# साइनोबैक्टीरियम *अनाबाना डॉलीओलियम* के विकास और शारीरिक चर पर लवणता और तांबे के संयुक्त प्रभाव का आकलन

# Assessment of the combined effect of salinity and copper on the growth and physiological variables of a cyanobacterium *Anabaena doliolum*

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# Assessment of the combined effect of salinity and copper on the growth and physiological variables of a cyanobacterium *Anabaena doliolum*

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# CERTIFICATE

This is to certify that the thesis entitled "**Assessment of the combined effect of salinity and copper on the growth and physiological variables of a cyanobacterium *Anabaena doliolum***" submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of Master of Science in Microbiology, embodies the results of bonafide research work carried out by **Mr. Shivaranjan C S, Roll No. 20833** under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma. The assistance and help availed during the course of investigation as well as source of information have been duly acknowledged by him.

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*This research work is dedicated*

To

My Parents

# CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS AND SYMBOLS

- µg – Microgram
- µM – Micromolar
- APX – Ascorbate Peroxidase
- $C_2H_4$ – Ethylene
- CAT – Catalase
- EDTA –Ethylene Diamine Tetra Aceticacid
- GC – Gas Chromatography
- GS – Glutamine Synthease
- mM – Micromolar
- NR – Nitrite Reductase
- ROS – Reactive Oxygen Species
- SOD – Superoxide Dismutase

# INTRODUCTION

According to estimates, the current world population of about 7.2 billion is expected to cross 9.6 billion by the end of year 2050. To feed this ever increasing population the annual production of cereals needs a leap of about 50%, i.e., from 2.1 billion tons per year to ~3 billion tons per year. This daunting task will put enormous pressure on agriculture sector and pose a serious threat to the food security. However, the target of feeding the increasing population could be achieved by enhancing the area of cultivation and by enhancing the productivity of crop plants. However, the application of synthetic fertilizers and pesticides, intensive tillage and over irrigation has resulted in serious deterioration of the productivity and quality of soil leading to reduced crop productivity. In a way the cost of agricultural production has increased tremendously due to deterioration of soil fertility, overuse of land and water resources and polluted environment. Therefore, to meet the present and future food requirements of the population within the available limited resources without deteriorating the environmental quality are going to be a huge challenge for the technocrats, policy makers and scientists. It is in this context that the sustainable agricultural practices are important. This has an emphasis on natural processes that conserve resources such as soil and water and efforts to minimize the cost of agricultural production. An important component of the sustainable agriculture is low-cost farming with the help of native microorganisms which is eco-friendly. All these sustainable agricultural management practices will help to agro-ecosystem to be more resilient to maintain the productivity and profitability.

Beneficial microbes are used in the sustainable management practices with considerable success and their contribution of microbes in enhancing the soil fertility has been recognized since long. In the recent times, bio-inoculants containing beneficial soil microbes have been used for enhancing crop productivity. In this contest, the cyanobacteria are important organisms that play an important role in the enhancement of agriculture productivity. Singh (1961) recognized the importance of cyanobacteria in sustaining rice production in tropical paddy fields has been long recognized. Venkataraman (1981) also documented the role of nitrogen fixing cyanobacteria in improving the fertility of the soil, particularly of rice paddy fields. Recently their role in enhancing the agricultural productivity and mitigation of GHG emissions has been proposed (Singh, 2011; Singh *et al.*, 2011a). Singh (2014) reported that the cyanobacteria play a vital role in the restoration of degraded lands. Cyanobacteria are photosynthetic organisms which can survive easily on bare minimum requirement of light, carbon dioxide ($CO_2$) and water (Woese, 1987; Castenholz, 2001). The cyanobacteria are mostly heterocystous however, non-heterocystous, filamentous and unicellular cyanobacteria that can fix nitrogen aerobically, micro aerobically and anaerobically have also been identified (Pearson *et al.*, 1979; Haselkorn, 1978). These organisms have been found to occur naturally in several agro-ecosystems like paddy fields and from Antarctica to Arctic poles (Pandey *et al.*, 2004). Bioinoculants such as cyanobacteria can improve the soil quality and plant growth as supplement for good crop management practices. The economic benefits of cyanobacterial application include nutrient cycling, $N_2$-fixation, bioavailability of phosphorus, water storage and movement, environmental protection and prevention of pollution and land degradation. Issa *et al.* (2014) reported that the cyanobacteria can contribute to about 20–30 kg N $ha^{-1}$ as well as the organic matter to the soil. Therefore, they are ideal for farmers from the economically weaker sections unable to invest for costly chemical nitrogen fertilizer.

In spite of the tremendous agricultural potential, the exploitation of cyanobacteria is constrained by a number of abiotic stress factors. Increasing incidence of abiotic stresses is one of the reasons that restrict the growth and productivity of cyanobacteria. In the natural environment, cyanobacteria may experience many kinds of stresses. Of the several abiotic stresses that challenge the survival of the cyanobacteria in the paddy fields is salinity. The problem of salinity is a serious concern globally and especially the tropical countries (Zhu, 2001). Large amount of salts are brought to the paddy field due to canal irrigation and water logging. Singh (1961) reported that the salinity decreases the cyanobacterial population of rice field significantly leading to reduced rice yield and soil fertility. Salinity exerts a negative effect on the growth and physiological aspects of cyanobacteria. In general the cyanobacteria exhibit considerable salt tolerance and limited success was obtained in the reclamation of saline and sodic lands using cyanobacteria (Thomas and Apte, 1984). High concentrations of salt adversely affect the various metabolic processes of the cyanobacteria such as growth, photosynthesis and enzyme activities (Moisander *et al.*, 2002; Srivastava *et al.*, 2005). Similarly, the increasing anthropogenic activities have also resulted in the generation of several heavy metals in the environment. Selection pressure is also exerted on the cyanobacteria through the contamination of rice paddy by heavy metals such as Cu. Poor agricultural practices such as use of contaminated water for irrigation, accelerated input of chemical fertilizer, use of copper sulfate for controlling pests and diseases lead to contamination of soils. Cao and Hu (2000) observed that water containing Cu when used for irrigation adversely affected the growth of rice plants. Adverse impact of heavy metals on cyanobacteria has also been established (Bhargawa *et al.,* 2008; Hudek *et al*., 2012). Heavy metals are one of the most common pollutants worldwide, being a serious hazard to the environment and public health. Use of contaminated water for irrigation, accelerated input of chemical fertilizers, use of copper sulfate for controlling insect pests in paddy and heavy metal-containing pesticides increase the load of copper in the environment. Increase in copper content in paddy soil irrigated with waste water has been reported by Luo *et al*. (2003). Therefore, the ever-increasing population is posing a negative pressure on all kinds of ecosystems. Of these stresses, the agro ecosystem is more severely affected by anthropogenic activities such as faulty irrigation practices and application of pesticides and chemical fertilizers, which in turn increase the salinity and metal content there by posing a threat to soil microflora including cyanobacteria.

However, in the natural environment the cyanobacteria may experience many kinds of stresses and we do not have a clear understanding about the combined effects of stresses such as salinity and heavy metals on cyanobacteria. At a given period of time cyanobacteria may experience one or more stresses. It has been demonstrated that susceptibility of organisms to a particular stress may be altered due to other stresses (Bhargawa *et al.,* 2008). Murali and Teramura (1987) observed that drought and mineral deficiency are capable of modifying the UV-B impact on plants. Enhanced tolerance to UV-B due to pre-exposure to UV-B has been reported in the cyanobacterium *Cylindrospermum* sp. (Chris *et al.,* 2006). Bhargawa *et al*. (2008) observed that pre-treatment with copper enhanced the UV-B tolerance in cyanobacterium *Anabaena doliolum*. Kumar and Gaur (2014) used cyanobacterium for the removal of copper from copper contaminated water bodies. Salinity is a serious threat to plants and cyanobacteria and there have been no reports on the effect of copper pre-treatment on the salinity tolerance in the cyanobacteria. Therefore, it is important to understand the modulation of stress response in the cyanobacterium exposed to salinity and copper stress and the modulation of stress response.

However, there have been no reports available on the enhancement of salinity tolerance in the cyanobacteria due to other environmental stress factors. Cyanobacteria occupy an important position in food web, and thus loss of cyanobacterial biomass may seriously affect soil fertility through nitrogen and carbon fixation. Further, they are also important as bioremediation agents in cleaning up the environment, and thereby reducing pollution load. Therefore, it is important to understand the modulation of stress response in the cyanobacterium exposed to salinity and copper stress and the modulation of stress response. Therefore, the present study has been proposed with following objectives:

- To assess the combined effect of salinity and copper on growth and physiological parameters of a cyanobacterium.
- To understand the modulation of salinity tolerance in cyanobacterium through copper pre-treatment.

# REVIEW OF LITERATURE

The cyanobacteria are photosynthetic organisms found in ecologically diverse habitats ranging from hot springs to Arctic and Antarctic (Stanier and Cohen-Bazine 1977). This ancient monophyletic group of the domain bacteria have colonized all light-exposed habitats on the Earth. Their ability to fix atmospheric nitrogen makes them important in any ecosystem (Bothe *et al*., 2010). They are among the oldest living organisms surviving on this planet existing since more than three billion years. . These organisms have thought to have originated more than 3.5 billion years ago (Brock, 1973). Fossil records harbouring these organisms dated as over three billion years old are available (Schopf, 1993). These organisms were probably responsible for the production of oxygen in the atmosphere during the Precambrian period of evolution (Schopf, 1970). According to a hypothesis, the chloroplasts of higher plants in fact originated through endosymbiosis of "cyanobacteria like microbe" with eukaryotic cells (Walsby, 1986). The cyanobacteria are considered to be progenitors of higher plant chloroplasts because of striking similarity with the chloroplasts (Fay, 1992). This is being supported by the resemblance in the genome organization, protein synthesizing machinery, photosynthetic pigments and their organization, the oxygenic photosynthesis, carbon fixation, and photorespiration in chloroplasts show close with cyanobacteria (Douglas, 1994) (Douglas, 1998). They were responsible for making the earth's atmosphere oxygenic and paved way for the evolution of aerobic organisms on earth (Schopf, 1975). The term cyanobacterium was used for Gram-negative prokaryotic autotrophs by Stanier (1970). They are considered as one of the ancient life forms to have evolved on Earth (Sheridan *et al*., 2003; Allwood *et al.,* 2006; Bosak *et al*., 2009). However, Stanier and Van Niel (1962) suggested that they be included under bacteria (photosynthetic bacteria. Stanier *et al.,* (1978) opined that that the nomenclature of cyanobacteria must be based on the bacteriological code of nomenclature.

The prokaryotic organism cyanobacteria was earlier known as blue green algae consist of a group of photosynthetic bacteria capable of performing oxygenic photosynthesis and therefore are similar to like higher plants and algae (Nelson and Ben-Shem, 2005) (Blankenship and Hartman, 1998). They are nearly ubiquitous in distribution and are found in extremes of environments ranging from hot springs to cold/frigid polar lakes, freshwater habitats to oceans, deserts and many terrestrial wastelands infested with man-made pollutants. Some cyanobacteria are provided with the capacity to fix free atmospheric nitrogen photosynthetically. These cyanobacteria include members (from the order Nostocales and Stigonematales) which develop unique cells known as heterocysts and house the nitrogenase enzyme responsible for $N_2$-fixation (Elhai and Wolk, 1990). However, the nitrogen-fixing cyanobacteria are especially abundant in wetlands and paddy fields of tropics and they contribute significantly to the fertility of the soil (Venkataraman 1981). Due to the ancient origin, these organisms have been exposed to several natural as well as manmade stress conditions during the course of their evolution and this is one of the reasons responsible for their ecological success in several habitats. The cyanobacteria play a significant role in biogeochemical cycles (De Ruyter and Fromme, 2008). Cyanobacteria generally exhibit high level of adaptive abilities and tolerance to a large number of environmental stresses. During the course of their evolution the cyanobacteria have survived several types of stresses and therefore, these organisms are considered as model system to decipher the fundamental aspects of adaptive responses to a variety of environmental stresses (Murata and Wada, 1995). Several strains of *Anabaena* resistant to different abiotic stresses like nutrient deficiency, salinity, drought, temperature up shift and desiccation have been identified and screened (Apte 2001; Singh *et al.*, 2010). Whitton and Potts (2000) also observed that these forms have wide distribution in several habitats and extreme habitats such as hot springs, hyper saline waters, deserts, and Polar Regions. There are reports on the exposure of cyanobacteria to heavy metal induced stress conditions (Srivastava *et al.*, 2005, 2008). Thajuddin and Subramanian (2005) reported that the cyanobacteria is composed of around 150 genera and 2,000 species. They consist of unicellular, colonial and filamentous to branched filamentous forms having a heterogeneous assemblage. Filamentous and non-filamentous cyanobacteria are further assigned to various orders based on the nature of the filaments as well the reproductive bodies. In fact the taxonomic status of cyanobacteria is complicated. The non-filamentous cyanobacteria mainly comprises of unicellular/non-filamentous aggregates of cells found within a matrix. The order Chroococcales is characterized by Binary division whereas the order Pleurocapsales reproduce through internal multiple fission. However, the filamentous cyanobacteria have one-seriate trichomes/filaments; trichomes sometime have false branches and have two orders such as Oscillatoriales and Nostocales. Another single order Stigonematales has multi-serriate trichomes/filaments.

The cyanobacteria have several uses in agriculture as well as industry. Free living and symbiotic associations of cyanobacteria with partners such as water fern *Azolla*, cycads and *Gunnera*, etc. are important in the nitrogen economy. Cyanobacteria have specialized cells known as heterocysts with thick walled and modified cells housing the enzyme which is the site of nitrogen fixation by nitrogenase enzyme. The enzyme complex catalyzes the conversion of the molecular $N_2$ into reduced form like ammonia (Elhai and Wolk, 1990). It has been reported that the fixed nitrogen is released in the form of ammonia, polypeptides, free amino acids, vitamins, and auxin-like substances after the cell death (Subramanian and Sundaram, 1986). Several Asian countries such as China, Vietnam, India have already been been utilizing cyanobacteria in paddy cultivation as the alternative to nitrogen fertilizers (Venkataraman, 1972; Lumpkin and Plucknett, 1982). Application of cyanobacteria in wheat crops enhanced growth parameters and yield (Spiller and Gunasekaran, 1990; Obreht *et al.,* 1993; Karthikeyan *et al.*, 2007, 2009). Species such as *Anabaena variabilis*, *Nostoc muscorum*, *Aulosira fertissima*, and *Tolypothrix tenuis* are the most commonly used biofertilizer strains. The cyanobacteria are integral components of rice fields and nitrogen fixing cyanobacterial forms play a significant role in sustaining and improving rice field productivity of nitrogen deficient paddy soils or soils with low nitrogen input (Roger and Kulasooriya, 1993). According to the estimates, cyanobacteria fix over 95 million tons of $N_2$ annually. Dubey and Rai (1995) showed the agronomic potential of cyanobacteria in increasing yield as well as reducing the dependence on chemical nitrogen by 25 percent.

According to Issa *et al.,* (2014) the cyanobacteria can contribute to about 20–30 kg N $ha^{-1}$ as well as the organic matter to the soil and ere economically feasible. Cyanobacteria play an important role in the reclamation of salt affected soils as they improve the physico-chemical quality of saline and alkali soils such as soil aggregation by lowering the pH, electrical conductivity, and hydraulic conductivity (Kaushik and Subhashini, 1985). There physiological advantages associated with cyanobacteria which enable them withstand salinity stress is due to its ability to curtail the influx of sodium (Apte *et al.,* 1987) and the accumulation of inorganic and organic osmoregulatory compounds such as sugars and quaternary amines etc. (Reed *et al.,* 1984). It was observed that the nitrogen added by the cyanobacteria in such soils helps binding of the soil particles thereby improving the soil permeability and aeration (Singh, 1961). Application of cyanobacteria in organically poor semi-arid soils can play a significant role in their reclamation. Nisha *et al.,* (2007) observed that the cyanobacteria develop a superficial network of the trichomes/filaments on the soil which binds the soil particles leading to enmeshing of the soil particles. Since these organisms are carbon and nitrogen fixers, they are able to contribute to the improvement of soil nutrient status. Cyanobacterial species such as *Anabaena oscillarioides* and *Microcystis aeruginosa* exhibit considerable salt tolerance (Coutinho and Seeliger, 1984; Moisander *et al.,* 2002). Another important property of the cyanobacteria is the production of EPS which help soil particles to bind together (Mazor *et al.,* 1996). This property has a major role in improvement of soil moisture. Exopolysaccharides from cyanobacteria also contribute to reclamation of the desert soils (Flaibani *et al*., 1989). Cyanobacteria release extracellular plant growth promoting substances and hormones such gibberellins (Singh and Trehan, 1973), cytokinin (Rodgers *et al*., 1979), auxin (Ahmad and Winter, 1968), or abscissic acids (Marsalek *et al*., 1992). Plant growth promoting effects of cyanobacteria involve enhance rice seed germination, root and shoot growth (Misra and Kaushik, 1989 a,b). Obreht *et al.,* (1993) observed that co-inoculation of cyanobacteria with wheat enhanced root dry weight and chlorophyll.

