures has been

reported by Geoffrey *et al.* (1993) and inorganic nitrogen source regulation of Cs+ in *Nostoc muscorum* (Singh *et al.*, 1994).

Bruins *et al* (1999) have reported that many microorganisms demonstrate resistance to metals in water, soil and industrial waste. Some metals, such as cobalt, copper, nickel, serve as micronutrients and are used for redox processes, to stabilize molecules through electrostatic interactions, as components of various enzymes, and for regulation of osmotic pressure. These toxic metals interact with essential cellular components through covalent and ionic bonding and could adapt themselves in the presence of both nutrient and nonessential metals by developing a variety of resistance mechanisms. Microorganisms growing in presence of toxic metals adapt themselves to grow by developing resistance against toxic compounds or such mutants could be isolated in laboratory and tested later for their survival and bioaccumulation of metal cations in field conditions. Resistance to growth inhibitory actions of toxic metals allow microbial mutants to grow and retain their biochemical and physiological activities (Baalen & Odonnel, 1978, Singh & Pandey, 1981). Chauhan *et al.* (2003) have isolated thallium resistant mutants in *Anabaena variabilis* which could resist high salt concentration. Verma *et al.* (2002) also reported application of mutant strain of Cd^{2+} for bioremediation. In the present study a thallium resistant mutants (Tl-R) in *Anabaena cycadae* was isolated with an aim to assess its capacity to bio-accumulate thallium from the outside medium. Investigation upon the mutant strains showed that it was able to grow at 0.8μM thallium concentration in the medium with normal thallium uptake activity. Its enzymatic activities GS, NR, NiR, O_2 evolution and consumption activities, heterocyst frequency determined by using cells grown in thallium supplemented medium were all comparable to wild type *Anabaena cycadae* grown in thallium

deficient medium. Since Tl-R phenotype showed normal Tl^{2+} uptake and enzymatic activity in thallium supplemented medium, such phenotypes could be used for bioremediation of toxic metal thallium after assessing its performance in the field condition in competition with natural microbial strains. Similar results on mutant strain of Ni^{2+} reported by Asthana *et al* (1993) showed effect of Ni^{2+} on various physiological processes like heterocyst frequency, nitrogenase activity, hydrogenase enzyme, O_2 evolution and consumption activities etc. These mutants strain developed envisage possibility for environmental biotechnology implications to detoxify the thallium polluted areas.

As on till date the US Environmental Protection Agency has cited threshold level for thallium to be present in the drinking water to be 2 ppb. In addition, EPA, USA has approved the following treatment methods for removing thallium from polluted areas: Activated alumina and Ion Exchange method, DOWA Iron treatment, EMD method and CABSORB methods (U.S.EPA, 2002) which are illustrated below.



The activated alumina and ion exchange method which was developed and patented by Sood (1988) removes impurities from aqueous solutions containing heavy metals ions by passing the impure solution through a bed of activated alumina adsorbent. The adsorbent must be further regenerated for reuse. Kikuchi *et al.* (1990) showed that thallium could be effectively removed from wastewater by DOWA Iron powder method, which uses iron metal powder and hydrogen peroxide at the pH range of 3–4. A slightly different method from DOWA iron powder method uses addition of lime or sodium hydroxide to obtain a pH between 8 and 10 and thallic hydroxide precipitates and circulated through the iron powder chamber (Schwartz, 1989). The EMD method for thallium removal using manganese oxides are considerably better heavy metal scavengers than iron oxides. The adsorption of thallium by electrolytic manganese dioxide (EMD) has been found to occur between pH 2 and 5 (Dahal & Lawrence, 1996). Rauws & Canton (1976) have suggested use of an insoluble inorganic pigment prussian blue as a possible way of eliminating

thallium from mining waste discharge. Manganese dioxide sludge produced during zinc electro winning has been shown to be very effective in removing thallous ions from zinc process waters and from lead smelting effluent wastewater. Thallium could also be removed by using water treatment technologies such as Zeolite (CABSORB ZS500RW) adsorption, ion exchange, solvent extraction, precipitation, activated carbon adsorption and potassium ferricyanoferrate (Prussian Blue) absorption. These few selected technologies appears to hold promise for application in mine waters for removing thallium to less than 2 ppb and these are recommended for further evaluation either in laboratory or pilot scale evaluation (Twidwell & Beam, 2002). The chemical based methods enlisted have been very useful for thallium removal but are extremely expensive. Therefore development of microbes based eco friendly technologies should be less expensive. The results of the present investigation do highlight the capacity of Tl-R phenotype of cyanobacteria for removal of thallium from the natural environment even at a very low thallium concentration; as an eco-friendly cost effective method.

