CHAPTER 4

DISCUSSION

4.1 PHYSICO-CHEMICAL PARAMETERS OF WATER SAMPLES OF SOME RELIGIOUS PONDS OF KURUKSHETRA, INDIA

Water is very vital for nature and can be a limiting resource to human and other living beings (Kohler 1994, Wang et al., 1999). It is well known that the physicochemical parameters such as temperature, salinity, pH, dissolved oxygen content, penetration of light and abundance of various nutrients in the water columns are of paramount importance for determining the biological productivity of potential resources (Munster and Chrost 1991). Thus, the ecological and physiological investigation of such fresh waters is of utmost importance (Millbrink 1977, Levine and Schindler 1999, Mianping 2001). The richness of nutrients supported luxuriant growth of phytoplanktons and promote productivity (Melack 1981, Montoya et al., 2004, Addico et al., 2009, Bhatnagar and Devi 2009) at the primary level. The climatic conditions affect the biodiversity of phytoplanktonic community in any ecosystem (Yoshimura and Kudo 2001). Human need for fresh water is physiological as well as physical. All kinds of ponds, lakes and rivers are being used as sinks of liquid, semisolid and solid wastes of direct or indirect disposes (Boyle 1986). The polluted water may be the store house of a variety of disease causing organisms and of chemicals causing toxic and carcinogenic effects (Heise 1949, McElhenny 1962, Mittal et al., 1979, Schwartz et al., 1990, Turner and Robinson 1995, Yoo et al., 1995, Uneno et al., 1996, Kuiper et al., 1999, Sivonen and Jones 1999).

It is emphasized for the decontamination of religious ponds of Kurukshetra by an Indian government agency like Kurukshetra Development Board. But the periodic examination of such fresh water ponds is essential to
check out the level of pollution in these ponds and to maintain their aesthetic value also. The selected study sites (*Brahma Sarowar* as site-I, *Sannihit Sarowar* as site-II and *Jyotisar Sarowar* as site-III) are very important in terms of their religious value (Table No. 1.1, Fig. 2.1, 2.3 and 2.3). The water quality is being affected due to very higher level of human activities therein. Regular monitoring of all the parameters became very difficult, lengthy and time consuming task even if adequate manpower and other laboratory facilities were available. Similar work has been supported by many workers (Vyas *et al.*, 2007, Bhatnagar and Sangwan 2009, Gupta *et al.*, 2011) also.

The various physicochemical properties (Ali *et al.*, 1999) like surface water temperature, pH value, transparency, turbidity, Dissolved Oxygen content, Electrical Conductance, free CO$_2$ content, nitrate content, ammonium, sulphate and chloride ion content of these selected three water bodies not only varied from season to season but also year wise (Table no. 3.1.1 to 3.1.11, Fig. 3.1.1 to 3.1.11) respectively (Sacks *et al.*, 1992). The seasonal effect of these parameters in different study sites has been explored through one-way ANOVA. In almost two-third cases, the result showed that seasonality has a significant (p<0.01) effect on the distribution of these parameters (Alfred and Thapa 1996).