The Cyanobacteria are also potential bio-control agents against various plant diseases. They have been reported to produce bioactive compounds which are antibacterial, antifungal, antialgal, and antiviral potential (Teuscher *et al.,* 1992; Burja *et al.,* 2001; Dahms *et al.,* 2006). Extracts from *N. muscorum* inhibited the in vitro growth of the fungal plant pathogens such as S. sclerotiorum (Cottony rot of vegetables and flowers) and *Rhizoctonia solani* (root and stem rots; Kulik, 1995). Dahms *et al.,* (2006) identified broad spectrum of antialgal compounds which inhibit growth of pathogens by disturbing their metabolic and physiological activities. De Caire *et al*., (1990) showed antifungal against soil fungi using extracts of the cyanobacterium, *Nostoc muscorum*. Natural pesticides against the fungi, insects and nematodes have been isolated from the cyanobacterium *Nostoc* sp. by Biondi *et al.* (2004). Cyanobacteria has the potential for the remediation of various types of environmental contaminates (Megharaj *et al.,* 1994), crude oil (Sokhoh *et al.,* 1992; Al-Hasan *et al.,* 1998, 2001), naphthalene (Cerniglia *et al.,* 1980a,b), phenanthrene (Narro *et al.,* 1992), phenol and catechol (Shashirekha *et al.,* 1997), heavy metals (Singh *et al*., 2011b). Kuritz and Wolk, (1995) observed that the biodegradation potential of cyanobacteria can be enhanced through genetic engineering and could be used as the economical and maintenance-free remediation technology for contaminated eco- systems. Artificial cultivation of cyanobacteria in wastewater may have tremendous potential in degrading the pollutants and pesticides and thereby reducing the pollution load to support growth of other microbes for reduction in the BOD and COD.

Cyanobacteria are also rich source as feed stock for the energy generation (Parmar *et al.,* 2011; Rosgaard *et al.,* 2012). Lawson *et al.* (2011) reported that the cyanobacterial biomass containing lignocellulosic compounds can be processed for syngas production. The quality and quantity of cyanobacterial biomass could be augmented through the manipulation of several physico-chemical parameters to achieve the desired level of cyanobacterial biomass having good quality bio-fuel products. Further, the cultivation of these cyanobacteria can be done at different scales, lesser space, time and under diverse conditions efficiently to achieve high value bio-fuel products. Cyanobacteria are a source of clean energy, hydrogen which is produced by two ways mediated either through the enzyme nitrogenase or hydrogenase (Pinzon-Gamez *et al.,* 2005). Several cyanobacterial genera such as *Anabaena, Calothrix, Oscillatoria, Cyanothece, Nostoc, Synechococcus, Microcystis, Gloeobacter, Aphanocapsa, Chroococcidiopsis,* and *Microcoleus* are studied for their ability to produce $H_2$ under various cultural conditions (Masukawa *et al.,* 2001; Parmar *et al.,* 2011; Nozzi *et al.,* 2013). Cyanobacterial $H_2$ production is a clean and green technology but the major limitation in this process is lesser amount of $H_2$ production that makes economically not feasible (Tiwari and Pandey, 2012). The cyanobacterial biomass can be used to produce biogas via anaerobic digestion or fermentation (Hankamer *et al.,* 2007). Cyanobacteria can also be used for the sequestration of carbon dioxide from the atmosphere (Jacob-Lopes *et al.,* 2008). Miller *et al.,* (2007) observed that thermophilic cyanobacteria such as *Synechococcus lividus* and *Mastigocladus laminosus* inhabiting range from 63–64°C and 73–74°C are ideal for the sequestration of $CO_2$. Converti *et al.* (2009) observed a carbon utilization efficiency of 90% in the cyanobacterium *A. platensis*. Cyanobacteria could be used to minimize the global warming (Cuellar-Bermudez *et al.,* 2014). Reduction in methane emission by the application of cyanobacteria without affecting the rice yield has been observed by Prasanna *et al.,* (2002).

Another important application of cyanobacteria is their potential use as food supplement. In the market different forms such as tablets and capsules are available for the consumption of human beings as health/dietary supplement. Liang *et al.*, (2004) reported that the cyanobacteria enhance the nutritive value of pastas, snack foods, candy bars or gums, and beverages. In addition to this, they can also be used as natural colorants in food industry (Bhaskar *et al.,* 2005). *Spirulina, Anabaena*, and *Nostoc* are consumed as human food in countries such as Chile, Mexico, Peru, and Philippines. In India also *Spirulina* (*Arthrospira*) is grown on large scale using either raceway ponds and is consumed as food supplement. *Spirulina* is used as food supplement because of it is rich in nutrients and has better digestibility (Brown et al., 1997; Bandaranayake, 1998; Sinha *et al.,* 1998). Further, it is rich in protein (more than 60%) and beta-carotene, thiamine, and riboflavin and vitamin $B_{12}$ (Plavsic *et al*., 2004). Kulshreshta *et al.*, (2008) reported that the cyanobacterium *Spirulina* has multifaceted uses and is a source of several unexplored bioactive compounds. Because of this several pharmaceutical companies have been established for research and development.

There is ample scope for the development of cyanobacterial bio-agents for sustainable agriculture. Keeping in view of the deteriorating soil health and productivity the maintenance of environmental sustainability is the challenging task and cyanobacteria may be employed for safe and eco- friendly agriculture and environmental sustainability. The cyanobacteria can be used to produce valuable chemicals including food supplement. They are excellent accumulators or degraders of environmental contaminants such as heavy metals, pesticides and hydrocarbons. Another way to exploit them is capturing and storage of $CO_2$ that may also lead to climate change mitigation. They are also the ideal source for an array of bioactive compounds having antagonistic properties. Undoubtedly, the cyanobacteria are “gold mine” for biological research. However, increasing incidences of abiotic stress factors hamper their exploitation. One of the most serious threats regarding the exploitation of cyanobacteria is the increasing incidence of abiotic stress to which they are constantly exposed to. The nitrogen-fixing cyanobacteria are generally used as bio-inoculants rice cultivation in tropical paddy fields. In this ecological habitat they are very often subjected to abiotic stresses such as salinity. It exerts a negative effect on the growth and physiological aspects of cyanobacteria. Otherwise the cyanobacteria are an interesting group of organisms inhabiting almost all types of diverse ecological habitat and their ability to survive under harsh conditions is unique. Singh (1961) reported that salinization of paddy fields in India adversely affected the nitrogen fixing cyanobacteria leading to decline in soil productivity. The problem of salinity is a serious concern globally and especially tropical countries (Zhu, 2001). Large amount of salts are brought to the paddy field due to canal irrigation and water logging. Salinity has been reported to negatively influence the plant growth and productivity (Pitman and Lauchli, 2002). Further, the salinity has been reported to affect the microbial metabolic processes, their diversity and community structure and the fertility of the soil as well (Casamayor *et al.*, 2002; Rietz and Haynes, 2003; Oren 2008). Another major problem due to salinity is secondary salinization caused by anthropogenic land use practices. Therefore, for the global food security it is pertinent to address the salt tolerance of agronomic beneficial microbes. The problem of secondary salinization is more serious as the water supplies always contain dissolved salts in it which on evaporation become more and more concentrated on the upper surface of the soil. This in turn will lead to deterioration of the quality of the soil making it unsuitable for cultivation (Rai and Rai, 2000). Salinity is a limiting factor for agricultural productivity and adversely affects 19.5% of the irrigated agricultural lands world over (FAO, 2007). There is an inverse relationship between the amount of salt and water in a solution. Therefore, increase in the salinity reduces the water availability leading to induction of osmotic stress. Generally, cyanobacterial cells maintain a constant cellular ionic and osmotic composition to maintain water uptake through osmosis and creation of turgor pressure necessary for cell enlargement and growth. A change in the external salt concentration and/or water availability therefore, poses a challenge for the cellular metabolism and cell survival. Faster and quick response to changes in salt and/or osmotic concentration is thus important for survival of the cyanobacteria in many habitats.

The cyanobacteria form a dominant group of organisms which influence influences the biogeochemical processes of the planet earth and account for about 30% of global primary production. Further, the long evolutionary record and occurrence in almost all habitats show case their ability to acclimatize to various environmental conditions (Tandeau de Marsac and Houmard 1993). Singh and Kshatriya (2002) reported salinity-induced decrease in chlorophyll content of the cyanobacterium *Anabaena doliolum*. High salt concentrations lower the water availability and increase the concentration of ions in the cells. The increase in the concentrations of inorganic ions in the cytoplasm can have toxic effects on cellular metabolism. Increasing salinity lowers the water potential of the cells which is immediately followed by the accumulation of intracellular ions disturbing ion homoeostasis. This in turn will lead to the alteration of the membrane permeability leading to perturbed uptake of essential ions leading to nutrient deficiency (Kochian and Lucas 1988; Swapnil *et al.,* 2015). The adaptive ability of cyanobacteria to salinity has already been studied by several workers (Jeanjean *et al.,* 1993; Pandhal *et al.,* 2009). Salinity exerts an adverse impact on growth, photosynthesis and nitrogen fixation in several cyanobacteria (Moisander *et al.,* 2002; Chris *et al*., 2006; Srivastava *et al*., 2005; 2008). However, the cyanobacteria are well equipped tom cope with such situations by the synthesis and accumulation of organic osmolytes Borowitzka, 1986; Reed, 1988; Roberts, 2005). Various mechanisms are responsible for conferring salt tolerance in cyanobacteria which includes up regulation of antioxidant enzyme machinery, synthesis of salt induced proteins and ion transport (Chris *et al*., 2006; Fulda *et al*., 2000; Swapnil *et al*., 2015). Wang *et al*., (2004) observed reduction in photosynthesis in cyanobacteria in response to salinity. Similar observations have also been made earlier by Sudhir and Murthy (2004) regarding impaired photosynthesis and reduction in the carbon pool in cyanobacteria exposed to salinity. Enhanced accumulation of $Na^+$ and inhibition in photosynthetic activity is a consequence of salinity in many cyanobacteria (Lu and Vonshak 2002). Salinity stress has resulted in reduction in PSI and PSII activity in the cyanobacterium *Anabaena* sp (Rai *et al*., 2014). Berera *et al*., (2012) observed that tight regulation of photosynthetic and respiratory electron transport activity is required to operate properly under salt stress conditions. Intracellular $Na^+$ accumulation beyond a level, leads to water deficiency, disturbances in ion homeostasis, changes in membrane potential and deficiency of nutrients leading to decreased growth (Stal *et al*., 1991; Rejili *et al*., 2007; Allakhverdiev *et al*., 2008). Therefore, maintenance of of high $K^+$ content is important in salt tolerance (Zhu *et al*. 2011). Therefore, maintenance of optimal $K^+/Na^+$ ratio is absolutely important for tolerance in plants exposed to salt (Munns and Tester, 2008). Torrecilla *et al.* (2001) observed that high intracellular calcium was responsible for enhancing the tolerance to salinity. Ions such as calcium play an important role in salinity tolerance by influencing ion channels and transport of nutrients (Lauchli and Schubert, 1989; Martinez and Lauchli, 1993; Kader and Lindburg, 2008). Further, in the cyanobacterium *Anabaena doliolum* and *Anabaena* PCC 7120 maintenance of high $Ca^{2+}/Na^+$ ratio was found to enhance the tolerance to salinity (Swapnil *et al*., 2015).

Salinity stress leads to oxidative stress conditions which in turn lead to the generation of super oxide, peroxide and hydroxyl radicals, singlet oxygen and other reactive oxygen species (ROS). The free radicals will cause severe damage to several cellular components such as DNA, protein, lipids and membranes (Banerjee *et al*., 2013). Therefore, osmotic stress due to salinity will also lead to cellular and photo-oxidative damage (Peltzer *et al*., 2002). Apel and Hirt (2004) reported that the ROS attack a variety of biomolecules, resulting in enzyme inhibition, chlorophyll degradation, DNA damage, and lipid peroxidation thereby resulting in irreparable metabolic dysfunction and cell death. Damage of cellular macromolecules due to generation of reactive oxygen species has been observed (Choudhury *et al*., 2013; Vallyathan and Shi, 1997). The nitrogenase complex is still vulnerable to oxygen and ROS toxicity due to permeation of oxygen into heterocysts from the adjoining vegetative cells despite the presence of such adaptations (Regelsberger *et al*., 2004; Zhao *et al*., 2007a). Although, toxic ROS and its byproducts are generated by all aerobic organisms, its production is further enhanced in photosynthetic organisms. Therefore, strategies involving both non-enzymatic and enzymatic antioxidants to detoxify ROS are under operation under these conditions. The antioxidant enzymes such as superoxide dismutases, catalases and peroxidases are of particular interest in the nitrogen-fixing cyanobacteria in the detoxification of free radicals (Alqueres *et al*., 2010; Regelsberger *et al*., 2004; Robson and Postgate, 1980). $H_2O_2$ is detoxified to water and molecular $O_2$ by catalases or to water using reductants like ascorbate, GSH, thioredoxins by (Bannister *et al.,* 1987; Cadenas 1989). Canini *et al.* (1991) reported both the Mn- and Fe-SOD isoforms in cyanobacteria. This defense mechanism are robust for imparting stress tolerance to the cyanobacteria under stress conditions. Accumulation of intracellular proline has been observed in the cells in response to stress conditions. Proline acts as osmo-regulator and is an important compatible solute (Wu *et al*., 1998, Molinari *et al*., 2004). The role of proline as a redox buffer in case of cells exposed to stress conditions has been reported by Hare and Cress (1997). Proline has multifaceted role in response to abiotic stress conditions. This include protection of enzymes and proteins (Nikolopoulos and Manetas, 1991; Delauney and Verma, 1993), regulation of cytosolic acidity (Venekemp, 1989), scavenging of free radicals (Matysik *et al*., 2002) and membrane stabilization by interactions with phospholipids (Wu *et al*., 1995). Differential levels of proline accumulation in stressed and non-stressed plant species was observed by Molinari *et al*. (2007). Therefore, the accumulation of proline is of paramount importance in relation to salinity stress conditions.

Heavy metals are one of the most common pollutants worldwide, being a serious hazard to the environment and public health. Environmental pollution by heavy metals has been known since long and exposure to heavy metals still continues and worsening, particularly in less developed countries. Use of contaminated water for irrigation, accelerated input of chemical fertilizers, use of copper sulfate for controlling insect pests in paddy and heavy metal-containing pesticides increase the load of copper in the environment. Although, copper sulfate ($CuSO_4$) has been used to control of algal growth its addition can lead to substantial changes on biotic and abiotic structure of the water ecosystem (Bressan *et al.,* 2008). There are reports on the impact of copper sulfate on the microbial community structure of a water bodies receiving copper (Shade *et al.,* 2012). Increase in copper content in paddy soil irrigated with waste water has been reported by Luo *et al*. (2003). Heavy metals such as copper exert a negative pressure on microorganisms including cyanobacterium through the contamination of rice paddies. Kumar and Gaur (2014) used cyanobacterium for the removal of copper from copper contaminated water bodies. Cao and Hu (2000) also reported an increase in the levels of copper in the brown rice, rice hull and rice straw. Increase in the food demand for the ever-growing population requires enormous use of fertilizers and pesticides. Some of these pesticides contain heavy metals which contaminate the paddy fields and the cyanobacteria inhabiting the paddy fields. Increase in the anthropogenic activities coupled with excess use of copper containing algicides, metal containing fertilizers, organic manures and fungicides leads to toxic levels of and have been reported in the aquatic ecosystems (Yruela 2005; Bona *et al.,* 2007). Mallick *et al.* (1990) reported inhibitory effect of copper on the growth of cyanobacteria. *Rai et al.* (1991) observed the adverse effect of copper on the photosynthetic efficiency of cyanobacteria. However, prolonged and repeated exposure of cyanobacteria to copper has been found to induce physiological tolerance despite its toxicity (Rai *et al.,* 1991; Shavyrina *et al.,* 2001). Copper, at low concentrations is required as a cofactor for the electron transfer and in thylakoid plastocyanin (Cavet *et al*., 2003; Scholnick and Keren 2006). However, copper has been shown to induce stress responses in *Synechococcus* and reduce cellular oxygen evolution (Ybarra and Webbb 1999). Copper is required for several essential biological processes such as respiration, through the cytochrome oxidase, or in photosynthesis through the electron transfer protein plastocyanin in cyanobacteria. Different enzymes including oxidases, monooxygenases, dioxygenases and superoxide dismutases also require copper as metal cofactor. Choudhary *et al.* (2007) attributed copper toxicity to its two oxidation states and the generation of free radicals. There are reports that copper induces production of reactive oxygen species via Haber–Weiss and Fenton reactions leading to damage of proteins, nucleic acids, amino acids, membrane lipids, and interfere with the transport processes and substitutes for Mg in chlorophyll (Küpper *et al.,* 2003). Sancenon *et al.* (2003) reported that a desired level of Cu in aerobic organisms is maintained by a highly efficient and conserved copper homeostasis system or by metal-chelating proteins (Sancenon *et al.,* 2003). Bhargawa *et al.* (2008) observed that excess copper induced anoxygenic photosynthesis in the cyanobacterium *Anabaena doliolum*. Hudek *et al*. (2012) also observed the adverse impact of heavy metals on cyanobacteria. Various strategies have been evolved by living systems to combat the negative impact of various heavy metals including copper on cellular metabolism. Protection of photosystems and electron transport complexes is important in such a scenario (Alia *et al.,* 1997). Accumulation of proline plays a vital role in scavenging reactive oxygen species (Sivakumar *et al.,* 2001). Further, the structural integrity of membranes, proteins, and nucleic acids need to be maintained (Alia and Pardha-Saradhi 1991; Sivakumar *et al.,* 2000; Sharmila and Pardha-Saradhi 2002). Similarly the regulation of cellular pH, NAD(P)+ to NAD(P)H ratio also plays an important role in combating the heavy metal stress (Alia and Pardha-Saradhi 1993; Greene *et al.,* 1992; Pardha-Saradhi *et al.,* 1996, 2014; Shabnam *et al.,* 2014). Therefore, understanding of the mechanisms responsible for the development of increased resistance to the toxic effect of copper in cyanobacteria is of practical importance.