CHAPTER - 5

SUMMARY

The investigations were carried out for identification of a novel approach involving cyanobacteria for removal of thallium from contaminated areas. A proper understanding of the regulation of thallium toxicity and mechanisms involved will provide better knowledge for developing meaningful strategy for thallium bioremediation. Therefore focus of the present study was to evaluate combined nitrogen sources regulation of thallium toxicity, thallium transport activity and effects of thallium on physiological and biochemical activity in cyanobacterial strains. Toxic effects of thallium was assessed by monitoring changes in photosynthetic pigments followed by its effects on heterocyst frequency, GS activity, NR activity, NiR activity, O_2 evolution, O_2 consumption, intracellular free proline levels in wild type strains and a Tl-R mutant cyanobacterial strain of *Anabaena cicadae*. Finally effects of combined nitrogen sources on thallium transport activity were also conducted to see if they regulate thallium toxicity by influencing thallium transport system in cyanobacteria. The results obtained are summarized as follows:

1. Thallium was found to be extremely toxic to the cyanobacterial strains *Nostoc* ANTH, *Anabaena cycadae*, *Nostoc muscorum, Anabaena variabilis* and *Mastigocladus s*p. The lethal dose was found to be 0.5μM to cells grown in N_2, nitrate and ammonium medium.
2. Exposure of cyanobacterial cells to 0.5μM thallium treatment caused more than 74% decrease in chlorophyll *a* level in cells grown in N_2 media, NO_3^- media and NH_4^+ media.

3. Thallium treatment to N_2 grown cyanobacterium caused decrease in phycobilisome contents as well. The range of inhibition was 68%-98%.
4. As cyanobacterial cells were sensitive to thallium, their heterocyst frequency decreased in response to thallium treatment.
5. Nitrogenase activity is severely inhibited in thallium treated conditions. The range of inhibition was 74%-90%.
6. The enzyme participating in assimilation of fixed/combined nitrogen also showed decreases in their activity. This includes glutamine synthetase activity (transferase) (> 60%), nitrate reductase (>56%), nitrite reductase (> 57%).
7. The inhibition of O_2 evolution and consumption activity was more than 80% and 54% respectively.
8. Thallium treatment to cyanobacterial cells enhanced intracellular proline level emphasizing role of proline under different stress conditions. However increase in intracellular proline level was not similar. The percentage increases were 45%, 9.3%, 9.1% and 12% in N_2 grown cells of *Nostoc* ANTH, *Nostoc muscorum*, *Anabaena variabilis* and *Anabaena cycadae* respectively. In nitrate grown culture increase in the proline levels was found to be 12% in *Nostoc muscorum*, followed by 18% in *Nostoc* ANTH, 14% in *Anabaena cycadae* and 12% both in *Nostoc muscorum* and *Anabaena variabilis* as compared with their respective controls. In ammonium supplemented medium, no significant difference in intracellular proline level was observed.
9. In contrast and comparison to inorganic nitrogen sources, amino acids alanine, glutamine, asparagines and proline protected cyanobacteria from thallium toxicity. The protection by rest of the amino acids was not significant and few

were found to be even toxic. The protection level was always more than 50% for chlorophyll a and 51% for phycobilisome contents.