Temperature enhances the rate of metabolism and growth of cyanobacteria as well as other algae inhabiting therein (McQueen and Lean 1987, Robarts and Zohary 1987). High temperature inhibits the process of photosynthesis by causing damage to enzymes and cell structure. Study Site-I showed maximum temperature during the summer season of 2010 (21.0±0.5°C) and minimum temperature during the winter season of 2009 (4.0±0.4°C). The decreasing order of variation in temperature was found as Summer Season > Rainy Season > Winter Season for all the Study Sites (Table no. 3.1.1, Fig. 3.1.1). The seasonal variation in measurement of temperature has been
observed in all the study sites (Table no. 3.1.1) (Modassir 1990, Santhanam and Perumal 2003). The upwelling of subsurface cold, nutrient rich waters during rainy season is also an important factor supporting the algal growth in these water ponds (Fig. 3.1.1). Light is the source of energy which enables photosynthesis. The transparency range of the three water bodies was recorded as 21.0±0.7 - 33.1±0.69 cm (Study Site-I), 21.0±1.1- 31.8±1.6cm (Study Site-II) and 19.0±1.3- 32.9±0.8cm (Study Site-III) respectively (Table no. 3.1.2). The quantity and quality of the ambient light has a great influence on the growth, distribution and productivity of cyanobacteria (Voros et al., 1998, Ghavzan et al., 2006). The penetration of light into the water bodies depend largely on the transparency(Table no. 3.1.2, Fig. 3.1.2) and turbidity (Table no. 3.1.3). The variation in measurements of turbidity of different study sites was recorded as Study Site-I (16.3±1.2 - 30.2±1.2 cm), Study Site-II (21.8±1.9-40.3±1.7cm) and Study Site-III (17.5±1.3-31.5±1.8 cm) (Table no. 3.1.3). In the monsoon or the rainy season, the transparency showed always low level as the rainfall makes the water more turbid (Sunkad and Patil 2004). It was also found that the Study Site-II (Sannihat sarowar) was the most turbid one in all the seasons, and the water of Study Site-I (Brahma sarowar) was more clearer as compared to that of Study Site-III (Jyotisar sarowar). In the present study, the pH also showed considerable variation (Fig. 3.1.5) in different study sites (Devi et al., 1996). The balance of an ecosystem is maintained when the pH is from 5.5 to 8.5. In the present investigation, the nature of the ponds was found alkaline, indicating that waters were well buffered and in high trophic states. The pH value of Study Site-I ranged from 5.4±0.69 (winter season of 2011) to 8.4±0.45 (rainy season of 2010). Similarly, Study Site-II ranged in pH values from a minimum of 5.6±0.42 (winter season of 2009) to a maximum of 8.1±0.48 (rainy season of 2010). The pH value of Study Site-III was recorded in a range of 4.92±0.44 (winter season of 2011) to 8.0±0.45 (rainy season of
2011) respectively (Table no. 3.1.5). The pH levels were within the limits set for protection of aquatic life (ISI 1974). The dissolved oxygen content of the selected ponds varied from a maximum of 8.8± 0.37 (rainy season of 2011, Study Site-I) to a minimum of 4.0±0.32 µg/l (summer season of 2010, Study Site-III) (Table no. 3.1.6). The seasonal variation in dissolved oxygen (Fig.3.1.6) was obvious in the present study (Haridas et al., 1973). The richness of nutrients (Smith 1982, 1983, Vymazal 1995, Shapiro 1997) like nitrate ions (Study Site-I: 0.54±0.02- 0.28±0.05 µg/ml; Study Site-II: 0.77±0.04-0.54±0.04 µg/ml; Study Site-III: 0.38±0.02- 0.6±0.03 µg/ml) (Table no. 3.1.8, Fig. 3.1.8), sulphate ions (Study Site-I: 26.0±1.9- 60.1±1.7 µg/ml; Study Site-II: 32.0±2.6- 56.9±1.9 µg/ml; Study Site-III: 26.9±1.4- 57.0±2.7 µg/ml) (Table no. 3.1.9, Fig. 3.1.9) and ammonium ions (Study Site-I: 1.47±0.12- 3.72±0.27 µg/ml; Study Site-II: 2.78±0.19- 4.63±0.23 µg/ml; Study Site-III: 2.72±0.26- 5.0±0.19 µg/ml) (Table no. 3.1.8, Fig. 3.1.11) supported luxuriant growth of phytoplanktons (Trimbee and Prepas 1987, Yoshimura and Kudo 2001, Panigrahi et al., 2009) and this growth and multiplication of primary and secondary producers (Ward and Wetzel 1980) results in the reduction of nitrate and nitrite values due to their utilization by primary producers (Venkatesan et al., 2001, Zahran and Willis 2003, Rajkumar 2004).

4.2 CYANOBACTERIAL AND ALGAL BIODIVERSITY OF STUDY SITES

For management of cyanobacterial hazards to human health, a basic understanding of the properties, the behavior in natural ecosystems and the environmental conditions which support the growth of certain species is helpful. This chapter provides information on the distribution of cyanobacterial and other algal genera in different ponds of Kurukshetra, as reported by their proliferation in aquatic ecosystems (Table no. 3.2.1, Fig. 3.2.1- 3.2.6).

In any ecosystem, not a single species grows independently and indefinitely, because all the species are interlinked and has cyclic
transformation of nutrients (Casamayor et al., 2000, Ernst et al., 2005). The physicochemical changes in the environment (Table no. 3.1.1 – 3.1.11) may affect particular species and induce the growth and abundance of other species (Table No. 3.2.1), which leads to the succession of several species in a course of time (Ilmavirta 1982, Jensen et al., 1994, Huisman and Hulot 2005). As reported in the publications addressing the persistence and stability of various organisms in fresh water ecosystems (Duncan and Blinn 1989, Scarsbrook 2002, Soininen and Eloranta 2004), cyanobacteria particularly forms their extreme blooms throughout the main part of the summer. We have also observed various algal bloom formations at different study sites i.e. Study Site-I (Synechocystis and Chroococcus bloom), Study Site-II (Merismopedia and Scenedesmus bloom) and Study Site-III (Microcystis bloom) respectively (Arjariya 2003, Pingale and Deshmukh 2005, Subha and Chandra 2005).