Most of the studies related to the abiotic stress tolerance were conducted by exposing the organism to the stresses individually. For example, to assess the impact of salinity or heavy metal, the test organism is exposed to salinity and the concerned heavy metal. However, in the natural environment the cyanobacteria may be exposed to multiple stress conditions. Interestingly, very few studies have been conducted to understand the effect of combined stress on the growth and physiology of cyanobacteria. We also do not have much information on the combined effects of stresses such as salinity and heavy metals such as copper on cyanobacteria. Bhargawa *et al.,* (2008) observed that susceptibility of organisms to a particular stress may be altered due to other stresses. Murali and Teramura (1987) observed that drought and mineral deficiency are capable of modifying the UV-B impact on plants. Similarly, enhanced tolerance to UV-B has been reported in the cyanobacterium *Cylindrospermum* sp. due to pre-exposure to UV-B (Chris *et al.,* 2006). In another study, it was observed that pre-treatment with copper enhanced the UV-B tolerance in the nitrogen fixing cyanobacterium *Anabaena doliolum* (Bhargawa *et al*., 2008). Since salinity and copper is a serious threat to cyanobacteria it is extremely important to understand and the combined effect of these stresses in the first place. Further, how the copper pre-treatment is going to modify the salinity tolerance in the cyanobacteria needs to be researched. Modulation of the stress response in the cyanobacterium exposed to salinity and copper stress is therefore important in understanding the stress response. However, systematic information is lacking on the growth, cellular constituents, nitrogen metabolism, antioxidant machinery of the organism under combined exposure to copper and salinity. Systematic documentation of the physiological and biochemical impairments in cyanobacteria under tress conditions induced by copper and salinity is therefore is important. Therefore, the study of the mechanism(s) underlying the stress tolerance in *Anabaena doliolum* is important not only to understand the physiological and biochemical basis of stress tolerance, but is also crucial for development of novel cyanobacterial inoculants under stress conditions. Since the cyanobacteria occupy an important position in food web, loss of cyanobacterial biomass may seriously affect soil fertility through nitrogen and carbon fixation. The loss of cyanobacterial productivity due to stress may adversely affect the soil nitrogen dynamics and increase our dependence on chemical nitrogen. Furthermore, they are important as bioremediation agents in cleaning up the environment, and thereby reducing pollution load. Therefore, the loss of productivity may also enhance the risk of pollution load in the ecosystem. Therefore, it is important to assess the combined effect of salinity and copper on growth and physiological parameters of a cyanobacterium.

# MATERIALS AND METHODS

## 3.1 Experimental organism and growth conditions

The diazotrophic cyanobacterium *Anabaena doliolum* was routinely established at the Centre for Conservation and Utilization of BGA, ICAR-Indian Agricultural Research Institute, New Delhi, India was used for the present study. This strain was regularly cultured in BG-11 medium without added nitrogen in unialagal form. The pH of the growth medium was adjusted to 7.5 and a light intensity of 72 $\mu$mol $m^{-2}$ $s^{-1}$ at a temperature of 28 $\pm$ $2^0$C for 16/8 h light and dark period.

## 3.2 Culture methods

Borosil grade glassware was used in the present investigation and the glass wares were washed by submerging them for 24 hrs in chromic sulphuric acid mixture. Washing in running tap water for at least an hour was done. Detergent washing was performed with tap water several times. Finally, the glassware was rinsed using double distilled water and oven dried. The culture vessels used in the investigation were plugged with non-absorbent cotton wool.

## 3.3 Chemicals

All the chemicals used in the present work were of Analytical grade and procured from standard companies.

## 3.4 Sterilization

Sterilization of the glassware as well as the nutrient solution was done by moist heat in an autoclave at $121^0$C, 15 lb/inch$^2$ pressure for 18 minutes.

## 3.5 Growth medium

*Anabaena doliolum* was routinely grown and maintained in BG 11 basal medium without combined nitrogen (Stanier *et al.*, 1971).

**Table 3.1: Composition of BG11 basal medium**

| Constituents | Concentration (g/L) |
|---|---|
| Sodium nitrate | 1.50 |
| Di potassium hydrogen phosphate | 0.04 |
| Magnesium sulphate | 0.075 |
| Calcium chloride | 0.036 |
| Citric acid | 0.006 |
| Ferric ammonium citrate | 0.006 |
| EDTA (di sodium magnesium salt) | 0.001 |
| Sodium carbonate | 0.02 |
| Micronutrient solution (A5 solution) | 1 ml |
| pH | 7.1- 7.3 |

### 3.6 Salinity and copper treatment

The selected cyanobacteria *Anabaena doliolum* was exposed to different concentrations of copper and salinity as mentioned below. Different concentrations of NaCl (50, 100, 150 and 200 mM) and Copper chloride (1, 3, 5, 7 and 10 µM) were used to determine the $LD_{50}$ concentration. Accordingly treatment combinations have been designed for the objectives mentioned in the study. The cultures that did not receive the copper and salinity treatment were taken as control.

**Estimation of Physiological response of the cyanobacterium to salinity and copper**

$T_1$ = Control
$T_2$ = 150 mM NaCl
$T_3$ = 5µM $CuCl_2$
$T_4$ = 150 mM NaCl +5µM $CuCl_2$

**To understand the modulation of salinity tolerance in cyanobacterium through copper pre-treatment.**

The cyanobacterium was pre-exposed Copper chloride (5µM) for different timings for 24 hrs, 48 hrs and 72 hrs. Subsequently, the pre-exposed cyanobacterium is treated with NaCl (150 and 200 mM). The following treatment combinations have been designed.

$T_1$ = Control (0 mM NaCl)
$T_2$ = 150 mM NaCl
$T_3$ = 200 mM NaCl

**24 hour pre-treatment with $CuCl_2$ (5 µM)**

$T_4$= 150 mM NaCl
$T_5$ = 200 mM NaCl

**48 hour pre-treatment with $CuCl_2$ (5 µM)**

$T_6$= 150 mM NaCl
$T_7$ = 200 mM NaCl

**72 hour pre-treatment with $CuCl_2$ (5 µM)**

$T_8$ = 150 mM NaCl
$T_9$ = 200 mM NaCl

### 3.7 Dry weight

A known quantity of the cyanobacterial suspension (10 ml) was taken, after adequate stirring was filtered using Whatman No.42 filter paper. The cultures were then oven dried at $60^o$ C and cooled until constant weight was achieved in desiccator. Dry weight was recorded as difference in the weights (Sorokin, 1973).

### 3.8 Pigment profile

### 3.8.1 Chlorophyll

To determine the total chlorophyll content the cold extraction method (McKinney, 1941) was used.

**Reagent:** Acetone (90%)

**(i) Procedure**

A known volume of the cyanobacterial suspension (5 ml) was homogenized and centrifuged at 3000 rpm for 10 min. To the pellet obtained, acetone (5 ml, 90%) was added and the tubes were incubated at $4^o$c overnight. After centrifugation of the suspension, the absorbance of the supernatant was recorded at 650 nm and 665 nm against 90% acetone as blank. The following formula was used to calculate the amount of chlorophyll and the chlorophyll content was expressed as µg/mg dry weight.

$$\text{Chlorophyll } (\mu g/mg \text{ dry weight}) = (20.2 \times A_{650}) + (8.02 \times A_{665})/1000$$

### 3.8.2 Carotenoids

The protocol of McKinney (1941) was used to estimate the carotenoid content of the cyanobacteria.

**(ii) Procedure**

Cyanobacterial suspension (5 ml) was centrifuged at 3000 rpm for 10 min and the pellet was washed with distilled water thoroughly. Acetone (5 ml, 90%) was added to the tubes and incubated overnight at 4$^o$C. The absorbance measured at 480 and 510 nm using 90% acetone as blank was used to determine the total carotenoid content.

$$\text{Carotenoids } (\mu g/ \text{ mg dry weight}) = 7.6 \times E_{480} - 1.49 \text{ x}510/1000$$

Where, $E_{480}$ and $E_{510}$ are absorbance at 480 and 510 nm, respectively. The carotenoid content was expressed as µg/mg dry weight.

### 3.8.3 Phycocyanin

Phycocyanin content of the cyanobacteria was estimated following the method of Bennett and Bogorad (1973).

**Reagent:** phosphate buffer (0.05 M, pH, 7.5)

**(iii) Procedure**

To the pellet left after the cold extraction for chlorophyll and carotenoid content, 5 ml of 0.05 M phosphate buffer, pH 7.5 (obtained by mixing equal volume of 0.1M $KH_2PO_4$ and 0.1 M $K_2HPO_4$) was added. The tubes were kept in deep freeze overnight. This was followed by repeated freezing and thawing till the pellet became colourless and the blue pigment was released in the supernatant. Absorbance of the samples was recorded at 562, 615 and 652 nm, respectively against a reagent blank and the amount of phycocyanin was calculated by using the following equation below. The amount of phycocyanin was expressed as µg/g dry weight.

$$\text{Phycocyanin (µg/ dry weight)} = \frac{A_{615} - 0.474 \times A_{652}}{5.34}$$

### 3.9 Estimation of average filament length

A small amount of the cyanobacterial suspension was taken out with a pipette and mounted on a clear grease free slide. The filaments were focused under low power of microscope. The filaments present in a field were all counted for their number of cells (vegetative cells as well as heterocysts). The average length of filament in a sample was obtained by adding up the number of cells in individual filament counted and dividing by their total number (Mishra, 2003).

### 3.10 Estimation of heterocyst frequency

The number of heterocyst per hundred vegetative cells is referred as heterocyst frequency and was calculated according to Mishra (2003) as follows:

$$\text{Heterocyst frequency} = \frac{\text{Total number of heterocyst}}{\text{Total number of vegetative cells}} \times 100$$

### 3.11 Estimation of Protein

Protein content of the cyanobacterium was estimated by the method of Lowry *et al.* (1951).

**Reagents:**

I. Sodium hydroxide (1 N)

II. (i) Sodium carbonate solution (5%)

(ii) Copper sulphate 0.5% in 1% of Sodium potassium tartarate.

- 2ml (ii) was mixed with 48ml of (i) prepared immediately before use.

III. Folin-ciocalteau reagent: Diluted the commercial reagent with an equal volume of water just before use.

IV. Standard protein: Bovine serum albumin solution was prepared in the range of 10-160 µg ml$^{-1}$.

**Procedure:**

To the protein sample of the cyanobacterium (0.5 ml) an equal amount of 1N NaOH was added. The tubes were then kept in a boiling water bath for 5 min and Reagent II (2.5 ml) was added after cooling and the samples were allowed to stand for 10 min at room temperature. Subsequently, reagent III (0.5 ml) was added rapidly and mixed thoroughly and incubated for15 min in room temperature. The absorbency of the solution was measured using a spectrophotometer at 650 nm. A standard curve of protein was prepared by using bovine serum albumin as standard.

### 3.12 Total Carbohydrate:

The total carbohydrate content of the cyanobacterial samples were estimated by the method of Spiro (1966).

**Reagents:**

(i) Standard glucose solution 10-100 µg $ml^{-1}$ in water
(ii) Anthrone reagent was prepared by mixing 0.1 g Anthrone reagent and thiourea (1 g) in 100 ml of 75 % $H_2SO_4$ and incubate at $85^0$ C for 20 minutes in water bath.

**Procedure:**

To a known volume of the cyanobacterial suspension (1 ml), 4 ml of Anthrone reagent was added. The samples were then incubated at $100^0$C for 15 minutes in a water bath and incubated and cooled in tap water. Absorbance of the samples was recorded at 620 nm using spectrophotometer. A calibration curve for the sugar was prepared using glucose as standard.

### 3.13 Assay of N- assimilation enzymes:

### 3.13.1 Nitrate reductase

The enzyme nitrate reductase is involved in the reduction of nitrate to nitrite. Assay of Nitrate reductase (NR) assay was performed according to Herrero *et al.* (1981).

**Reagents:**

(i) Sodium nitrate ($NaNO_3$)
(ii) Sulphanilamide (1g in 100 ml of 1:4 HCl and water)
(iii) α – (N-1)) napthyl ethylene diaminedihydrochloride (NEDD), (0.2% W/V)

**Enzyme assay**

A known volume of algal suspension was centrifuged and washed with sterile water and incubated in basal medium containing $NaNO_3$ (10 mM, pH 7.0). The samples (1 ml) were taken out after the incubation period of 24 h and Sulphanilamide (2 ml) was added. Contents were thoroughly mixed followed by the addition of 2 ml α – (N-1) napthyl ethylene diamine dihydrochloride (NEDD). The tubes containing the samples were incubated at room temperature for 30 minutes. Absorbance of the cultures was recorded at 540 nm and the enzyme activity was expressed as as µmol $NO_2$ $mg^-$ of protein. A standard curve was prepared using known concentrations of sodium nitrite. Nitrite was estimated by the method of Snell and Snell (1949).

### 3.13.2 Glutamine Synthetase activity

Cyanobacterial glutamine synthetase enzyme (L-glutamate: ammonia lyase, ADP- forming EC 6.3.1.2) basically catalyzes the incorporation of ammonia into glutamate and requires ATP:

$$NH_4^+ + \text{glutamate} + ATP \xrightarrow[Mg^{2+}/Mn^{2+}]{} \text{glutamine} + ADP + Pi$$

The glutamine synthetase also catalyzes the formation of $\gamma$- glutamyl hydroxamate by the transferase reaction.

$$\text{Glutamine} + NH_2OH \xrightarrow[\text{ADP, Pi, arsenate}]{} \gamma \text{ glutamyl hydroxamate} + NH_4^+$$

In the present experiment the transferase activity was determined by measuring the amount of $\gamma$ –glutamyl hydroxamate formed using the method of Shapiro and Stadtman (1979).

**Reagents**

(a) Glutamine : 0.1 M, pH 7.0
(b) Sodium arsenate : 0.1M, pH 7.0
(c) Sodium ADP : 0.01M, pH 7.0
(d) Mn $Cl_2$ : 0.1 M
(e) Hydroxylamine – Hcl : 2.0 M
(f) Imidazole HCl buffer : 100 mM, pH 7.0
(g) $\gamma$- glutamyl hydroxamate : 10 mM standard
(h) Stop mixture: Ferric chloride (10% w/v), Trichloroacetic acid (24% w/v), 6N HCl and double distilled water in the ratio of 8:2:1:13

Reaction mixture was prepared by mixing the following volumes of the above solutions.

I. Imidazole buffer : 2.0 ml
II. Glutamine : 1.5 ml
III. $MnCl_2$ : 1.5 ml
IV. Sodium ADP : 2.0 ml
V. Sodium arsenate : 1.0 ml
VI. Hydroxylamine- HCl : 1.5 ml
VII. Water : 2.0 ml

Hydroxylamine hydrochloride was neutralized with 15 ml of 2N NaOH before adding other components of the mixture.