10. Thallium uptake activity is not inhibited by inorganic and organic combined nitrogen sources showing a biphasic uptake pattern with a rapid first phase lasting for about 10 min followed by a slower second phase. Extrusion of intracellular thallium subsequent to addition of 2mM K^+ in the uptake reaction mixture suggests existence of common transport system for Tl^{2+} and K^+.
11. Inhibition of growth of cyanobacteria at 0.5μM thallium concentration decreased with increasing K^+ concentration in medium with almost complete protection in presence of 25mM K^+ in medium.
12. Decrease in thallium toxicity in presence of K^+ suggest that Tl^{2+} exert its toxicity by inhibiting/affecting K^+ dependent metabolic activities.
13. Thallium resistant *Anabaena cycadae* strains grew well at 0.8μM thallium concentration and showed very little changes in the measured physiological and biochemical activities.
14. The thallium uptake activity in Tl-R phenotype, like wild type strains, exhibited a similar biphasic pattern.
15. The normal uptake of toxic heavy metal thallium by Tl-R *Anabaena cycadae* mutant phenotypes can be exploited for bioremediation of thallium by generating and using such thallium resistant strains of *Anabaena cycadae* and other photoautotrophic cyanobacterial strains.

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List of Publications

Refereed Journals

National Syiem, M.B., Singh, A.K., Rai, A.N., Nonibala, K., Singh, R.K.S., **Adhikari S.** and Bhattacharjee, A. (2007) Nitrogen metabolism, artificial association study in two cyanobacterial isolates and assessment of their potential as biofertilizer. *Indian J Biotechnol.* Vol 6, 397-403

International Singh, A.K., Syiem M, Singh, R.K.S., **Adhikari, S.** and Rai A. N. (2007) A common transport system for methionine, L-methionine-DL-sulfoximine and phosphinothricin (PPT) in the cyanobacterium *Nostoc muscorum. Curr Microbiol,* 10, 9112-2.

Review Article ***Samrat Adhikari***, Prashant Sarkhel, Arvind kumar Singh and M.B Syiem (2008); "CTD – a pioneer tool for Environmental biotechnology";published at the proceedings of National Seminar on "*Toxicity of Chemicals and their hazards with special reference to heavy `metals*" Oct 23rd – 24th , 2008

Abstracts in Symposium/Conferences

2008 Mayashree B Syiem, **Samrat Adhikari,** Prashant Sarkhel and A.K Singh "Toxicity of gadolinium and ruthenium on the growth of diazotrophic cyanobacteria *Anabaena cycadeae*" in the National seminar on "*Toxicity of chemicals and their hazards with special reference to heavy metals*" held on -23rd – 24th October, St. Edmund's college, Shillong, India.

2008 **Samrat Adhikari,** Mayashree B Syiem, A.K Singh and A.N Rai; Nitrogen regulation of thallium to diazotrophic cyanobacterium *Anabaena cycadeae* and *Nostoc muscorum*" in the National seminar on "*Current trends in biomedical research*" held on 22nd -23rd, 2008 March, NEHU (under UGC-SAP Programme), Shillong, India.

2008 Amrita Bhattacharjee, Natasha Nongrum, Mayashree B Syiem, **Samrat Adhikari** and A.N Rai (2008); Role of cyanobacteria in cadmium bioremediation in rice fields; proceedings of National seminar on *"Toxicity of chemicals and their hazards with special reference to heavy metals"* held on -23rd – 24th October, 2008, St. Edmund's college, Shillong, India. Abstract on pp-161.

2008 Nawaz Islam, B. Manners, Mayashree B Syiem, **Samrat Adhikari,** Prashant Sarkhel and Arvind kumar Singh; Evaluation of gadolinium on the growth of diazotrophic cyanobacterium *Anabaena cycadae*; proceedings of National seminar on *"Toxicity of chemicals and their hazards with special reference to heavy metals"* held on -23rd – 24th October, 2008, St. Edmund's college, Shillong, India. Abstract on pp-164.

2008 Roshan Choudhury, , Mayashree B Syiem, **Samrat Adhikari,** Prashant Sarkhel and Arvind kumar Singh; Evaluation of ruthenium on the growth of diazotrophic cyanobacterium *Anabaena cycadae*; proceedings of National seminar on *"Toxicity of chemicals and their hazards with special reference to heavy metals"* held on -23rd – 24th October, 2008, St. Edmund's college, Shillong, India. Abstract on pp-168.

2008 Pooja Chakraborty, Usha barkakoti, Mayashree B Syiem, **Samrat Adhikari,** Prashant Sarkhel and Arvind kumar Singh (2008); Evaluation of salt toxicity on the growth of diazotrophic cyanobacterium *Anabaena cycadae* and *Nostoc muscorum*; proceedings of National seminar on *"Toxicity of chemicals and their hazards with special reference to heavy metals"* held on -23rd – 24th October, 2008, St. Edmund's college, Shillong, India. Abstract on pp-172.