The interest towards the study of the cyanobacterial communities present in various water reservoirs of Kurukshetra fuelled with the richness in cyanobacterial biodiversity (Table no.3.2.1), because a lot of work has been already done at many places in the world (McDaniel et al., 1962, Margalef 1972, Stulp and Stam 1982, 1984, 1985, Tilman et al., 1986, Thajuddin and Subramanian 1992, 2005, Takami et al., 1997, Turner 1997, Otsuka et al., 2001, Taton et al., 2003, Oren 2004, Ochman et al., 2005, Tringe and Rubin 2005, Tringe et al., 2005, Addico et al., 2009, Yamamoto et al., 2010) yet, a place like Kurukshetra is still untouched and needs to be explored (Biban and Singh 2011). Such freshwater habitats are the breeding grounds for numerous algal and cyanobacterial blooms. These were the organisms (cyanobacteria and desmids) which were prevalent during the entire course of investigation (Table no. 3.2.1). However their population fluctuates according to the seasonal variations like rainy season, winter and autumn etc. in their respective site of occurrence. The climatic conditions have affected the biodiversity of
phytoplanktonic community (Panigrahi et al., 2009). Our findings suggest that there was a change in the community structure of all sites (Table no. 3.1.1-3.1.11); this could be attributed to the deterioration of the quality of water (Rai 2008). During summer season, low diversity of cyanobacteria was attributed to a massive bloom of *Microcystis* at Jyotisar sarowar, *Merismopedia* and *Scenedesmus* bloom at Sannihit sarowar and Synechocystis and *Chroococcus* bloom at Brahma sarowar (Table no. 3.2.1) respectively (Pingale and Deshmukh 2005, Rani et al., 2005, Subha and Chandra 2005). *Microcystis* is one of the dominant organisms that is associated with almost permanent blooms in tropical freshwaters that are exposed to constant sunshine, warmth, and nutrients. Formation of cyanobacterial blooms in freshwater bodies is essentially due to buoyant nature of these organisms (Jeyaraman 1972, Jones et al., 1998). Chaudhary and Meena (2007) enlisted various phytoplanktons from Udaipur Lakes, These Lakes were dominated by a toxic cyanobacterium *Microcystis*.e.g. *M.aeruginosa* Kütz (Kütz), *M.robusta* (Clark) Nygaard, *M.flos-aquaes* (Wittr.) Kirchner, *M.protocystis* Crow. Mohan et al., (2006) identified thirty eight species of blue green algae from Hadeera Tal (Kannuji) U.P. Their distribution was markedly influenced by the physicochemical nature of the aquatic system. Pandey and Dungarwal (2004) gave the taxonomic account of the freshwater algae collected from the tank of Eklingji temple in Udaipur. The algae belonged to Cyanophyta, Bacillariophyta and Chlorophyta. Suseela and Kumar (2004) described freshwater algal variation at three sites of river Gomti in India during winter. A phytoplankton and water quality survey was conducted by Zhao et al., 2004 during flood and dry seasons in 2000 in 19 typical reservoirs in Guangdong Province, China, in order to evaluate the trophic state and cyanobacterial pollution.

The cyanobacteria collected were tried to culture in laboratory using various growth media (Kraus 1966, Allen and Stanier 1968, Venkataraman
1969, Heaney and Jaworski 1977, Hellebust and Craigie 1978, Vaara et al., 1979, Van 1982, Kaushik 1987, Reuter and Petersen 1987, Castenholz 1988, Thiel et al., 1989, Waterbury 1991, Sigee 2005, Mohapatra 2006a; b). Some attempts were successful and we have isolated a few cyanobacterial genera like Nostoc muscorum, Anabaena variabilis, Merismopedia, Oscillatoria, Phormidium, Lyngbya and other genera like Cladophora, Chlorella etc. The Allen and Arnon’s medium was found most suitable to isolate and maintain the strains like Anabaena variabilis, Nostoc muscorum and Merismopedia. However Chlorella and Scenedesmus were better maintained in Chu-10 medium (Chu 1942). The isolated algal samples of Anabaena variabilis were analyzed for their physiological and biochemical activities under the influence of heavy metals like mercury, zinc, cupper and cadmium under laboratory conditions.

The ponds were also covered by some higher plants like Eichhornia, Hydrilla and Nelumbo etc. with very high population. The Diatoms like Navicula, Nitzschia, Pinnularia, Fragillaria were also in abundance in all the ponds throughout the observations. During the sampling time of rainy season, the dominant cyanobacterial genera were the filamentous forms like Oscillatoria, Anabaena, Lyngbya etc. Totally 19 genera of cyanobacteria (Fig. 3.3.8 and Fig. 3.3.9) (Frankelin 1972), 8 genera of green algae (Fig. 3.3.10) and 7 species of diatoms (Fig. 3.3.11) were recorded in the ponds (Krammer and Lang-Bertalot 1986, 1988).