**Enzyme assay**

The cell suspension (0.5 ml) was treated with 0.25 ml toluene and incubated overnight at $4^0$C. Subsequently, these cells were centrifuged and the toluene layer was removed and cell pellet was suspended in imidazole buffer (0.5 ml). 0.5 ml of reaction mixture was added to start the reaction and the samples were incubated at $37^0$C for 30 min. 3.0 ml of stop mixture was added to terminate the reaction and absorbance was recorded at 540 nm. Finally the transferase activity was expressed as nmol mg protein $^{-1}$

### 3.13.3 Estimation of Nitrogenase Activity

The nitrogenase enzyme catalyzes the conversion of molecular nitrogen to ammonia, a reaction which is dependent on reduced ferredoxin and obligatorily coupled to reduction of protons resulting in formation of molecular hydrogen:

$$N_2 + 8\ Fd_{red} + 8\ H^+ + 16\ ATP \longrightarrow 2\ NH_3 + H_2 + 8\ Fd_{ox} + 16\ ADP + 16\ Pi$$

Besides $N_2$ several compounds with a triple bond are reduced by Nitrogenase. For example, acetylene ($C_2H_2$) is also reduced to ethylene ($C_2H_4$). In order to estimate nitrogenase activity, acetylene reduction assay of Stewart *et al.* (1968) was used. The assay was performed in calibrated triplicate serum bottles of about 8.5 ml capacity. Cyanobacterial suspension (5 ml) was taken in each bottle and the acetylene concentration was kept at 10% and the bottles were incubated at $28\pm1^0$C at 2500 lux intensity with intermittent shaking for 4 hour. The reaction was terminated by injecting

0.8 ml of 15% (w/v) trichloroacetic acid. Finally, the ethylene produced in reaction bottle was analyzed by a gas chromatograph (Nucon 5765 GC) fitted with a poropak R column and a hydrogen flame ionization detector. The nitrogenase activity is expressed as nmol $C_2H_4$ formed mg chlorophyll$^{-1}$ h$^{-1}$.

### 3.14 Assay of enzymatic and non-enzymatic antioxidant enzymes

### 3.14.1 Superoxide dismutase (EC 1.15.1.1)

**Preparation of samples**

Cyanobacterial samples collected from the cultures after appropriate treatment were suspended in cell lysis buffer (pH 7.0) consisiting of EDTA (1 mM) and polyvinyl pyrrolidone (10%). Samples were centrifuged at 15000xg for 30 min at 4º C and the resulting supernatant was used for the antioxidant enzyme activity.

**Reagents**

1. Methionine (200 mM): L-Methionine 0.298 g was dissolved in water and the volume was made up to 10 ml with double distilled water.
2. Nitro-blue tetrazolium chloride (NBT) (2.25 mM): NBT 0.0184 g was dissolved in water and the volume was made up to 10 ml with double distilled water.
3. EDTA (3.0 mM): EDTA 0.0558 g was dissolved in water and the volume was made up to 50 ml with double distilled water.
4. Riboflavin (60 μM): Riboflavin 0.0023 g was dissolved in water and the volume was made up to 100 ml with double distilled water.
5. Sodium carbonate (1.5 M): Sodium carbonate 7.95 g was dissolved in double distilled water and the volume was made up to 50 ml with double distilled water.
6. Phosphate buffer (100 mM, pH 7.8):

Sol A: Potassium dihydrogen phosphate 6.8 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Sol B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Buffer was prepared by mixing 8.5 ml of sol. A and 91.5 ml of sol. B and final pH was adjusted with the help of pH meter.

**Enzyme assay**

Superoxide dismutase activity (SOD) was estimated by recording the decrease in optical density of formazone made by superoxide radical and nitro-blue tetrazolium dye (Dhindsa *et al*., 1981). Reaction mixture (3 ml) contained 13.33 mM methionine (0.2 ml of 200 mM), 75 µM nitro- blue tetrazolium chloride (NBT) (0.1 ml of 2.25 mM), 0.1 mM EDTA (0.1 ml of 3 mM), 50 mM phosphate buffer (pH 7.8) (1.5 ml of 100 mM), 50 mM sodium carbonate (0.1 ml of 1.5 M) and 0.05 to 0.1 ml enzyme, 0.9 to 0.95 ml of water (to make a final volume of 3.0 ml). Riboflavin (2 mM, 0.1 ml) was added to start the reaction and the tubes were placed under two 15 W fluorescent lamps for 15 min. The control consisted of complete reaction mixture without enzyme, which gave the maximal colour. The reaction was stopped by switching off the light to maintain dark conditions. A complete reaction mixture which is non-irradiated served as a blank. The absorbance was recorded at 560 nm. One unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme and is expressed as units mg protein$^{-1}$.

### 3.14.2 Ascorbate peroxidase (EC 1.11.1.11)

**Reagents**

1. Ascorbic acid (3.0 mM): Ascorbic acid (0.0265 g) was dissolved in water and the volume was made up to 50 ml with double distilled water.
2. EDTA (3.0 mM): EDTA (0.0558 g) was dissolved in water and the volume was made up to 50 ml with double distilled water.
3. Hydrogen peroxide (1.5 mM): 16 µl of standard hydrogen peroxide (30%) was dissolved in water and the volume was made up to 100 ml with double distilled water.
4. Phosphate buffer (100 mM, pH 7.0)

Sol A: Potassium dihydrogen phosphate 6.80 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Sol B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Buffer was prepared by mixing 39 ml of sol. A and 61 ml of sol. B, and final pH was adjusted with the help of pH meter.

**Enzyme assay**

Ascorbate peroxidase (APX) was assayed by the method of (Nakano and Asada, 1981). Decrease in optical density due to ascorbic acid at 290 nm was recorded. The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.0) (1.5 ml of 100 mM), 0.5 mM ascorbic acid (0.5 ml of 3.0 mM), 0.1 mM EDTA (0.1 ml of 3.0 mM), 0.1 mM $H_2O_2$ (0.2/0.6 ml of 1.5 mM), 0.1 ml enzyme and water 0.6 ml (to make a final volume of 3.0 ml). Reaction was started with the addition of 0.2 ml of hydrogen peroxide and decrease in absorbance for a period of 30 seconds was measured at 290 nm in an UV-visible spectrophotometer (Halo DB 20, USA). The enzyme activity was expressed as units mg protein$^{-1}$ that determines the amount of enzyme necessary to decompose 1 μmol ascorbate per mg protein.

**3.14.3 Catalase (EC 1.11.1.6)**

**Reagents**

1. Phosphate buffer (0.1 M, pH 7.0)

Sol A: Potassium dihydrogen phosphate 6.8 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Sol B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Buffer was prepared by mixing 39 ml of solution A and 61 ml of solution B, and final pH was adjusted.

2. Hydrogen Peroxide (75 mM): A solution of 75 mM $H_2O_2$ was prepared by dissolving 775 μl of 30% $H_2O_2$ in 100 ml of double distilled water.

**Enzyme assay**

Catalase enzyme activity was assayed by measuring the disappearance of $H_2O_2$ according to Aebi (1984). Reaction mixture (3 ml) consisted of 0.5 ml of 75 mM $H_2O_2$ and 1.5 ml of 0.1 M phosphate buffer (pH 7) in cuvette. The reaction was initiated by adding 50 μl of diluted enzyme extract and the decrease in absorbance at 240 nm was observed for 1 min in a UV-visible spectrophotometer (Halo DB 20, USA). The initial and final contents of $H_2O_2$ were measured. Enzyme activity was computed by calculating the amount of $H_2O_2$ decomposed. The results were expressed as units on the basis of the amount of enzyme required to decompose 1 µmole $H_2O_2$ per milligram protein and expressed as units mg protein$^{-1}$.

### 3.14.4 Proline content

The proline content of the cyanobacterial samples were estimated according to the method of Bates *et al*. (1973).

**Reagents**

1. Sulfosalicylic acid: 3% aqueous solution.
2. Acid ninhydrin: 2.48 g ninhydrin was dissolved in 60 ml glacial acetic acid and 40 ml of 6N phosphoric acid, kept in 4 ºC.
3. Toluene
4. Glacial acetic acid

**Assay**

The cyanobacterial pellets collected from the cultures were ground using 3% sulfosalicylic acid (10 ml). The extract was filtered through Whatman's filter paper. Filtrate (2 ml) was then mixed with 2 ml acid ninhydrin and 2 ml of glacial acetic acid in a test tube. The mixture was heated at 100 ºC in a water bath for 1 h and the tubes were removed from water bath and quickly placed in ice to stop the reaction. This was followed by the addition of Toluene (4 ml) to the mixture and vortexing of the samples for 15-20 sec. Absorbance of the chromophore from the toluene phase was measured at 520 nm using UV-visible spectrophotometer (Halo DB 20, USA). A reagent blank was also prepared. The proline content was calculated using the standard curve prepared using L-proline.

### 3.15 Statistical analysis

All the experiments were performed in triplicate using triplicate samples. The data recorded on various parameters were subjected to statistical analysis.

# RESULTS

## 4.1 Effect of salinity and copper on the growth and cellular constituents of the cyanobacterium *Anabaena doliolum*

Based on the growth experiments using various concentrations of NaCl and copper chloride, it was observed that the cyanobacterium was able to grow well upto a NaCl concentration of 150 mM and copper chloride concentration of 5 µM (results not shown). Therefore, these two concentrations have been used for further experiments. The rice field cyanobacterium *Anabaena doliolum* was exposed to NaCl (150 mM) and copper chloride (5 µM) individually and together. Growth was recorded as increment in the dry weight over a period of time. The cyanobacterium exposed to NaCl showed a reduction in the dry weight by 16.85% whereas 40.44% reduction was observed in response to copper chloride on the twelfth day of treatment (Fig. 4.1). However, the cells exposed to salinity and copper together showed further reduction in the dry weight by 52.98%.

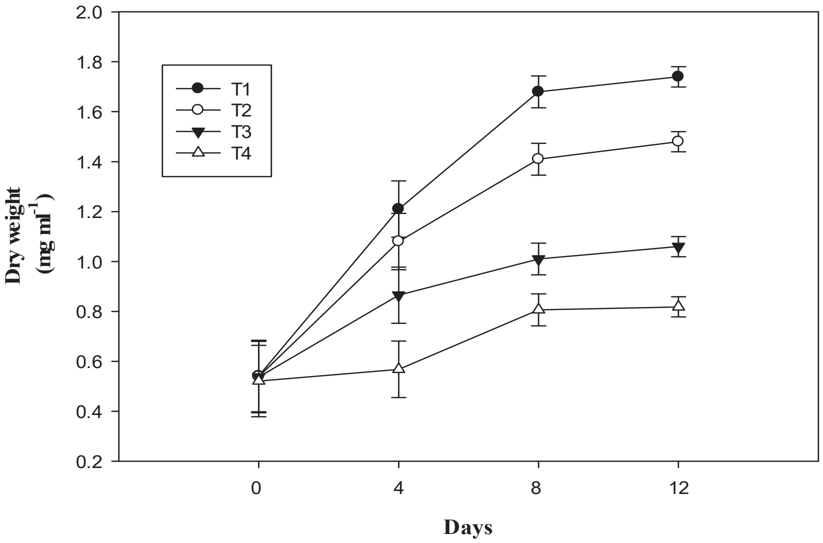

**Figure 4.1. Dry weight accumulation in the cyanobacterium *A. doiolum* in response to exposure to salinity and copper**

After treatment with salt and copper, the protein content of the cyanobacterium showed significant decrease (Fig. 4.2). The decrease in protein content observed was 39.55% and 46.69% in response to salinity and copper treatment. However, further reduction in the protein content (61.03%) was observed in response to combined exposure on twelfth day after treatment.

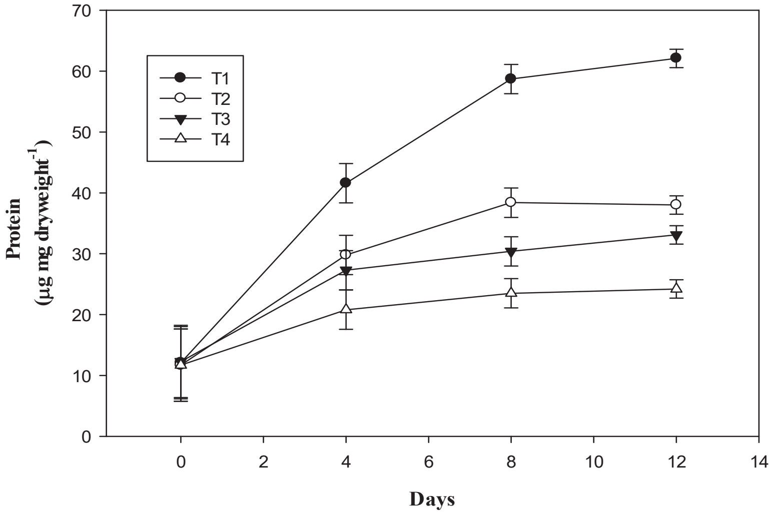

**Figure 4.2. Salinity and copper induced changes in the protein content of the cyanobacterium *A. doliolum*.**

In cyanobacteria the chlorophyll content may be considered as an important index for estimating the productivity. The cyanobacterium showed reduction in chlorophyll content with salinity and copper treatment (Fig. 4.3). On twelfth day of incubation with salinity, a reduction by 51.72% in the chlorophyll content was noticed whereas a reduction of 64.48% was observed in response to copper treatment. On the other hand, combined exposure of salinity and copper resulted in a decrease of 80.34% in the chlorophyll content. Similarly, one of the important photosynthetic accessory pigment phycocyanin also showed significant decrease in response to salinity and copper treatment (Fig. 4.4). The phycocyanin content was found to decrease by 32.39 and 52.11% in response to the stress induced by exposure to salinity and copper. Another accessory photosynthetic pigment, carotenoid also showed significant decrease in response to salinity and copper treatment (Fig. 4.5). The observed increase in the carotenoid content was 36.95 and 39.94% due to exposure to salinity and copper, respectively. Fig 4.6 and 4.7 depict the reduction due to salinity and copper treatment in the phycoerythrin and allophycocyanin content of the test cyanobacterium *Anabaena doliolum*. It appears that salinity and copper treatment leads to severe decrease in the pigment profile of the cyanobacterium *A. doliolum*.