2007 Arundhati Gogoi, Arvind kumar Singh, **Samrat Adhikari** and Amar Nath Rai (2007), 'Analysis of molecular diversity of cyanobacterial strains isolated from

local rice fields', Paper presented in the National seminar on "*Adaptation Biochemistry*" held on 22nd -23rd, 2007 March, NEHU (under UGC-SAP Programme), Shillong, India.

2006 Arvind Kumar Singh, Sven Becker, R.K.S Singh, **Samrat Adhikari** and Amar Nath Rai, (2006), "Molecular Fingerprinting of cyanobacterial strains from rice fields and fresh water lakes"; Paper presented in the International conference on "*Current trends in algal bioresources utilization*" organized by Assam University, Silchar, 4th -6th December, 2006. Abstract on pp no O-3.

2006 Arvind Kumar Singh, Nonibala Khumanthem, R.K.S Singh, **Samrat Adhikari**, M.B Syiem and Amar Nath Rai, (2006), "Colonization of roots and submerged shoots of rice by Nostoc ANTH and Mastigocladus laminosus""; Paper presented in the International conference on "*Current trends in algal bioresources utilization*" organized by Assam University, Silchar, 4th -6th December, 2006. Abstract on pp no O-20.

2006 Arvind Kumar Singh, **Samrat Adhikari**, M.B Syiem and Amar Nath Rai, (2006), "Evaluation of thallium toxicity to diazotrophic cyanobacteria Nostoc ANTH, Anabaena cycadeae and Nostoc muscorum"; Poster presented in the International conference on "*Current trends in algal bioresources utilization*" organized by Assam University, Silchar, 4th -6th December, 2006. Abstract on pp no P-49.

2002 Shome B.R, Rajeswari .S, Kumar. A, **Adhikari.** S, Paul. S, Rahman. M, Muragkar. H.V, Rahman. H, Chakraborty. S, Das. A and Bajarbaruah K.M. (2002). "Detection of Shiga like toxin genes (stx 1, stx 2) and enterohemolysin

gene (hlyA) in E. coli strains isolated from Pig and poultry by Polymerase Chain Reaction.". Paper presented in the "*National seminar on veterinary Biotechnology*", Pondicherry University on 12th – 14th December 2002.

2002 Rajeswari .S, Shome B.R, Chakraborty. S, Kumar. A, Das. A, **Adhikari. S**, Paul. S, Rahman. M, Muragkar. H.V, Rahman. H and Bajarbaruah K.M. (2002). "RAPD analysis of selected *E. coli* 0157 and 0149 strains isolated from pig and poultry in Meghalaya". Paper presented in the "National seminar on veterinary Biotechnology," Pondicherry University, 12th – 14th December, 2002.

2001 Shome B. R., Rajeswari Shome, Das, Mazumder Y, Rahman, M A., **Adhikari S.** Kumar A, Gogoi U.C, Rajkhowa C, and Verma, N. D. (2001). "Characterization of *Aeromanas hydrophila* isolated from the Indian major carp *Catla catla* suffering from ulcerative syndrome in Meghalaya". Paper presented at National seminar on "*Approaches for increasing agricultural productivity in hill and mountain ecosystem*" on 18th -20th Oct', 2001.Abstract on pp-169.

2001 Shome B. R., Rajeswari Shome, **Adhikari S**, Rahman, M A., Mazumder Y, Kumar A, Gogoi U.C, Rajkhowa C, and Verma, N. D. (2001). "Characterization of *Bordetella bronchiseptica* isolated from the pigs suffering from atrophic rhinitis in Meghalaya". Paper presented at National seminar on "*Approaches for increasing agricultural productivity in hill and mountain ecosystem*" on 18th -20th Oct', 2001.Abstract on pp-165.

2000 **Adhikari S**, Paul S and Syiem. M.B. " Effect of carbon and nitrogen sources on the growth and heterocyst frequency of free living cyanobacteria *Nostoc* sp" paper presented at *National seminar on strategies for agricultural research in the North East*, on 10th to 12th Nov', Barapani, Shillong. Abstract on pp-178.