Cyanobacterial diversity was firstly explored during the 19th century when cyanobacteria were recognized as a separate group of organisms (Ludwig and Klenk 2001). The classification was based entirely on morphology of isolated strains and specimens (Kukkonen et al., 1997, Kolmonen et al., 2004). The most important morphological traits were cell dimension, cell/filament morphology, type of cell division and presence of sheath/envelope (Komarek and Anagnostidis 1989, 1999, 2005, Doolittle 1999, Fisher and Triplett 1999).
This trend continued to the second half of the 20th century. From that time, molecular markers (especially 16S rRNA) have revolutionized cyanobacterial systematic (Liu et al., 1997, Lan and Reeves 2000, Gurtler and Mayall 2001, Lyra et al., 2001, Kurmayer et al., 2002, Liu and Stahl 2002, Konstantinidis and Tiedje 2005). However, it should be emphasized that the importance of morphological features is eminent. For this reason, a combination of morphological, ecological and molecular data was used and this led to the concept of “polyphasic approach” (Doers and Parker 1988, Cohen and Gurevitz 1991, Palenik and Haselkorn 1992, Pace 1997, Komarek and Kastovsky 2003, Komarek et al., 2004, Giovannoni and Stingl 2005) which is today the most respected route to the practical determination and description of cyanobacterial taxa (Klenk and Zillig 1994, Kondo et al., 2000, Jing et al., 2005). In the light of recent research, the real biodiversity is underestimated using the criterion of morphological variability. There are numerous examples of species entities which are morphologically indistinguishable but do not share a common evolutionary history, i.e. their molecular phylogeny is more diverse than morphological diversity (Palenik and Swift 1996, Palinska et al., 1996, Jain et al., 1999, Gordon et al., 2000, Rudi et al., 2000, Gogarten et al., 2002, Komarek 2003, Philippe and Douady 2003, Jenkins et al., 2004).

Traditionally, cyanobacterial taxa were described according to the International Code of Botanical Nomenclature due to their original classification as plants. Later, application of the International Code of Bacteriological Nomenclature was applied which stressed cyanobacterial evolutionary relations to bacteria (Castenholz and Waterbury 1989, Herdman et al., 2001, Rossello-Mora and Amann 2001, Hoffmann 2005, Oren and Tindall 2005). However, a simple transfer of taxa from botanical to bacteriological code is problematic as cyanobacteria possess unique evolutionary, ecological, and physiological features (Reynolds 1984, Castenholz 1992, Ishida et al.,
2001, Reynolds et al., 2002, Komarek 2003, Komarek et al., 2004, Castenholz and Norris 2005). The definition of a cyanobacterial species is still blurred. However more confusing is delimiting higher taxonomical units. The often accepted system described in Bergey’s Manual of Systematic Bacteriology (Castenholz 1988, Santos and Ochman 2004) separates cyanobacteria into 5 groups based on thallus characteristics. Cyanobacteria are a more than 150 genera and 2000 species, which play diverse yet significant roles in aquatic and terrestrial ecosystems. The first two groups possess coccoid thallus; the third group is filamentous, non-heterocytous; the fourth group is filamentous with heterocysts; and the fifth group is filamentous with heterocysts and true branching. Such distinctive morphological features, may give the impression of stability during evolution. However, these groups apart from the fourth and fifth have a polyphyletic origin. It follows that multicellularity and also unicellularity have evolved several times during evolution. Their diversity ranges from unicellular to multicellular, coccoid to branched filaments, nearly colorless to intensely pigmented, autotrophic to heterotrophic, psychrophilic to thermophilic, acidophilic to alkylphilic, planktonic to barophilic, freshwater to marine including hyper saline (Yoo et al., 1995, Thajuddin and Subramanian 2005).

They show specific growth pattern in a specific environment and therefore the distribution, ecology, periodicity, qualitative and quantitative occurrence of cyanobacteria differ widely (Table no. 3.2.1, Fig. 3.2.8 – 3.2.11). Indeed, it is the luxuriant growth of cyanobacteria in these environments which has earned their undistinguished nickname of “pond scum” (Jeyaraman 1972, Carpenter et al., 1985, Jasser and Arvola 2003, Chellappa et al., 2004). It can flourish well either in nutrient rich and warm water or at times in water with apparently low nutrient concentrations, subjected to higher temperature and bright light conditions (May 1975, Mez et al., 1998, Scheffer 1998).
Cyanobacterial blooms may have far-reaching ecological effects on aquatic ecosystems. Formation of cyanobacteria blooms typically leads to a reduction of light penetrating through the water column, causing a shading effect (Meffert 1987, Oliver and Ganf 2000). This lowered transparency (Table no. 3.1.2) causes poor growing conditions for epiphytes, phytoplankton, and benthic algae (Scheffer et al., 1993, Mlouka et al., 2004). Increases in pH (Table no. 3.1.5) due to carbon dioxide depletion (Table no. 3.1.7) by cyanobacteria blooms and/or anoxia resulting from a collapsed bloom could lead to massive fish kills (Paerl and Ustach 1982, Ochumba 1990, Vos and Roos 2005). Additionally, when these blooms die off they sink to the bottom, where they decompose causing a depletion of bottom water oxygen (Table no. 3.1.6) or hypoxic conditions (Schreurs 1992, Hecky et al., 1994, Magdaleno et al., 2001).