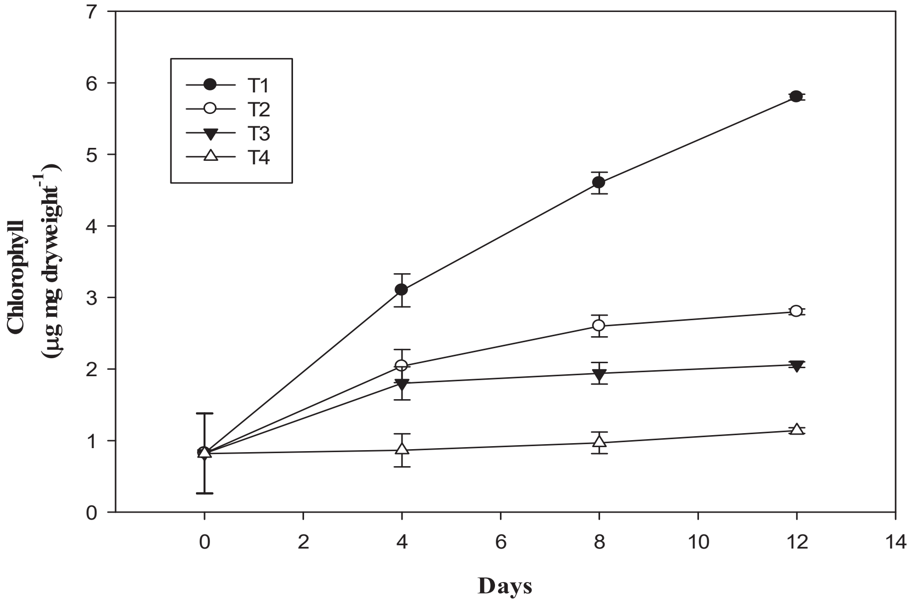

**Figure 4.3. Chlorophyll content of the cyanobacterium *A. doliolum* in response to salinity and copper treatment.**

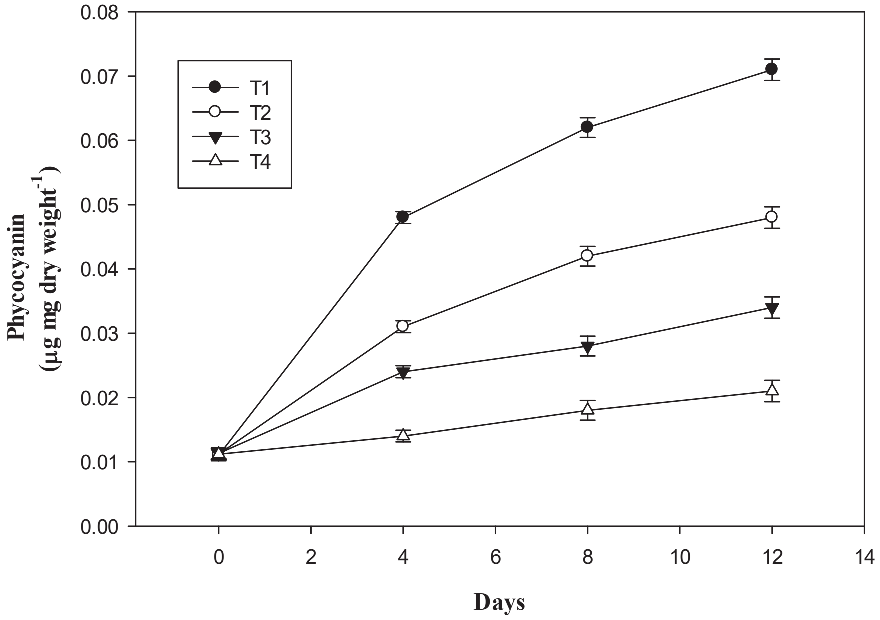

**Figure 4.4. Phycocyanin content of the cyanobacterium *A. doliolum* in response to salinity and copper treatment.**

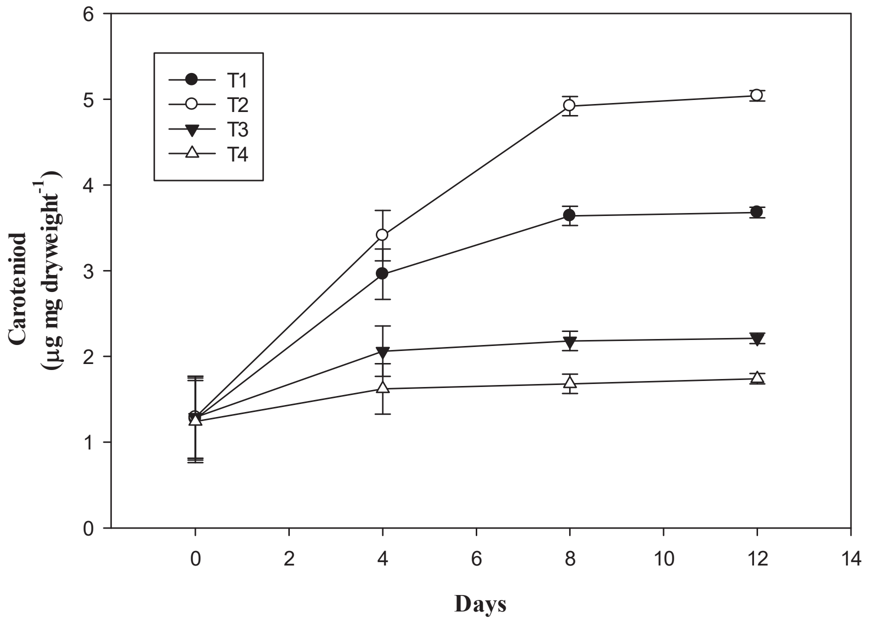

**Figure 4.5. Carotenoid content of the cyanobacterium *A. doliolum* in response to salinity and copper treatment.**

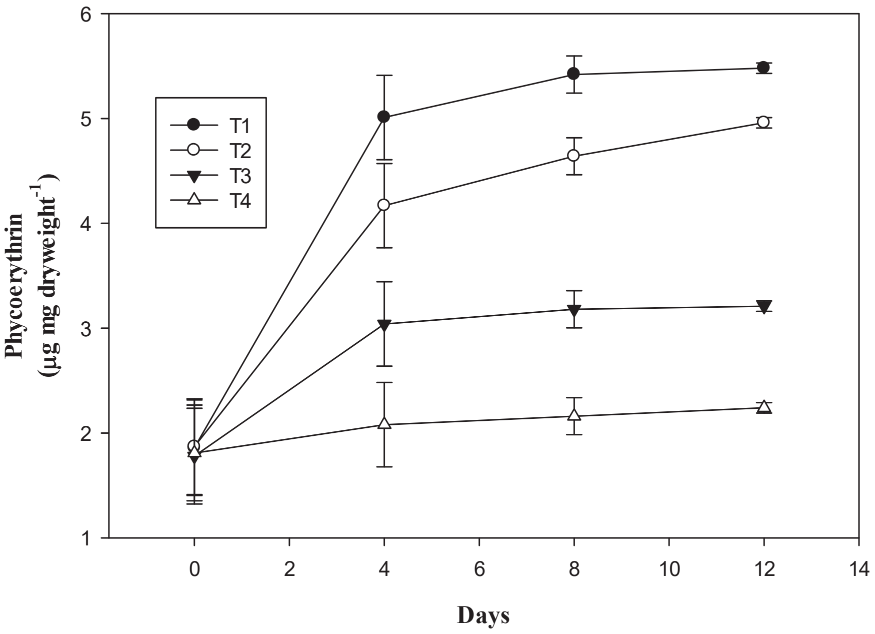

**Figure 4.6. Phycoerythrin content of the cyanobacterium *A. doliolum* in response to salinity and copper treatment**

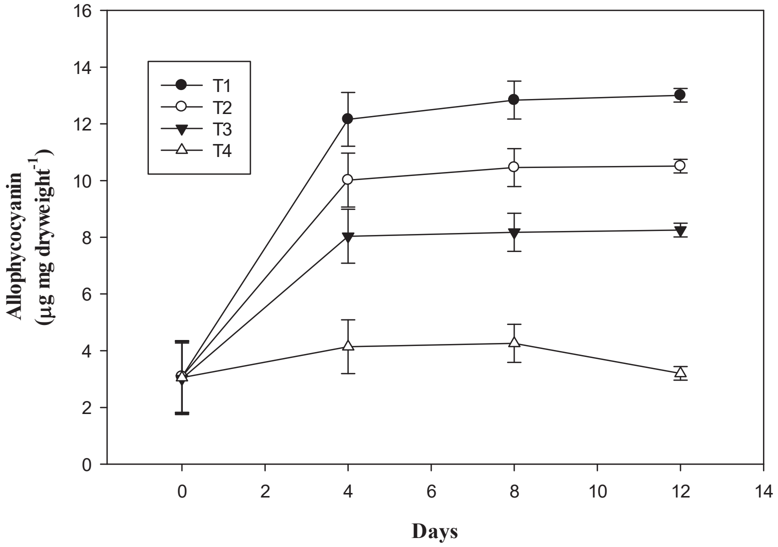

**Figure 4.7. Allophycocyanin content of the cyanobacterium *A. doliolum* in response to salinity and copper treatment**

On the contrary, the sugar content of the cyanobacterium *A. doliolum* sp. exposed to salinity increased significantly by 26.79% on twelfth day after the salinity treatment (Fig. 4.8). However, exposing the organism to copper as well as a combined exposure to copper and salinity resulted in decrease in the sugar content by 33.75% and 44.93%. An increase in the sugar content is considered as a general response by the cyanobacterium to cope with the osmotic imbalance.

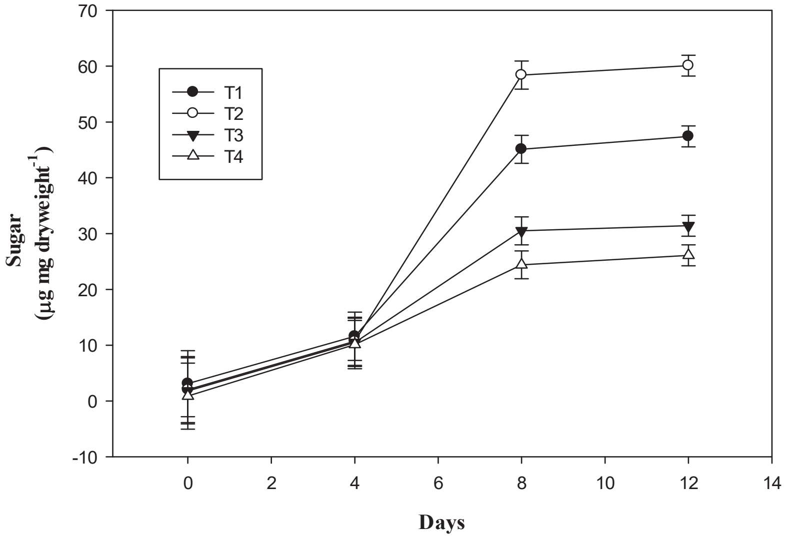

**Figure 4.8. Sugar contents of the cyanobacterium *A. doliolum* in response to salinity and copper exposure.**

## 4.2. Effect of salinity and copper exposure on the average filament length, heterocyst frequency and enzymes of nitrogen assimilation in the cyanobacterium *A. doliolum*.

We have calculated the changes in the average filament length of the cyanobacterium *A. doliolum* in response to salinity and copper exposure (Fig. 4.9). The filament length was maximum in control (51.6). However, at salinity treatment the filament length decrease in the filament was observed (40.1). Further the filament length reduced to 30.1 in response of copper treatment. Maximum reduction in the average filament length (20.6) was observed in response to the combined exposure to salinity and copper.

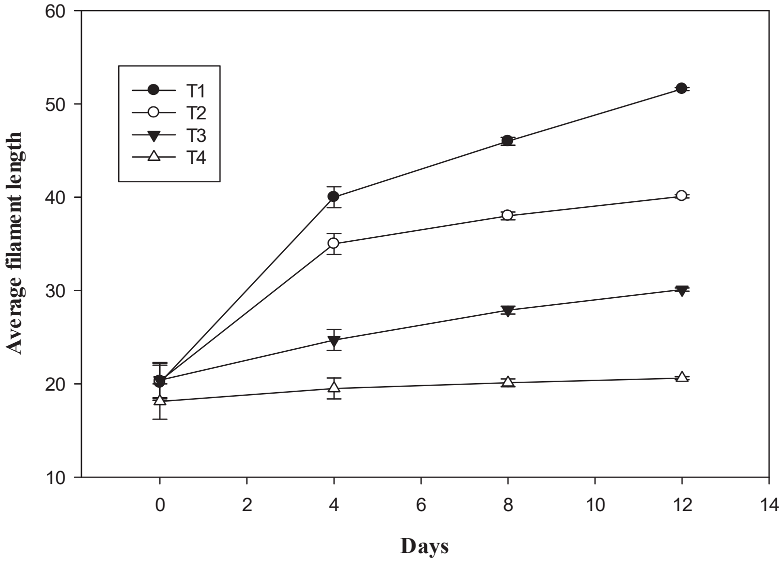

**Figure 4.9. Effect of salinity and copper on the average filament length of the cyanobacterium *A. doliolum*.**

The heterocyst frequency of the cyanobacterium *A. doliolum* exposed to salinity and copper was (Fig. 4.10). The control cells recorded the highest frequency of heterocysts (12.1%) followed by the cells treated with salt (10.4%) and copper chloride (6. 8%).However, cells treated with salt and copper chloride recorded the lowest frequency of heterocysts (3.1%).

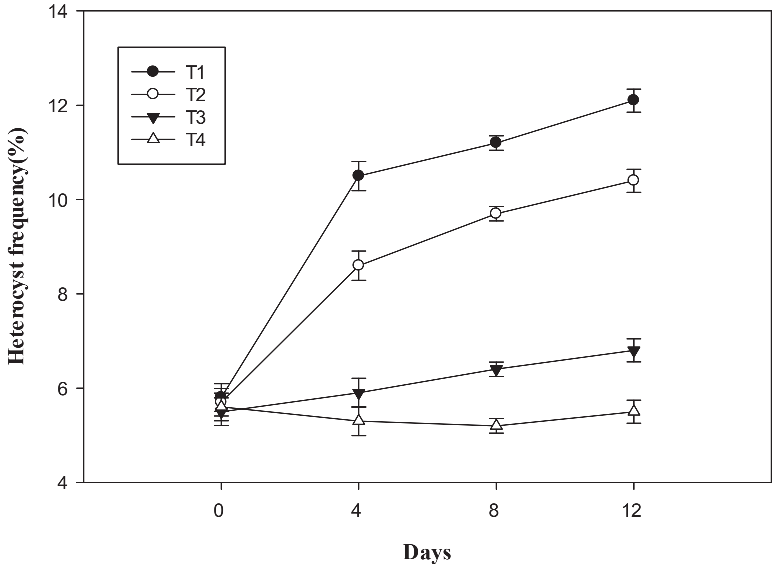

**Figure 4.10. Effect of salinity and copper on the heterocyst frequency of the cyanobacterium *A. doliolum.***

A study of the nitrogen assimilation enzymes was conducted in the cyanobacterium *A. doliolum* exposed to salinity and copper (Table 4.1). Salinity treatment resulted in an increase in the nitrate reductase activity of *A. doliolum*. Considerable increase in the nitrate reductase activity was observed in the test organism in response to salinity treatment. However, exposure to copper increased the nitrate reductase activity by 88.10%. Maximum increase in the nitrate reductase activity was noticed due to combined exposure to salinity and copper (82.78%). The enzyme, glutamine synthetase is the primary enzyme responsible for the assimilation of ammonia. Salinity treatment decreased the glutamine synthetase activity in *A. doliolum* by 12.81%. Further reduction in the glutamine synthetase activity by 25.64% was observed when the cells were exposed to copper. The reduction in the glutamine synthetase activity was severe (38.09%) in response to the combined exposure of salinity and copper. Acetylene reduction assay (ARA) was performed to assay the nitrogenase enzyme activity of the cyanobacterium *A. doliolum* exposed to salinity and copper. The control cells showed nitrogenase activity of 35.8 ± 0.252 µmoles $C_2H_4$ mg chl$^{-1}$ h$^{-1}$. Further, the nitrogenase activity declined to 29.7 ± 0.256 µmoles $C_2H_4$ mg chl$^{-1}$ h$^{-1}$ in case of cells exposed to 150 mM salinity. Copper treated cells showed nitrogenase activity of 18.4 ± 0.254 µmoles $C_2H_4$ mg chl$^{-1}$ h$^{-1}$. However, the nitrogenase activity further declined to 10.6 ± 0.153 µmoles $C_2H_4$ mg $chl^{-1}$ $h^{-1}$ in response to combined exposure of salinity and copper.

**Table 4.1. Effect of salinity and copper exposure on the N assimilation enzymes of the cyanobacterium *A. doliolum*.**

| Treatments | Nitrate Reductase (µmol mg $protein^{-1}$) | Glutamine Synthase (nmol mg protein $^{-1}$) | Ethylene formed (µmol $C_2H_4$ mg $chl^{-1}$ $h^{-1}$) |
|---|---|---|---|
| **T1** | 5.173±0.041 | 163.9±0.513 | 35.8±0.252 |
| **T2** | 11.43±0.231 | 142.6±0.458 | 29.7±0.256 |
| **T3** | 9.66±0.0153 | 121.8±0.0577 | 18.4±0.254 |
| **T4** | 9.45±0.0252 | 101.4±0.351 | 10.6±0.153 |

### 4.3 Effect of salinity and copper on the enzymatic and non-enzymatic antioxidant activity of the cyanobacterium *A. doliolum*.

The enzymatic as well as the non-enzymatic antioxidants play an important role in any organism exposed to stress conditions. We have also studied the antioxidant enzyme profile of the cyanobacterium *Anabaena* sp. exposed to salinity and copper. Exposure of the cyanobacteria to salinity, copper and combined exposure to both the stresses induced the activity of superoxide dismutase, ascorbate peroxidase and catalase. The increase in the superoxide dismutase activity was 18.18% on the 4th day of NaCl treatment (150 mM) whereas it was found to enhance by 41.17% upon exposure to 5 µM copper chloride. Highest increase (58.28%) in the superoxide dismutase activity was observed when the organism was exposed to both NaCl and copper together (Fig. 4.11). Similarly, the increase in the ascorbate peroxidase activity was highest due to combined exposure to NaCl and copper (82.52%). Salinity treatment had resulted in an increase in the ascorbate peroxidase activity by 47.61% (Fig. 4.12) whereas the copper treatment enhanced the activity by 62.12%. Regarding the catalase activity, salinity treatment increased the activity significantly (Fig. 4.13). Copper chloride treatment also was found to enhance the activity whereas the maximum activity was observed in response to combined application of NaCl and copper chloride.