Curriculum Vitae

Mr. SAMRAT ADHIKARI. *B.SC, MSC, NET, BITP*

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stedmundc.btisnet@nic.in

ACADEMIC:

EDUCATION

2003	BITP Industrial fellow, DBT, Govt. of India.
	Specialization: Diagnostic Protein Engineering
2002	M.Sc (Biotechnology)
	University: Bangalore university, Bangalore.
	Specialization: Molecular Microbiology.
	Division: FIRST.
1999	B. Sc (Biotechnology)
	University: NEHU, Shillong
	Specialization: *Honours.*
	Division: FIRST.
	Position: TENTH
1996	H.S.S.L.C
	Board: MBOSE, Shillong
	Division: FIRST.
1994	S.S.L.C
	Board: MBOSE, Shillong
	Division: FIRST.

Scholarships/Fellowships/ Honours/ Awards/ Nominations/ Recognitions

2004	Qualified NET (ARS) in Animal Biotechnology Roll No. 170273.
2003	Selected on all India basis for the Biotech Industrial Training Programme (BITP) sponsored through BCIL, on behalf of DBT, Govt. of India
2002	Won the Best Biotech Student Award, 2002 from AMC, Department of Biotechnology, Post Graduate center, Bannerghatta, Ban galore.
2001	Won Ist Prize in MICROCOSM on the model exhibition of "Sewage Treatment Plant" held on 25th Nov' St. Joseph College, Bangalore
2001	Merit holder of Stipend of Rs 9400/-p.a for two years during the M.Sc (Biotechnology), from NEC, Shillong.
1999- 2000	Merit holder of Stipend of Rs 9400/-p.a for two years during the B.Sc (Biotechnology), from NEC, Shillong
1997	Placed among the Top 10% in the Part A examination from Indian Association of Physics Teachers, Mumbai at the National Standard Examination In Physics.
1994	Won Ist Prize in "Science and environmental fair" at State Level Science exhibition on the topic "Production of artificial Rayon through Biological Waste" organized by DST, Govt. of Meghalaya

Positions Held

2 010 onwards	Head, Department of Biotechnology, St. Edmund's College, Shillong.
2008 onwards	Deputy coordinator, Bioinformatics Facility, SEC, Shillong
2006-2009	Lecturer, Deptt. of Biotechnology, St. Edmund's College, Shillong.
2004 onwards	Research Fellow, Deptt. of Biochemistry, NEHU, Shillong.
2002	NATP Project Fellow
2003	BCIL Industrial fellow

Title of thesis

PHD "A study on the effect of thallium on cyanobacteria (Supervisor: Dr. A.K Singh, Madam Curie Fellow, New Castle University, U.K, & Associate Professor, Deptt of Biochemistry, NEHU, and Shillong).

M.Sc : A study on the molecular characterization of *Bordetella bronchiseptica* isolated from pigs suffering from atrophic rhinitis.
(Supervisor: Dr. B.R Shome, Principal Scientist, Deptt. of Animal Biotechnology, IAHVB, (ICAR) Bangalore).

B.Sc "Effect of fixed carbon sources (Glucose, Fructose, Sucrose) on the growth and heterocyst frequency of free living Cyanobacteria, *Nostoc.* spp Supervisor: Dr. M.B Syiem, Deptt. of Biochemistry, NEHU, Shillong)

Seminars Organized

2010 Organized a workshop on "Bioinformatics – a computational approach to biological information" on 27th – 29th July, 2010.

2008 Organized as JOINT CONVENER the Seminar on "*Toxicity of Chemicals and their hazards with special reference to heavy metals*" Oct 23rd – 24th, 2008.

2002 Assisted Dr. B.R Shome, Principal Scientist, IHAVB, Bangalore and Dr. H. Rahman, Director, Division of Animal Sciences, IVRI, Izatnagar, U.P, in organizing a 5 day workshop on " Development of DNA Based Diagnostic", held at ICAR Research complex for NEH Region, Barapani, Shillong, during tenure as senior research fellow

Technical Proficiencies

DGGE (BioRad, USA),

FPLC system (Pharmacia Biotech, Sweden)

Acquainted in handling all types of Peristaltic pump (Electro lab, India; Gilson, France).

Gas Chromatogram (Chemito India Ltd.).