After having detected mercury in various samples (Table no. 3.3.1 – 3.3.4), our aim was modified as an attempt to look for the cyanobacterial strains as early warning bioindicators of mercury pollution among these Experimental sites (Table no. 3.3.3, Fig. 3.3.3). For this, the effect of different concentrations of Hg^{2+} as HgCl_{2} on the proposed indicator organisms (Anabaena variabilis) has also been studied under laboratory conditions. However, further work is required to be tested for the efficacy of such indicator organisms under different ecological niche having varied levels of metal contamination (Mona et al., 2011, Kiran and Kaushik 2012).

4.3 MERCURY CONTAMINATION IN DIFFERENT COMPONENTS OF STUDY SITES

Heavy metals constitute a special group of contaminants to aquatic ecosystems (Singh et al., 2005). They occur naturally in the ecosystem, with large variations in concentration (Nriagu and Pacyna 1988, Anon 1992). Their distribution in the water-sediment system is controlled by many physico-
chemical interactions and equilibria (Qu and Kelderman 2001, Wunderlin et al., 2001), largely governed by pH, concentration, type of ligands and chelating agents, oxidation state of the mineral components and the redox conditions of the ecosystem (Nriagu 1989). Metal contaminated sediments may release heavy metals back to the overlying water column (Qu and Kelderman 2001) and pose threat to aquatic as well as terrestrial life and hence deserve special attention (Rai et al., 1981). Now-a-days anthropogenic sources of heavy metal pollution are on increase. Waste derived fuels are especially prone to contain heavy metals, so they should be a central concern in a consideration of their use (Crump and Barlow 1980). The most probable source of mercury contamination to these religious fresh water ponds is the introduction of flowers, fruits, leaves, garlands, twigs etc. (different plantlets) by the people for many rituals and other occasions (Biban and Singh 2012). It could be possible that these plantlets might be earlier treated with the pesticides or the insecticides containing mercury as one of their constituents. These plants had bioaccumulated that mercury and introduced it into the clean water of such religious ponds.

Living organisms require varying amounts of “heavy metals”. Iron, cobalt, copper, manganese, molybdenum, and zinc are required by humans. Excessive levels can be damaging to the organisms, other heavy metals such as mercury, plutonium and lead are toxic metals that have no known vital or beneficial effect on organisms (Sorentino 1979). Their accumulation over time in the bodies of animals can cause serious illness, certain elements that are normally toxic for certain organisms under certain conditions, beneficial (Shumate and Strandberg 1985). Examples include vanadium, tungsten, and even cadmium. Metal pollution of the world’s waters continues to pose a serious threat to the health of man. Some workable systems for the extraction of these metals from the environment must be devised. These are known to
cause several health problems (Pilotto et al., 1997, Wagemann and Muir 1984, Zevenhoven and Kilpinen 2001). Higher concentration could be lethal also.

These heavy metals are released into aquatic environment from herbicides, fungicides, petroleum products, automobile exhausts, industrial wastes from the iron, steel and pesticides factories and other industrial complexes involving a cascade of biochemical processes by life support systems for their biotransformation, bioaccumulation and biomagnification. The ambient macrophytes of the environment act as bioabsorbants and bioaccumulators of heavy metals (Nasu and Kugimota 1981). They also act as bioindicators of the heavy metal pollution. Thus, bioremediation of the heavy metals from the aquatic environment can be done by the use of the algae (Biban and Singh 2012) and other macrophytes (Nasu and Kugimota 1981) inhabiting therein. Bioremediation is considered an efficient and environmentally safe technology for inexpensive decontamination of polluted systems (Mohapatra and Mohanty 1992, Radway et al., 2001). Application of bioremediation using indigenous microorganisms for decontamination of water systems polluted with organic contaminants provides a viable and sustainable approach for environmental resources. However, certain advantages of blue-green algae, like low nutrient requirement, nitrogen fixation, large biomass and exopolysaccharide production, provide them an edge over other inhabitants for their use as an attractive, economic and effective biosorbant (Dubey 1993). Mercury is among the highest priority environmental pollutants (Ramamoorthy et al., 1983, Johnson and Shubert 1986). Mercury and mercury compounds have no known beneficial biological function (Mora and Fabregas 1980). It presents a potential risk to the environment and humans being mutagenic and teratogenic.