Intracellular accumulation of proline is related to the stress tolerance of the organism. The proline content of the cyanobacterium *A. doliolum* showed a differential response to salinity and copper treatment. Significant increase in the proline accumulation was observed in the cyanobacterium *A. doliolum* exposed to salinity, copper as well as combined exposure to salinity and copper (Fig. 4.14). Increase in the proline content was 93.09% in response to the stress induced by NaCl whereas it enhanced to 35.52 % due to copper treatment. Combined exposure to copper and salinity on the other hand enhanced the proline content by 55.26%

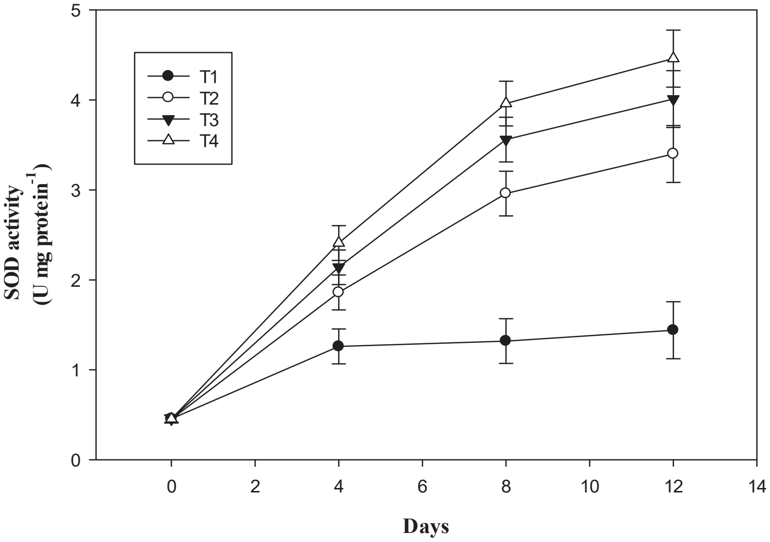

**Figure 4.11. Effect salinity and copper on the superoxide dismutase (SOD) activity of the cyanobacterium *A. doliolum*.**

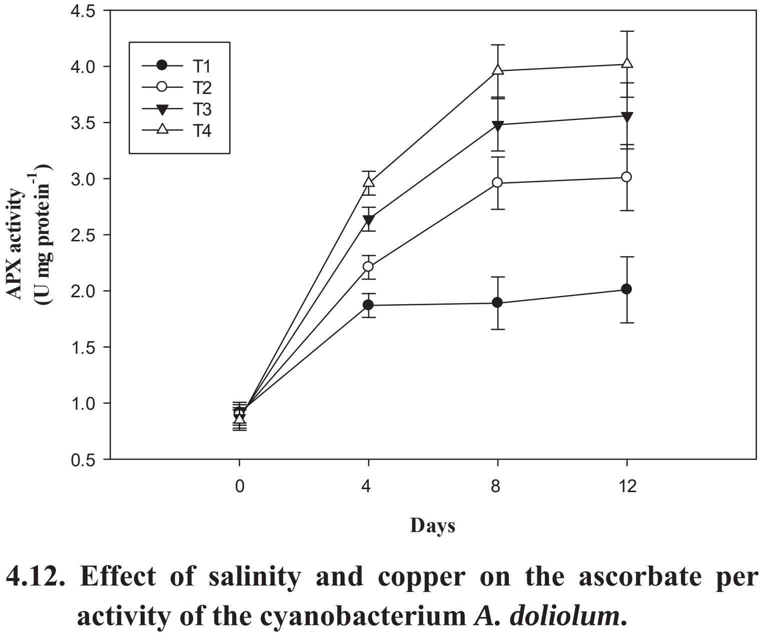

**Figure 4.12. Effect of salinity and copper on the ascorbate peroxidase (APX) activity of the cyanobacterium *A. doliolum*.**

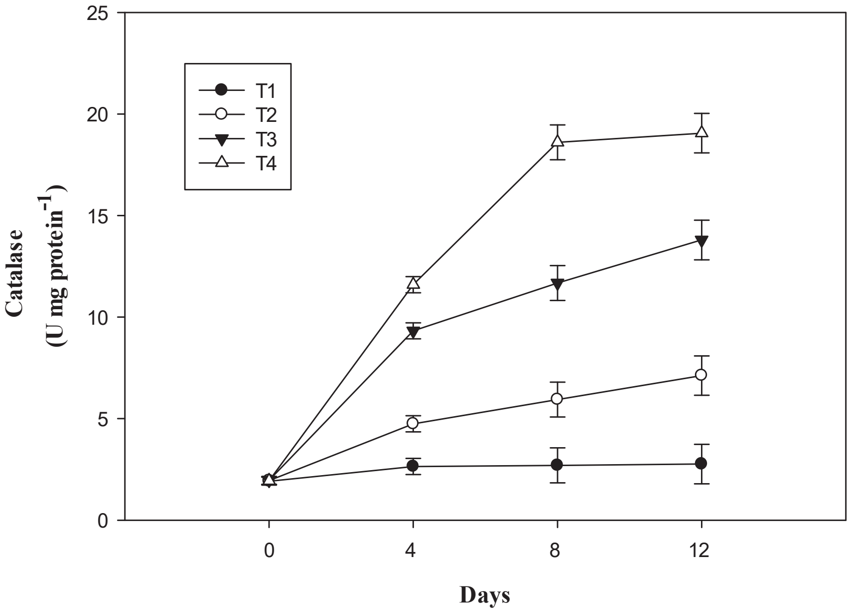

**Figure 4.13. Effect of salinity and copper on the catalase (CAT) activity of the cyanobacterium *A. doliolum*.**

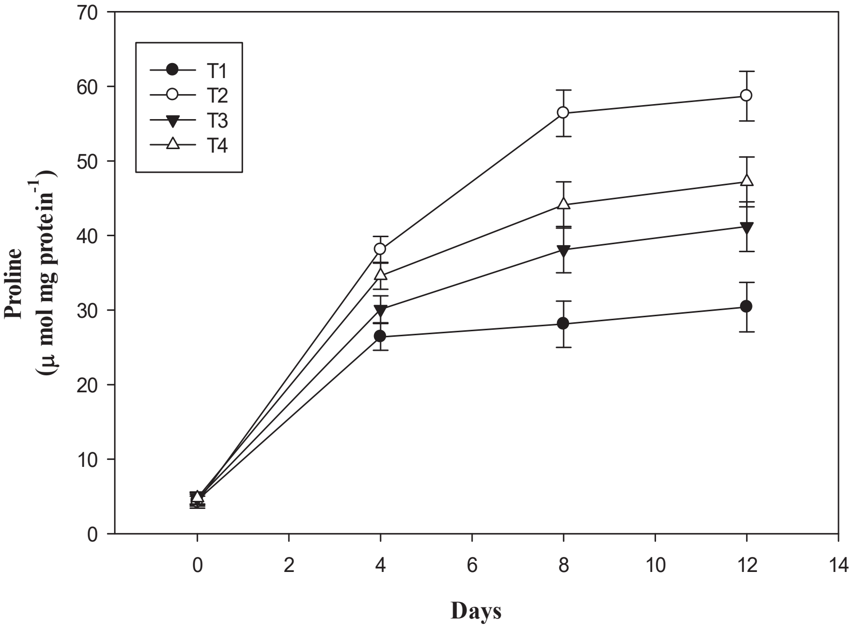

**Figure 4.14. Proline content of the cyanobacterium *A. doliolum* in response to salinity and copper treatment**

## 4.4. Effect of copper pre-treatment on the growth, cellular constituents of the cyanobacterium *A. doliolum*.

In the present set of experiments the importance of pre-exposure of the organism to copper chloride and its role in enhancing the salinity tolerance of the cyanobacterium *A. doliolum* was studied. For this, the test cyanobacterium was pre-exposed Copper chloride (5µM) for different time intervals for 24, 48 and 72 hrs. The pre-exposed cyanobacterium was subsequently exposed to NaCl concentration of 150 and 200 mM. The growth and cellular constituents of the cyanobacterium *A. doliolum*

Table 4.2 shows the effect of the pre-exposure to copper on the growth of the cyanobacterium *A. doliolum* estimated as increase in dry weight. It was observed that pre-exposure to copper for 24 hrs resulted in slight increase in dry weight as compared to cells directly exposed to 150mM NaCl. Similar increase was also observed with 48 hrs of pre-treatment with copper. However pre-exposure to copper for 72 hrs did not influence the growth significantly. The protein content of the cyanobacterium *A. doliolum* pre-exposed to copper treatment showed increase in the protein content (Table 4.3). On the other hand, the pre-treatment of 72 hrs resulted in poor response as compared to direct exposure to NaCl.

Photosynthetic pigments chlorophyll a also was found to respond positively to pre-exposure of 24hrs and 48hrs, although the response was not very significant (Table 4.4). However, the assessor pigments such as phycocyanin, phycoerythrin and allophycocyanin showed slight increase in response to pre-exposure with copper (Tables 4.5-4.7). Interestingly the carotenoid content showed a favourable and positive response to pre-exposure (Table 4.8). The heterocyst frequency and average filament length was not affected by pre exposure irrespective of the duration (Table 4.17). Table 4.10 depicts the sugar content of the cyanobacterium *A. doliolum* in response to pre-exposure. It was observed that the sugar content increased significantly at 24hrs and 48 hrs of pre-exposure. Pre-exposure to 72 hrs also resulted in increase of sugar content.

Effect of pre-exposure to copper on the enzymes of nitrogen assimilation was studied (Table 4.11). Although direct exposure resulted in increase in the nitrate reductase activity, pre-exposure did not influence the nitrate reductase activity. The glutamine synthase and nitrogenase activity however was not found to be influenced by the pre-exposure. The pre-exposure as profound influence on the antioxidant profile of the test cyanobacterium (Table 4.12). Pre-exposure to copper for 24hrs and 48hrs with copper significantly enhanced the activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidise and catalase. The accumulation of proline was also found to be influenced significantly by the pre-exposure. Increase in the proline content was noticed due to pre-exposure as compared to direct exposure.

Therefore, the results of the present study clearly indicated that pre-exposure to copper definitely had role in conferring salt tolerance to the cyanobacterium *A. doliolum.* Cyanobacterial cells exposed to short duration pre-exposure of 24 hrs and 48 hrs was found to be favourable whereas exposure to 72hrs had not much role in relation to enhancing the salinity tolerance of the cyanobacterium. Although there was not much increase in the growth and some cellular constituents and the antioxidants enzymes responded significantly to pre-exposure and subsequent treatment of salinity.

**Table 4.2. Effect of pre-exposure to copper on the dry weight accumulation (mgml$^{-1}$) of the cyanobacterium *Anabaena doliolum* further exposed to salinity.**

| Treatments | 4th day | 8th day | 12th day |
|---|---|---|---|
| **T1** | 1.36±0.0153 | 1.86±0.02 | 1.98±0.0152 |
| **T2** | 1.16±0.0152 | 1.36±0.0153 | 1.39±0.0153 |
| **T3** | 0.868±0.00208 | 0.614±0.0115 | 0.426±0.03 |
| **T4** | 1.18±0.01 | 1.48±0.767 | 1.56±0.0152 |
| **T5** | 0.876±0.0208 | 0.624±0.00252 | 0.434±0.01 |
| **T6** | 1.26±0.153 | 1.56±0.0148 | 1.64±0.020 |
| **T7** | 0.872±0.03 | 0.628±0.00208 | 0.438±0.035 |
| **T8** | 1.12±0.0135 | 1.41±0.0115 | 1.46±0.015 |
| **T9** | 0.856±0.0153 | 0.616±0.00153 | 0.618±0.005 |

T1 = Control (0 mM NaCl)

T2 = 150 mM NaCl

T3 = 200 mM NaCl

24hrs pre-treatment with 5µM$CuCl_2$

T4= 150 mM NaCl

T5 = 200 mM NaCl

48 hrs pre-treatment with 5µM$CuCl_2$

T6= 150 mM NaCl

T7 = 200 mM NaCl

72 hrs pre-treatment with 5µM$CuCl_2$

T8 = 150 mM NaCl

T9 = 200 mM NaCl

**Table 4.3. Effect of pre-exposure to copper on the Protein (µg $mg^{-1}$ dry weight) content of the cyanobacterium *Anabaena doliolum* further exposed to salinity.**

| Treatments | 4th day | 8th day | 12th day |
|---|---|---|---|
| **T1** | 46.1±0.30 | 64.4±0.37 | 66.4±0.17 |
| **T2** | 32.4±0.20 | 40.1±0.20 | 44.4±0.15 |
| **T3** | 12.6±0.15 | 18.1±0.26 | 18.6±0.15 |
| **T4** | 34.6±0.24 | 46.8±0.66 | 48.1±0.31 |
| **T5** | 12.8±0.43 | 18.6±0.15 | 19.1±0.15 |
| **T6** | 37.1±0.26 | 50.1±0.21 | 51.4±0.23 |
| **T7** | 13.2±0.26 | 18.8±0.28 | 19.4±0.26 |
| **T8** | 30.6±0.27 | 32.4±0.24 | 34.1±0.16 |
| **T9** | 11.8±0.15 | 12.6±0.17 | 13.1±0.27 |

T1 = Control (0 mM NaCl)

T2 = 150 mM NaCl

T3 = 200 mM NaCl

24hrs pre-treatment with 5µM$CuCl_2$

T4= 150 mM NaCl

T5 = 200 mM NaCl

48 hrs pre-treatment with 5µM$CuCl_2$

T6= 150 mM NaCl

T7 = 200 mM NaCl

72 hrs pre-treatment with 5µM$CuCl_2$

T8 = 150 mM NaCl

T9 = 200 mM NaCl

**Table 4.4. Effect of pre-exposure to copper on the Chlorophyll a ($\mu g\ mg^{-1}$ dry weight) content of the cyanobacterium *Anabaena doliolum* further exposed to salinity.**

| Treatments | 4th day | 8th day | 12th day |
|---|---|---|---|
| **T1** | 3.06±0.02 | 3.66±0.021 | 4.04±0.025 |
| **T2** | 2.46±0.04 | 2.64±0.013 | 2.84±0.012 |
| **T3** | 0.806±0.002 | 0.821±0.04 | 0.828±0.335 |
| **T4** | 2.58±0.026 | 2.76±0.036 | 2.84±1.489 |
| **T5** | 0.808±0.028 | 0.826±0.04 | 0.834±0.052 |
| **T6** | 2.64±0.025 | 2.88±0.031 | 2.86±0.153 |
| **T7** | 0.810±0.031 | 0.816±0.034 | 0.818±0.041 |
| **T8** | 2.42±0.033 | 2.46±0.035 | 2.48±0.351 |
| **T9** | 0.801±0.035 | 0.804±0.041 | 0.807±0.02 |

T1 = Control (0 mM NaCl)
T2 = 150 mM NaCl
T3 = 200 mM NaCl

24hrs pre-treatment with 5µM$CuCl_2$
T4= 150 mM NaCl
T5 = 200 mM NaCl

48 hrs pre-treatment with 5µM$CuCl_2$
T6= 150 mM NaCl
T7 = 200 mM NaCl

72 hrs pre-treatment with 5µM$CuCl_2$
T8 = 150 mM NaCl
T9 = 200 mM NaCl

**Table 4.5. Effect of pre-exposure to copper on Phycocyanin (µg $mg^{-1}$ dry weight) content of the cyanobacterium Anabaena doliolum further exposed to salinity.**

| Treatments | 4th day | 8th day | 12th day |
|---|---|---|---|
| **T1** | 0.032±0.02 | 0.042±0.05 | 0.046±0.03 |
| **T2** | 0.026±0.02 | 0.032±0.05 | 0.036±0.01 |
| **T3** | 0.012±0.01 | 0.016±0.01 | 0.018±0.015 |
| **T4** | 0.030±0.01 | 0.038±0.02 | 0.044±0.0153 |
| **T5** | 0.014±0.02 | 0.018±0.005 | 0.019±0.02 |
| **T6** | 0.037±0.05 | 0.046±0.02 | 0.048±0.03 |
| **T7** | 0.012±0.01 | 0.014±0.01 | 0.015±0.015 |
| **T8** | 0.032±0.028 | 0.036±0.01 | 0.037±0.02 |
| **T9** | 0.009±0.001 | 0.012±0.01 | 0.012±0.015 |

T1 = Control (0 mM NaCl)

T2 = 150 mM NaCl

T3 = 200 mM NaCl

24hrs pre-treatment with 5µM$CuCl_2$

T4= 150 mM NaCl

T5 = 200 mM NaCl

48 hrs pre-treatment with 5µM$CuCl_2$

T6= 150 mM NaCl

T7 = 200 mM NaCl

72 hrs pre-treatment with 5µM$CuCl_2$

T8 = 150 mM NaCl

T9 = 200 mM NaCl

**Table 4.6. Effect of pre-exposure to copper on phycoerythrin (µg mg$^{-1}$ dry weight) content of the cyanobacterium *Anabaena doliolum* further exposed to salinity.**

| Treatments | 4th day | 8th day | 12th day |
|---|---|---|---|
| **T1** | 4.82±0.0153 | 4.96±0.0265 | 5.01±0.0208 |
| **T2** | 4.18±0.0208 | 4.32±0.0153 | 4.36±0.0153 |
| **T3** | 1.16±0.01 | 1.18±0.01 | 1.21±0.0252 |
| **T4** | 4.20±0.0764 | 4.34±0.02 | 4.38±0.0208 |
| **T5** | 1.14±0.0153 | 1.16±0.0153 | 1.20±0.0551 |
| **T6** | 4.21±0.0306 | 4.38±0.01 | 4.36±0.0153 |
| **T7** | 1.16±0.03 | 1.20±0.0208 | 1.21±0.0153 |
| **T8** | 4.16±0.0306 | 4.18±0.01 | 4.21±0.0252 |
| **T9** | 1.12±0.01 | 1.14±0.01 | 1.16±0.01 |

T1 = Control (0 mM NaCl)