Liquid Scintillation counter.

PCR (Biorad, Applied Biosystem, eppendorf),

Centrifuge (Hareus, Remi, eltek),

Super centrifuge or Sharple (Pennwalt India Ltd.),

Thermal Cycler (Bio Rad, USA),

Gel Doc system (American Advanced Biotechnology, USA),

UV Spectrophotometer (Systronic, Spectronic Unikem),

ELISA reader (BioRad, USA with software for analysis),

Prep Cell Electrophoresis apparatus (BioRad, USA),

Isoelectric focusing (Multiphor II, Hoefer) , Ultra filtration (Millipore, USA)

Millipore ultrafiltration equipment

Seminar/ workshop attended:

- All India XXI Bioinformatics Coordinator meeting on BTIS NET, on 2-4th February, 2010, CARI, Port Blair, Andaman & Nicobar Island.
- NEBINET Bioinformatics Coordinator meeting on BTIS NET, on 13-14th November, 2009, AAU, Assam.
- All India XXth Coordinator meeting on BTIS NET, on 2-4th February, 2009 at NEHU, Shillong.
- National seminar on "*Adaptation Biochemistry*" held on 22nd -23rd, 2007 March, NEHU (under UGC-SAP Programme), Shillong, India.
- Regional workshop on "*Safety assessment and regulation of GM crops with a special focus on Cartagena protocol on Biosafety*", organized by BCIL (New Delhi), Ministry of Environment & Forest (Govt. of India). and AAU (Assam), on Aug 28th -29th, 2006, India
- Seminar on the "*Trends in Biochemical Research*", 31st march, 2006, NEHU, Shillong, India.
- International conference on "*Current trends in algal bio resources utilization*"

organized by Assam University, Silchar, 4th -6th December, 2006, India.

- National Seminar on "*Advances in Biochemical Education and Research*" Feb 25 & 26th, 2005, NEHU, Shillong, India.
- Training on "*Application of filtration techniques in purification of Diagnostic Proteins*" held in 3rd – 7th July, 2003, Bangalore
- Seminar on "*Challenges in the development and production of Monoclonal antibodies, vaccines and other Biologics*" organized by Amersham Biosciences, Sweden on 3rd April 2003, at Hotel Orchid, Mumbai, India.
- 72nd meeting of National academy of Sciences (NASI) and National Seminar on "*Biodiversity*", 25-26th October, 2002, NEHU, Shillong
- National Roving Seminar on "*Patenting in Biotechnology*" 27th Oct, 2002 NEHU, Shillong.
- National seminar on "*Microbiology, Biotechnology and allergy*"28th -29th, October, 2002, Indian Academy of Allergy and Astra- IDL held at St. Joseph College, Bangalore.
- National Seminar on "*Approaches for increasing agricultural productivity in hill and mountain ecosystem*" 18th –20th October' 2001. ICAR, Barapani, Shillong
- National Seminar on "*Strategies for increasing agricultural productivity in North Eastern Region*" 11th -14th Nov' 1999, ICAR, Barapani, Meghalaya.
- Actively participated in the Intensified Pulse Polio Programme organized by the Govt. of India (Ministry of Health & Family welfare) on 25th of October, 1999.
- Workshop on the '*INTERNET*", held at St Anthony's College, Shillong, 23rd – 28th August, 1999.
- Certificate course on the "*Personal computer software*" held at St Anthony's College, Shillong, 7th – 9th September, 1999.
- Attended the "*Crash course on Self employment*" at Shillong, 20th – 22nd September, 1999.
- Workshop on "*Development of Communication Skills, Public speaking, Group discussion and Interviews*" held at held at Students Development services cell, St Anthony's College, Shillong, 4th – 15th October, 1999.
- Awareness programme on "*Applications of Radioisotopes and Radiation Technology*", organized by NAARI, Mumbai & RSIC, Shillong, 26th-27th, November, 1999.

Personal Information:

Father Name: Shri Sudhamoy Adhikari

Date of Birth:	16th FEB, 1979
Caste:	Brahmin
Blood Group:	B Positive
Marital Status:	Married
Nationality:	Indian.
Hobbies:	Music, singing, cooking, writing Scientific articles & Carpentry

Referee:

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