The present study has been taken up to analyze the presence of the most hazardous heavy metal mercury ($\text{Hg}^{2+}$) along with some ecophysiological
characteristics of water samples from the selected religious ponds of Kurukshetra. All of the selected ponds were found contaminated with toxic levels of Hg. However, the presence of contamination by other heavy metals (not worked out at present) may not be ruled out. The mercury content of the water samples of different study sites was recorded in the decreasing order as Winter Season > Rainy Season > Summer Season (Table no. 3.3.1). The mercury content of the water samples of Study Site-I ranged between 0.04±0.01 – 0.19±0.01 μmol/ml, Study Site-II (0.137±0.009 – 0.29±0.013 μmol/ml) and Study Site-III (0.11±0.011 – 0.21±0.007 μmol/ml) respectively (Table no. 3.3.1). It had become interesting when we detected high levels of mercury pollution in pond waters (Table no. 3.3.1, soil samples (Table no. 3.3.2), crude algal biomass (mainly the cyanobacterial community, Table no. 3.3.3) and in other macrophyte plants (Table no. 3.3.4). During the course of our investigations, among all these observations, the maximum level of Hg²⁺ (0.92 μmol/ml) were recorded in the macrophyte plants at site-III during winter season of 2011 (Table no. 3.3.4). A minimum value of 0.01 μmol/ml was detected in the soil sample collected from the corners of the site-II during the rainy season of 2011. Several significant changes have been noticed according to varying seasons. The values are significant at the probability level of 0.05. Such a high concentration of Hg²⁺ detected may be attributed to the spurt of human activities, as the plant parts/products like twigs, leaves, flowers, fruits, seeds, wood, flour etc. are used to worship God during Pind-Daan, Shraadh etc (Biban and Singh 2011, 2012). Also the waste materials are regularly disposed of by the tourists visiting from entire India on the occasions of Solar Eclipse, annual Geeta Jayanti Festival and other occasions likewise (Fig 2.3, 2.4 and 2.5) respectively. It is also likely to happen that the environmental factors changing rapidly season wise may affect the potential of inhabiting cyanobacteria and other macrophytes to bioaccumulate Hg²⁺ at the study sites.
due to interactive potential of environmental variables on metal toxicity. Hence, there is a possibility of using some metal tolerant species of cyanobacteria (like *Anabaena variabilis*) in developing metal specific bioindicators.

### 4.4 IMPACTS OF INTRACELLULAR ACCUMULATION OF MERCURY ON PHYSIOLOGY OF ANABAENA VARIABILIS

The biomarker approach has been used extensively by ecotoxicologists for detecting exposure to and effects of environmental contamination. Cyanobacteria are characterized by high tolerance and can exist in various extreme conditions. Simultaneously, they are very sensitive and quickly respond to metals and other stress conditions (Murugesan and Ruby 2005, Biban and Singh 2012). Hence, cyanobacteria, are excellent models of bioaccumulation studies (Arunakumara *et al.*, 2008).

Therefore, the present enquiry was aimed with determining the actual level of intracellularly accumulated Hg$^{2+}$ responsible for damaging the physiology of the diazotrophic cyanobacterium *Anabaena variabilis* Breb., isolated from an ancient sacred pond (*Brahma sarowar*, Site-I) of the holy city of Kurukshetra, India (Biban and Singh 2011, 2012) (Table no. 3.2.1).

Heavy metal toxicity/uptake in cyanobacteria has been extensively reviewed (Whitton 1970, Sorentino 1979, Stratton *et al.*, 1979, Huntsman and Sunda 1980, Rai *et al.*, 1981, Whitton *et al.*, 1981, Shehata and Whitton 1982, Singh *et al.*, 1987, Singh 1987, Singh and Singh 1987, 1992a, 1992b, Murthy *et al.*, 1989, Wilkinson *et al.*, 1989, Pant 1993, 2000). They may bind up to 10% of their biomass as metals (Murugesan and Ruby 2005). To assess the potential biological impact of heavy metals in the environment, it is critical to know both the total and the biologically available levels of heavy metals. The toxic effect of heavy metal ions on aquatic organisms is mainly dependent on the metal ion species which in turn, may be determined by pH (Eide *et al.*, 1980, Schecher
and Driscoll 1985), chemical composition and concentrations of organic solutes (Hall and Fisher 1983), naturally occurring ligands (Guy and Kean 1980), synthetic complaxans (Van Baalen and O’Donnell 1978, Allen et al., 1980), thiols (Singh and Yadava 1985) and population size and other culture conditions (Allen and Gorham 1981, Heng et al., 2004, Satoha et al., 2005, Greger et al., 2007). Mercury (Hg\(^{2+}\)) occupies the prime position in the list of toxic heavy metals (Rai et al., 1981, Luoma 1983, Vijver et al., 2004, Kumudhaveni et al., 2013, Donnachie et al., 2014). It is used in thermometers, barometers, dental fillings, batteries and fluorescent lamps. In addition, a number of organomercurials have also been deployed as effective fungicides. The comprehensive study on its interaction with the cyanobacterial cell certifying the actual level of intracellular Hg\(^{2+}\) responsible for the observed effects (Fig. 3.4.1, 3.4.2 and 3.4.3) as well as its transport across cyanobacterial cell membrane is scant and is an active research area in the field of heavy metal phycology. Many prokaryotic and eukaryotic algae have been shown to accumulate organic mercury and inorganic salts as chlorides or sulphates, including lead acetate, tetraethyl lead and lead nitrate (DeFilippis and Pallagh 1976a, b; Mora and Fabregas 1980, Hassett et al., 1981, Johnson and Shubert 1986, Pant et al., 1992).