T2 = 150 mM NaCl

T3 = 200 mM NaCl

24hrs pre-treatment with 5µM$CuCl_2$

T4= 150 mM NaCl

T5 = 200 mM NaCl

48 hrs pre-treatment with 5µM$CuCl_2$

T6= 150 mM NaCl

T7 = 200 mM NaCl

72 hrs pre-treatment with 5µM$CuCl_2$

T8 = 150 mM NaCl

T9 = 200 mM NaCl

**Table 4.7. Effect of pre-exposure to copper on Allophycocyanin (µg $mg^{-1}$ dry weight) content of the cyanobacterium *Anabaena doliolum* further exposed to salinity.**

| Treatments | 4th day | 8th day | 12th day |
|---|---|---|---|
| **T1** | 10.6±0.208 | 12.4±0.3 | 13.1±0.208 |
| **T2** | 8.2±.153 | 8.8±0.208 | 9.4±0.208 |
| **T3** | 3.62±0.0208 | 3.81±0.02 | 3.92±0.0252 |
| **T4** | 8.6±0.265 | 8.9±0.01 | 9.1±0.11 |
| **T5** | 3.60±0.153 | 3.66±0.0321 | 3.72±0.0153 |
| **T6** | 8.4±0.265 | 8.6±0.208 | 8.8±0.1 |
| **T7** | 3.52±0.153 | 3.58±0.01 | 3.64±0.043 |
| **T8** | 8.1±0.122 | 8.4±0.1 | 8.7±0.015 |
| **T9** | 3.46±0.0265 | 3.51±0.0153 | 3.54±0.02 |

T1 = Control (0 mM NaCl)

T2 = 150 mM NaCl

T3 = 200 mM NaCl

24hrs pre-treatment with 5µM$CuCl_2$

T4= 150 mM NaCl

T5 = 200 mM NaCl

48 hrs pre-treatment with 5µM$CuCl_2$

T6= 150 mM NaCl

T7 = 200 mM NaCl

72 hrs pre-treatment with 5µM$CuCl_2$

T8 = 150 mM NaCl

T9 = 200 mM NaCl

**Table 4.8. Effect of pre-exposure to copper on Carotenoid (μg $mg^{-1}$ dry weight) content of the cyanobacterium *Anabaena doliolum* further exposed to salinity.**

| Treatments | 4th day | 8th day | 12th day |
|---|---|---|---|
| **T1** | 2.41±0.01 | 2.68±0.04 | 2.82±0.02 |
| **T2** | 3.16±0.04 | 3.89±0.02 | 4.28±0.02 |
| **T3** | 2.86±0.0155 | 3.16±0.015 | 3.24±0.03 |
| **T4** | 3.86±0.0153 | 4.26±0.01 | 4.68±0.025 |
| **T5** | 3.14±0.025 | 3.72±0.015 | 3.90±0.0208 |
| **T6** | 3.46±0.025 | 3.92±0.017 | 4.14±0.02 |
| **T7** | 3.18±0.153 | 3.28±0.01 | 3.62±0.03 |
| **T8** | 3.21±0.252 | 3.20±0.0208 | 3.25±0.036 |
| **T9** | 2.90±0.03 | 2.96±0.03 | 3.02±0.04 |

T1 = Control (0 mM NaCl)

T2 = 150 mM NaCl

T3 = 200 mM NaCl

24hrs pre-treatment with 5μM$CuCl_2$

T4= 150 mM NaCl

T5 = 200 mM NaCl

48 hrs pre-treatment with 5μM$CuCl_2$

T6= 150 mM NaCl

T7 = 200 mM NaCl

72 hrs pre-treatment with 5μM$CuCl_2$

T8 = 150 mM NaCl

T9 = 200 mM NaCl

**Table 4.9. Effect of pre-exposure to copper on Heterocyst frequency and Average filament length of the cyanobacterium *Anabaena doliolum* further exposed to salinity.**

| Treatments | Heterocyst frequency (%) | Average filament length |
|---|---|---|
| **T1** | 15.8±0.1 | 54.2±0.208 |
| **T2** | 10.1±0.2 | 42.1±0.2 |
| **T3** | 3.6±0.153 | 16.4±0.1 |
| **T4** | 10.4±0.1 | 41.6±0.208 |
| **T5** | 3.4±0.153 | 16.2±0.265 |
| **T6** | 9.1±0.208 | 41.8±0.208 |
| **T7** | 3.8±0.265 | 16.1±0.306 |
| **T8** | 9.6±0.1 | 40.8±0.208 |
| **T9** | 3.1±0.1 | 15.8±0.2 |

T1 = Control (0 mM NaCl)

T2 = 150 mM NaCl

T3 = 200 mM NaCl

24hrs pre-treatment with 5µM$CuCl_2$

T4= 150 mM NaCl

T5 = 200 mM NaCl

48 hrs pre-treatment with 5µM$CuCl_2$

T6= 150 mM NaCl

T7 = 200 mM NaCl

72 hrs pre-treatment with 5µM$CuCl_2$

T8 = 150 mM NaCl

T9 = 200 mM NaCl

**Table 4.10. Effect of pre-exposure to copper on the sugar (µg $mg^{-1}$ dry weight) content of the cyanobacterium *Anabaena doliolum* further exposed to salinity.**

| Treatments | 4th day | 8th day | 12th day |
|---|---|---|---|
| **T1** | 32.1±0.231 | 48.4±0.361 | 50.6±0.153 |
| **T2** | 40.4±0.265 | 60.1±0.306 | 62.4±0.153 |
| **T3** | 38.6±1.353 | 52.4±0.3 | 54.5±0.217 |
| **T4** | 44.5±0.231 | 64.1±0.208 | 65.1±0.243 |
| **T5** | 40.2±0.263 | 54.8±0.252 | 56.1±0.306 |
| **T6** | 48.6±0.208 | 68.1±0.306 | 70.1±0.608 |
| **T7** | 44.1±0.306 | 56.4±0.209 | 58.4±0.252 |
| **T8** | 42.5±0.153 | 57.2±0.361 | 59.4±0.153 |
| **T9** | 40.1±0.309 | 51.7±0.208 | 52.6±0.153 |

T1 = Control (0 mM NaCl)

T2 = 150 mM NaCl

T3 = 200 mM NaCl

24hrs pre-treatment with 5µM$CuCl_2$

T4= 150 mM NaCl

T5 = 200 mM NaCl

48 hrs pre-treatment with 5µM$CuCl_2$

T6= 150 mM NaCl

T7 = 200 mM NaCl

72 hrs pre-treatment with 5µM$CuCl_2$

T8 = 150 mM NaCl

T9 = 200 mM NaCl

**Table 4.11. Effect of pre-exposure to copper on the N assimilation enzymes of the cyanobacterium *Anabaena doliolum* further exposed to salinity.**

| Treatments | Nitrate reductase (µmol mg protein $^{-1}$) | Glutamine synthetase (nmol mg protein $^{-1}$) | Ethylene formed (µmol $C_2H_4$ mg chl$^{-1}$ h$^{-1}$ ) |
|---|---|---|---|
| **T1** | 6.21±0.047 | 172.4±0.557 | 32.4±0.351 |
| **T2** | 14.71±0.041 | 148.4±0.36 | 20.6±0.1 |
| **T3** | 10.8±0.1 | 120.1±0.71 | 11.2±0.208 |
| **T4** | 14.2±0.208 | 146.4±1.27 | 20.8±0.153 |
| **T5** | 9.6±0.1 | 121.4±0436 | 11.8±0.2 |
| **T6** | 14.8±0.2 | 144.5±0.208 | 21.2±0.321 |
| **T7** | 10.2±0.252 | 118.2±0.529 | 12.4±0.153 |
| **T8** | 15.1±0.351 | 148.8±0.265 | 22.8±0.265 |
| **T9** | 10.4±0.1 | 124.2±0.153 | 10.8±0.154 |

T1 = Control (0 mM NaCl)

T2 = 150 mM NaCl

T3 = 200 mM NaCl

24hrs pre-treatment with 5µM$CuCl_2$

T4= 150 mM NaCl

T5 = 200 mM NaCl

48 hrs pre-treatment with 5µM$CuCl_2$

T6= 150 mM NaCl

T7 = 200 mM NaCl

72 hrs pre-treatment with 5µM$CuCl_2$

T8 = 150 mM NaCl

T9 = 200 mM NaCl

**Table 4.12. Effect of pre-exposure to copper on the antioxidant profile of the cyanobacterium *Anabaena doliolum* further exposed to salinity.**

| Treatments | Superoxide dismutase (U mg protein$^{-1}$) | Ascorbate peroxidase (U mg protein$^{-1}$) | Catalase (U mg protein$^{-1}$) | Proline (µ mol mg protein$^{-1}$) |
|---|---|---|---|---|
| **T1** | 1.36±0.15 | 1.94±0.01 | 2.78±0.0153 | 32.6±0.252 |
| **T2** | 1.96±0.1 | 2.46±0.0252 | 4.92±0.0321 | 46.8±0.208 |
| **T3** | 2.01±0.20 | 2.96±0.0153 | 4.96±0.01 | 40.4±0.473 |
| **T4** | 2.46±0.2 | 2.68±0.03 | 5.16±0.0252 | 54.6±0.3 |
| **T5** | 2.12±0.265 | 3.04±0.03 | 5.04±0.0173 | 42.8±0.436 |
| **T6** | 2.86±0.152 | 3.12±0.0321 | 5.28±0.0208 | 60.4±0.643 |
| **T7** | 2.16±0.153 | 3.06±0.0252 | 5.08±0.0404 | 44.2±0.351 |
| **T8** | 2.04±0.252 | 2.44±0.0208 | 5.20±0.0252 | 50.1±0.416 |
| **T9** | 2.06±0.153 | 2.86±0.0252 | 5.01±0.0361 | 41.4±0.513 |

T1 = Control (0 mM NaCl)

T2 = 150 mM NaCl

T3 = 200 mM NaCl

24hrs pre-treatment with 5µM$CuCl_2$

T4= 150 mM NaCl

T5 = 200 mM NaCl

48 hrs pre-treatment with 5µM$CuCl_2$

T6= 150 mM NaCl

T7 = 200 mM NaCl

72 hrs pre-treatment with 5µM$CuCl_2$

T8 = 150 mM NaCl

T9 = 200 mM NaCl

*Chapter 5*

# DISCUSSION

The cyanobacteria are distributed widely in a number of ecological niches and therefore they are ideal organisms to investigate for stress response (Friedmann and Galun 1974). Moreover, these organisms have a very long evolutionary history and have the adaptability to respond to a variety of abiotic stress conditions (Murata and Wada 1995). One of the major areas of current research interest in cyanobacteria is to understand the tolerance mechanism to different environmental stresses. In the natural environment, the cyanobacteria may experience many kinds of stresses and salinity is one of the most serious abiotic stresses that challenge the survival of the cyanobacteria in the paddy fields. Singh (1961) reported that the salinity decreases the cyanobacterial population of rice field significantly leading to reduced rice yield and soil fertility. The problem of salinity is a serious concern globally especially tropical countries (Zhu, 2001). Large amount of salts are brought to the paddy field due to canal irrigation and water logging. Salinity exerts a negative effect on the growth and physiological aspects of cyanobacteria. Increasing anthropogenic activities coupled with rapid industrialization and urbanization has also resulted in the generation of several heavy metals in the environment. Heavy metals are one of the most common pollutants worldwide, being a serious hazard to the environment and public health. Use of contaminated water for irrigation, accelerated input of chemical fertilizer, use of copper sulfate for controlling pests and diseases lead to contamination of soils. Increase in copper content in paddy soil irrigated with waste water has been reported by Luo *et al.*, (2003). Heavy metals such as Cu exert selection pressure on the cyanobacteria affecting their growth and productivity. However, in the natural environment at a given period of time the cyanobacteria may experience one or more stresses. It has been demonstrated that susceptibility of organisms to a particular stress may be altered due to other stresses (Bhargawa *et al.,* 2008). How exposure to one stress modify the response to the other stress is important. However, very few studies have been conducted in the past to understand the physiological and biochemical responses of the cyanobacterium in relation to salinity and copper exposure.

It was observed that in the present study, the cyanobacterium *Anabaena doliolum* exposed to individual as well as combined exposure to salinity showed a decrease in the growth. Stress tolerance in general reflects the sum total of various simple and complex interactions that take place at the structural, physiological and molecular levels in the organism Reduction in the growth may be considered as a general response to salinity and copper treatment. Rogers *et al.,* (1993) reported salt induced growth in cyanobacteria. Decrease in the growth of the cyanobacterium *Anabaena doliolum* due to salinity stress was reported earlier by Srivastava *et al.,* (2008). Rai *et al.,* (1991) observed the adverse effect of copper on the photosynthetic efficiency of cyanobacteria. Similarly, the effect of copper on the reduction in growth was reported by Bhargawa *et al.* (2008). However, the exposure to combined salinity and copper treatment has resulted in further drastic reduction in growth. Chris *et al.,* (2006) reported severe reduction in the growth of a cyanobacterium *Cylindropsermum* sp. due to combined stress conditions. Reduction in growth could also be attributed to loss of cell water and turgor coupled with toxicity by the accumulated ions (Apte, 2001). Reduction in the content of soluble protein was also observed in the cyanobacterium *A. doliolum* under similar conditions. Apte and Bhagawat (1989) observed reduction in the protein content in the cyanobacteria exposed to salinity. There are conflicting reports on the impact of salinity on the increase or decrease in the cellular protein content in different organisms (Assche *et al.*, 1988; Jusu *et al.*, 2004). Zhang *et al.* (2013) reported decrease in the protein content of the cyanobacterium *Microcystis aerginosa*. Huertas *et al.,* (2014) observed protein degradation and perturbations in the protein function in cyanobacteria due to exposure to copper. However, the greater reduction in the protein content due to combined exposure to salinity and copper may be due to an additive effect.

Decrease in the photosynthetic pigments such as chlorophyll, phycocyanin. allophycocyanin and phycoerytherin was observed in response to salinity and copper exposure. Todd *et al.* (2008) observed that the chlorophyll content varies with the physiological status as well as environmental perturbations. Decrease in chlorophyll content in the cyanobacterium *Anabaena* sp. due to osmotic stress was reported by Sharma *et al.,* (2012). Kumar and Gaur (2014) also reported decrease in the chlorophyll content of the cyanobacterium *Phormidium bigranulatum* due to exposure to copper. Similar results have been observed with the accessory pigments such as phycocyanin, phycoerytherin and allophycocyanin. The localization of phycocyanin on intracellular thylakoid membrane makes it highly sensitive to stress conditions (Grossman *et al.*, 1993; Prasad *et al.*, 2005). Lin and Wu (2014) also observed inhibition of pigment synthesis in cyanobacteria due to osmotic stress conditions. Decrease in the accessory pigments is also supported by the findings of Sundaram and Soumya (2011). However, the gravity of the reduction in the accessory pigments irrespective of the type was observed in the test cyanobacterium *A. doliolum* due to a combination of salinity and copper treatment. The pigments play an important role in photosynthesis and decrease in their content in turn may lead to reduced photosynthetic activity under stress conditions in the test cyanobacterium leading to reduction in growth. Rai *et al.* (1991) observed the adverse effect of copper on the photosynthetic efficiency of cyanobacteria. Ybarra and Webb (1999) observed that copper induces stress responses in *Synechococcus* and in turn reduce cellular oxygen evolution. Bhargawa *et al.* (2008) observed that excess copper induced anoxygenic photosynthesis in the cyanobacterium *Anabaena doliolum*. On the other hand,

We observed increase in the carotenoid content of the cyanobacterium exposed to salinity. However, copper treatment as well as combined exposure to salinity and copper decreased the carotenoid content of the test cyanobacterium. Lohscheider *et al.* (2011) reported that the cyanobacteria tend to accumulate carotenoids in response to conditions of oxidative stress. Enhanced accumulation of carotenoids in the cyanobacterium is correlated with enhanced tolerance to drought stress conditions (Lin and Wu 2014). Kumar and Guar (2014) reported decrease in the carotenoid content of the cyanobacterium due to elevated levels of copper.

The total soluble sugar content of the test organism exposed to salinity showed significant increase in the sugar content as compare the control. However, the copper exposure as well as the exposure to a combination of salinity and copper did not result in any significant increase in the sugar content. Under salinity, the cyanobacteria have been reported to synthesize and accumulated sugars for osmotic adjustment. Sucrose and trehalose are accumulated by the least salt tolerant strains (Reed *et al.,* 1986).Salinity leads to osmotic stress conditions in the cells and accumulation of sugars was observed in cyanobacteria due to drought stress (Sakamoto *et al*., 2009). Higo *et al.,* (2006) observed that dehydrated cells of *Nostoc sp.* PCC 7120 accumulated sugars in response to osmotic stress. Osmotic stress induced accumulation of sugars was also observed in the cyanobacterium *Anabaena sp.* PCC 7120 (Singh 2013b). Therefore, it was reported that excess accumulation of sugars is a response to osmotic stress to overcome the water deficit conditions and the sugars, prevent *in vivo* aggregation of protein (Fisher, 2006).

A study was also performed to assess the activity of the antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase and catalase. Superoxide dismutase, the primary antioxidant enzyme is involved in the conversion of the superoxide anion to $H_2O_2$ whereas the ascorbate peroxidase is involved in the reduction of $H_2O_2$ using ascorbate as the electron donor and is well known to be important in the detoxification of $H_2O_2$ (Asada *et al.,* 1987). Scandalios, (1993) reported that catalase activity along with superoxide dismutase as the most effective strategy in preventing cellular damage. In the present study, the activities of superoxide dismutase, ascorbate peroxidase and catalase significantly increased in the cells exposed to salinity and copper individually. However, the maximum activity was observed when the organism was exposed to salinity and copper together. In general, the antioxidant enzymes have a key role in the main tolerance mechanism in response to various abiotic stress conditions. SOD is the first line of defence against oxidative stress (McCord and Fridovich 1969). Both Mn- and Fe-SOD are reported in cyanobacteria (Canini *et al.,* 1991). In stress conditions, several algae and cyanobacteria show enhancement in the antioxidant enzyme activities (Mallick and Mohn, 2000; Collen and Davison 2001; El-Baky *et al*., 2004). Srivastava *et al.* (2008) reported increase in the antioxidant enzyme activity in response to salinity in the cyanobacterium *Anabaena doliolum*. Swapnil and rai (2018) observed enhanced activity of antioxidant enzymes in the salt stressed cyanobacterium *Anabaena fertilissima*. Therefore, increase in the enzymatic activity is correlated with the ability to tolerate salinity and copper.

Similarly, exposure of the cyanobacterium *A. doliolum* to salinity and copper resulted in significant increase in the proline. Increased accumulation of proline in response to stress was noticed in several organisms. Wu *et al.* (1998) reported that proline act as an osmo-regulant and protect enzymes and proteins. The present study also revealed increase in the accumulation of proline. Increased accumulation of proline conferred salinity tolerance in the cyanobacterium *Scenedesmus* sp. (Tripathi and Gaur 2004). Yadav *et al.* (2016) observed increase in the accumulation of proline due to salinity stress conditions in the cyanobacterium *Anabaena* sp. Sharmila *et al.* (2017) also observed enhanced accumulation of proline in response to heavy metal stress. Proline protects various cellular components/complexes induced by heavy metal or any other abiotic stress-induced damage. It also scavenges the reactive oxygen species produced in plants exposed to heavy metal stress. There are several reports that the synthesis of proline has a significant role in regulating $NAD(P)^+$ to NAD(P)H ratio

(Alia and Pardha-Saradhi 1993; Greene *et al.,* 1992; Pardha-Saradhi *et al.*, 1996, 2014; Shabnam *et al.*, 2014; Sharmila *et al.,* 2008).Increased intracellular proline content therefore shows the ability of the organism to tolerate the stress due to salinity and copper. Salinity and copper treatment also resulted in decrease in the heterocyst frequency and average filament length. The nitrogenase enzyme is located in the heterocyst. Rai and Abraham (1992) observed reduction in the heterocyst frequency and average filament length of cyanobacteria due to salinity stress.

Nitrate is a major source of nitrogen in the environment and the enzyme nitrate reductase converts it to ammonium. In cyanobacteria stress induced accumulation of nitrate has been reported earlier (Rai *et al*., 1995). This leads to reduction in the toxicity induced by nitrate. Therefore, the increase in nitrate reductase in response to may help the cyanobacteria to tide over the adverse impact of stress conditions. On the other hand, stress due to salinity and copper resulted in decrease in the GS and nitrogenase activity. Warr *et al*. (1984) reported decrease in the glutamine synthetase activity under stress conditions. Stress conditions have also resulted in decrease in the glutamine synthetase activity of the cyanobacterium *Anabaena* sp. as has been reported by Swapnil *et al.* (2015). Exposure to stress conditions inhibited the activity of nitrogenase. Disturbed electron transport resulted in inhibition of nitrogenase activity (Tel-Or and Melhamed-Harel, 1981). Decrease in nitrogenase activity in the cyanobacterium was observed due to stress conditions (Moisander *et al.,* 2002). Inhibition in the nitrogenase activity due to copper exposure was reported by Bhargawa *et al.* (2008). Yadav *et al.* (2016) also reported the inhibition in nitrogenase activity under the influence of salinity. The results show the adverse impact of stress conditions on the inhibition of nitrogen assimilation enzymes in the cyanobacteria.

Our findings show that the cyanobacterium *Anabaena doliolum* is sensitive to both salinity and copper stress. Decrease in the growth and cellular constituents was observed in response to stress conditions. The same was true with the individual stress application as well as combined exposure to stress. However, to understand whether the pre-exposure to one stress has any effect in relation to the other stress was studied by pre-exposing the cells to copper for various time intervals. The results on the pre-treatment revealed a contrasting pattern with respect to the growth variables as well as the antioxidants. Pre-treatment of 24 hours with copper chloride and subsequent exposure to salinity resulted in slight improvement in dry weight, protein, chlorophyll, phycocyanin, phycoerythrin and allophycocyanin. More or less similar response was observed when the cyanobacterium was pre-exposed to copper chloride for 48 hours and then exposed to copper chloride. Interestingly, the sugar and the carotenoid content increased significantly in response to pre-exposure to 24 and 48 hours of pre-exposure. However, the pre-exposure also did not have much influence on the enzymes of nitrogen assimilation. It has been demonstrated that susceptibility of organisms to a particular stress may be altered due to other stresses (Bhargawa *et al.,* 2008). Murali and Teramura (1987) observed that drought and mineral deficiency are capable of modifying the UV-B impact on plants. Enhanced tolerance to UV-B due to pre-exposure to UV-B has been reported in the cyanobacterium *Cylindrospermum* sp. (Chris *et al.,* 2006). Bhargawa *et al.* (2008) observed that pre-treatment with copper enhanced the UV-B tolerance in cyanobacterium *Anabaena doliolum*. In case of the antioxidant enzymes the pre-exposure had a positive influence. Enhanced salt tolerance has been correlated with hyperactivity of antioxidant enzymes (Sreenivasulu *et al.,* 2000). Signalling molecules such as $H_2O_2$ induce genes for enzymes involved in controlling oxidative stress such as APX and SOD and trigger cross adaptation in maize seedlings (Gong *et al.,* 2001). Hernandez *et al.* (1995) suggested enhanced m-RNA expression and activity of APX and SOD in relation to salinity stress. Pre-treatment with copper for 72 hours had no response on any of the parameters studied. On the basis of our studies, it is concluded that individual as well as combined salinity and copper treatment resulted in strong inhibitory effects on the growth, pigments and nitrogen assimilation enzymes of the cyanobacterium *A. doliolum*. However, pre-exposure with copper seems to have some adaptive significance probably through the antioxidant enzymes and proline accumulation. The proline accumulated due to pre-treatment with copper as well as the hyper activity of the antioxidant enzyme activities may suppress free radical production and decrease the lipid peroxidation.

Our data showed that important physiological parameters related to antioxidant defense and nitrogen metabolism improved in cells pre-exposed to copper for 24 and 48 days. This clearly indicates that the cyanobacterium pre-exposed to mild concentrations of copper underwent priming, which is considered as an important adaptive strategy. Pre-exposure in fact leads to priming effects. It has been reported that during priming, the cyanobacterium was prepared to improve the defensive capacity for subsequent challenge. Physiological, transcriptional, metabolic, and epigenetic changes in organisms such as modifying the membrane composition, induction of certain transport processes (intracellular homeostasis) and appropriate metabolic changes crucial for their defense are transmitted to the cellular machinery through the stimulus of priming (Mauch-Mani *et al.,* 2017). Martinez-Medina *et al.,* (2016) and Mauch-Mani *et al.* (2017) observed that these defenses were more robust with better performance compared to the directly induced defenses and involved negligible to minimal cost enabling the cyanobacterium to grow normally. Further, it was demonstrated that priming is durable and can be passed on from one generation to the next (Mauch-Mani *et al.,* 2017). Therefore, it appears from the results that the priming stimuli transmit signals to the cellular machinery for the induction of appropriate cellular processes to cope with the stress by warning them probably.

*Chapter 6*

# SUMMARY AND CONCLUSION

The prokaryotic organism cyanobacteria was earlier known as blue green algae consist of a group of photosynthetic bacteria capable of performing oxygenic photosynthesis and therefore are similar to like higher plants and algae. They are found in ecologically diverse habitats ranging from hot springs to Arctic and Antarctic. Some cyanobacteria are endowed with the capacity to fix free atmospheric nitrogen to ammonia using solar energy. Because of this property, the cyanobacteria are used as bioinoculants in agriculture. During the course of their evolution the cyanobacteria have survived several types of stresses such as extreme heat, cold, anaerobiosis, salinity, drought, pesticides, heavy metals etc. Therefore, the cyanobacteria generally exhibit high level of adaptive abilities and tolerance to a large number of environmental stresses. The exploitation of cyanobacteria is constrained by a number of abiotic stress factors. Of the several abiotic stresses that challenge the survival of the cyanobacteria in the paddy fields is salinity. Selection pressure is also exerted on the cyanobacteria through the contamination of rice paddy by heavy metals such as Cu. Therefore, increase the salinity and metal content pose a serious threat to the cyanobacteria. However, in the natural environment at a given period of time cyanobacteria may experience one or more stresses and we do not have a clear understanding about the combined effects of stresses. It has been demonstrated that susceptibility of organisms to a particular stress may be modified due to other stresses. Therefore, in the present study we have assessed the combined effect of salinity and copper on growth and physiological parameters of the nitrogen fixing rice field cyanobacterium *Anabaena doliolum.* An attempt was also made to understand the modulation of salinity tolerance in cyanobacterium through copper pre-treatment.

It was observed that salinity and copper exposure resulted in decrease in growth and cellular constituents such as protein, chlorophyll and phycobiliproteins. Further, the stress induced by salinity and copper was evident from the reduction in the average filament length and heterocyst frequency of the test organism. Although, salinity treatment enhanced the activity of the nitrogen assimilation enzyme nitrate reductase the other two enzymes such as glutamine synthetase and nitrogenase showed reduction in their activity. It was also observed that the copper treatment decreased the activity of all the three major nitrogen assimilation enzymes. The effect of combined exposure of salinity and copper on the growth, cellular constituents and the enzymes of nitrogen assimilation was studied and it was more pronounced on the growth, cellular constituents and the enzymes of nitrogen assimilation. The antioxidant enzymes play a crucial role in in the detoxification of free radicals. Individual as well as simultaneous exposure of the cyanobacterium *A. doliolum* to salinity and copper enhanced the activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase and catalase. Similar results have been observed in response to proline accumulation by the cyanobacterium.

The cyanobacterium was pre-exposed to copper chloride (5µM) for different timings for 24 hrs, 48 hrs and 72 hrs and then treated with NaCl concentration of 150 and 200 mM to study as to how pre-exposure modulates the physiological activities of the organism. Pre-exposure of the organism to copper chloride (5µM) for 24 and 48 hours resulted in insignificant increase in the cellular constituents. However, the pre-exposure had no impact on the enzymes of nitrogen assimilation. On the other hand, the antioxidant enzymes showed considerable enhancement in their activity. Intracellular proline accumulation was also found to increase under similar conditions. However, pre-exposure to 72 hours failed to have any positive impact. Our data showed that important physiological parameters related to antioxidant defense and nitrogen metabolism improved in cells pre-exposed to copper for 24 and 48 days. This clearly indicates that the cyanobacterium pre-exposed to mild concentrations of copper underwent priming, which is considered as an important adaptive strategy.

# Assessment of the combined effect of salinity and copper on the growth and physiological variables of a cyanobacterium *Anabaena doliolum*

## ABSTRACT

In the present study the effect of salinity and copper on the growth and physiological variables has been studied in the rice field nitrogen fixing cyanobacterium *Anabaena doliolum*. Salinity and copper exposure resulted in decrease in growth and cellular constituents such as protein, chlorophyll and phycobiliproteins. The stress induced by salinity and copper was also evident from the reduction in the average filament length and heterocyst frequency of the test organism. Salinity treatment enhanced the activity of the nitrogen assimilation enzyme nitrate reductase whereas the other two enzymes such as glutamine synthetase and nitrogenase showed considerable inhibition in their activity. However, the copper treatment has resulted in decrease in the activity of all the three nitrogen assimilation enzymes. The reduction in growth, cellular constituents and the enzymes of nitrogen assimilation was more pronounced when the cyanobacterium *A. doliolum* was exposed to simultaneous exposure to salinity and copper. Increase in the activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase and catalase was observed in the cyanobacterium due to individual as well as combined exposure to salinity and copper. Exposure of the cyanobacterium to salinity and copper also resulted in increased accumulation of proline. The cyanobacterium was pre-exposed to Copper chloride (5µM) for different timings for 24 hrs, 48 hrs and 72 hrs and then treated with NaCl concentration of 150 and 200 mM to understand how pre-exposure modulates the physiological activities of the organism. The results indicate the pre-exposure of the cyanobacterium with copper chloride for 24 and 28 hours had insignificant increase on the cellular constituents of the test cyanobacterium. However, the pre-exposure resulted in better profile of the antioxidant enzymes and proline. The results showed the adverse impact of salinity and copper on the growth of the cyanobacterium *A. doliolum* and the importance of pre-exposure in the modulation of antioxidant machinery of the cells.

Keyowrds : ***Anabaena doliolum*** , Copper , Salinity, Pre-exposure

# साइनोबैक्टीरियम *अनाबाना डॉलीओलियम* के विकास और शारीरिक चर पर लवणता और तांबे के संयुक्त प्रभाव का आकलन

## सार

वर्तमान अध्ययन में वृद्धि और शारीरिक चर पर लवणता और तांबे के प्रभाव का अध्ययन चावल क्षेत्र नाइट्रोजन फिक्सिंग साइनोबैक्टीरियम अनाबाना डॉलीओलियम में किया गया है। लवणता और तांबा एक्सपोजर के परिणामस्वरूप प्रोटीन, क्लोरोफिल और फाइकोबिलिप्रोटिन जैसे विकास और सेलुलर घटकों में कमी आई है। लवणता और तांबा द्वारा प्रेरित तनाव औसत फिलामेंट लंबाई और परीक्षण जीव की हेटरोसाइट आवृत्ति में कमी से भी स्पष्ट था। लवणता उपचार ने नाइट्रोजन एसिमिलेशन एंजाइम नाइट्रेट रेडक्टेज की गतिविधि को बढ़ाया जबकि ग्लूटामाइन सिंथेथेस और नाइट्रोजेनेस जैसे अन्य दो एंजाइमों ने अपनी गतिविधि में काफी अवरोध दिखाया। हालांकि, तांबे के उपचार के परिणामस्वरूप सभी तीन नाइट्रोजन एसिमिलेशन एंजाइमों की गतिविधि में कमी आई है। विकास, सेलुलर घटकों और नाइट्रोजन एसिमिलेशन के एंजाइमों में कमी अधिक स्पष्ट थी जब साइनोबैक्टीरियम ए डॉलिओलम को लवणता और तांबे के साथ-साथ संपर्क में उजागर किया गया था। एंटीऑक्सीडेंट एंजाइमों की गतिविधि में वृद्धि जैसे कि सुपरऑक्साइड डिमूटेज, एस्कॉर्बेट पेरोक्साइड और कैटलस साइनोबैक्टीरियम में व्यक्तिगत और साथ ही लवणता और तांबे के संयुक्त संपर्क के कारण मनाया गया था। लवणता और तांबे के लिए साइनोबैक्टीरियम के एक्सपोजर के परिणामस्वरूप प्रोलिन का संचय बढ़ गया। साइनोबैक्टीरियम 24 घंटे, 48 बजे और 72 बजे के लिए अलग-अलग समय के लिए कॉपर क्लोराइड (5μM) से पहले सामने आया था और फिर यह समझने के लिए कि पूर्व-एक्सपोजर जीव के शारीरिक सक्रियण को कैसे नियंत्रित करता है, को समझने के लिए 150 और 200 मिमी के NaCl एकाग्रता के साथ इलाज किया जाता है। परिणाम 24 और 28 घंटों के लिए तांबे क्लोराइड के साथ साइनोबैक्टीरियम के प्री-एक्सपोजर को परीक्षण साइनोबैक्टीरियम के सेलुलर घटकों पर महत्व में वृद्धि दर्शाते हैं। हालांकि, प्री-एक्सपोजर के परिणामस्वरूप एंटीऑक्सीडेंट एंजाइमों और प्रोलिन की बेहतर प्रोफ़ाइल हुई। परिणामों ने साइनोबैक्टीरियम ए डॉलिओलम के विकास पर कोशिकाओं की एंटीऑक्सीडेंट मशीनरी के मॉड्यूलेशन में पूर्व-एक्सपोजर के महत्व पर लवणता और तांबा के प्रतिकूल प्रभाव को दिखाया।

मुख्य शब्द :*अनाबाना डॉलीओलियम,* कॉपर, लवणता, प्री-एक्सपोजर

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