The bioassay studies on the cyanobacterium, Anabaena variabilis has clearly indicated that the organism to be very sensitive to very low concentrations of mercury (Table no. 3.4.1, 3.4.2) (Fig. 3.4.1) (Hofner et al., 1986, Humdy 2000, Murugesan and Ruby 2005). Metal accumulation by algae (Table no. 3.4.1, 3.4.2) is influenced by a number of biotic (e.g. cellular activity, algal biomass concentrations, extracellular products) factors (Fathi and Omair 2006, Macfle and Welbourn 2000) and abiotic factors like the presence of chelating agents. Uptake of metals and metalloids can be harmless (i.e. no
effects), toxic or even beneficial (Awasthi 2005). The effect of the metal ion is dependent on the varying degree of metal concentration (Fig. 3.4.2, $F_{\text{Hg}^{2+}, 5,40} = 7.607$, $p<0.05$; $F_{\text{days}, 8,40} = 7.38$, $p<0.05$; $r= 0.91$, $p< 0.025$) in the cell and also the amount of ion that can transfer the cell membrane (Vallee and Ulmer 1972, Sinkins 1979). Biosorption studies on the Hg$^{2+}$ uptake on a short term exposure (Table no. 3.4.1) showed its dependence on the metal concentration in the ambient medium (Fig. 3.4.1, $F_{\text{Hg}^{2+}, 5,35} = 32.34$, $p<0.01$; $F_{\text{min}, 7,35} = 22.62$, $p<0.01$; $r= 0.877$, $p< 0.025$). The uptake and toxicity of heavy metals by the target cells of Anabaena variabilis, is regulated by a complex mechanism conforming to physiological status of cells (Dral et al., 1985, Pettersson et al., 1985a). Once taken in, such cations adversely affect the cyanobacterial physiology and biochemistry (Asthana et al., 1990, 1992, Pandey et al., 1992). The rapidness in the uptake of Hg$^{2+}$ ions by A. variabilis during the first 30 min exposure (Fig. 3.4.1) to all Hg$^{2+}$ concentrations indicates towards the energy-dependent nature of the event, as reported for Cu$^{2+}$ uptake by some cyanobacterium (Verma and Singh 1990, Fathi et al., 2000) and Cd$^{2+}$ uptake by Anacystis nidulans (Singh and Yadava 1985). The consistent influx of Hg$^{2+}$ ions during this period enhanced the intracellular Hg$^{2+}$ build-up within first 30 min in a concentration dependent manner (Table no. 3.4.1). There are reports that organic mercury is highly toxic over its inorganic counterpart (Roderer 1983, Singh and Singh 1992a). Also the former (organic) is accumulated very rapidly by algae like Chlorella, Scendesmus and Microcystis, and transferred to inorganic mercury following 48 h of uptake (Havlik et al., 1979, Stratton et al., 1979). Cyanobacterial cells, in line with eukaryotic algae, accumulate heavy metals (Vallee and Ulmer 1972, Van 1982) through a faster initial reaction (adsorption), followed in sequence by relatively slower, metabolism-dependent intracellular cation uptake, as observed for zinc (Shehata and Whitton 1982), cadmium, copper and zinc (Les and Walker 1984), cadmium (Singh and
Yadava 1985), copper (Singh 1985), aluminium (Pettersson et al., 1985b) nickel (Campbell and Smith 1986) and zinc (McHardy and George 1990). Since nature is never unimetallic and heavy metals rarely occur singly in natural environments, it became imperative to account for toxicity potential of Hg²⁺ in presence of other interacting metal cations.

The growth inhibitory effects of Hg on plants have been directly linked to ultrastructural damage (Gadd 1988). Growth inhibition (Table no. 3.4.4-3.4.7) and chlorosis (Table no. 3.4.12, 3.4.13) are common symptoms of metal toxicity, in which photosynthesis is probably the most affected metabolic process (Brookes and Rumsby 1965, Barbara and Michael 1994, Overnell 1975, Fathi and Falkner 1997, Ali et al., 2006). Alterations in morphology and ultra structure have also been frequently reported (Choudhury and Panda 2005). Growth inhibition in micro algae is well known for metal toxicity (Fry 1990) and found to be related to the amount of metal bound to the algal cell surface in some cases, to the amount of intracellular metal (Franklin et al., 2000, 2001, Ma et al., 2003) and to the chemical nature of the metal (Fathi and Omair 2006, Tripathi and Gaur 2006). The general growth of the cyanobacterial cells could be negatively correlated with mercury uptake (Fig. 3.4.3, 3.4.4). Here the data are discussed in the light of the physiological efficiency. The viability of the cyanobacterial cells was of prime concern during exposure to the elevated Hg²⁺ levels (Fig. 3.4.2).

Cyanobacteria and algae possess a wide range of coloured components including carotenoids, chlorophylls and phycobiliproteins (Jeffrey and Humphrey 1975, Jenson 1978, Giovannoni et al., 1988, Sarada et al., 1999, Gerla et al., 2004). The principal phycobiliproteins are phycocyanin, allophycocyanin and phycoerythrin. Phycocyanin is an important light harvesting photosynthetic pigment in cyanobacteria (Gantt 1980, 1981). Phycocyanin is used as a colourant in food (chewing gums, dairy products, ice
scherbaths, gellies etc.) and cosmetics such as eyeliners in Japan, Thailand and China (Muthulakshmi et al., 2012). It was also shown to have therapeutic value (immune modulating activity and anticancer activity) (Romay et al., 2003). The prevailing environmental conditions such as temperature and ionic strength of the medium, considerably affect the aggregation and assembly of phycocyanin into phycobilisomes (Cohen-Bazire and Bryant 1982, Almog and Berns 1984). It has been a general observation that cyanobacteria extrude phycobiliproteins in the ambient medium (Table no. 3.4.17) when the cultures are sufficiently old, or even during log phase, if subjected to environmental stress (Table no. 3.4.18) (Allen 1968). The responses of the mercury stress events were also examined in the presence of diluted spent medium (a natural chelator) and EDTA (a known synthetic chelator) (Table no. 3.4.18) (Fig. 3.4.16, F$_{chelators \ 4, \ 28}$ = 28.89, p<0.025; F$_{hours \ 7,28}$ = 106.37, p<0.01; r= 0.86, p< 0.01 ) (Bailey and Taub 1980, Biban and Singh 2013). These prokaryotes are excellent source of a wide range of biologically active compounds of significant values that has fascinated researches for their pharmaceutical, biotechnological and biomedical exploitation (Sarada et al., 1999, Rastogi and Sinha 2009). e.g. polyunsaturated fatty acids (PUFAs), β- carotene and other photopigments (carotenoids, chlorophyll and phycocyanin) that function as antioxidants, anti-inflammatory, neuro and hepatoprotective (Romay et al., 2003) and antitumor compounds (Liu et al., 2000, Pardhasaradhi et al., 2003), polysulfated polysaccharides as antivirals (Ghosh et al., 2004), sterols as antimicrobials and mycosporine-like amino acids (MAAs) and scytonemin as photoprotectants (Rastogi and Sinha 2009). Not only this, they are also used as ingredients of food, cosmetics, natural dyes and fluorescent markers (Glazer et al., 1976, Kronik and Grossman 1983) for immunodiagnosis.

Since the data on Hg- inhibited photoautotrophic growth (Table no. 3.4.4- 3.4.7) was based on the whole cell suspension absorbance at 650 nm
(Fig. 3.4.3-3.4.7), it was of interest to go for the changes in different photopigments, if any (Jenson 1978). Hg treatment reduced the amount of chlorophyll (Table no. 3.4.12, 3.4.13) (Fig. 3.4.11) and resulted in breakdown of thylakoids (Kamp-Nielsen 1971, Jones 1984, Kuwabara 1985, Lenti et al., 2002). The metals were found to cause disruption of the thylakoid membranes in *Anabaena flos-aquae*, resulting in the degradation of the light harvesting pigment and thus decreasing their contents of the cells (Rai and Dubey 1989). Such observations suggest that the association of phycocyanin with lamellar proteins was so strong that it took a minimum of 4 h for Hg$^{2+}$ ions to disrupt its association as well as assembly into phycobilisomes, and to make it free to move. It also permeabilized the cell membrane and enhanced membrane potential, as observed in *Cylindrospermum* (Singh et al., 1989). As a consequence of this, the cell membrane was disrupted and the intracellularly accumulated Hg$^{2+}$ ions were also released into the ambient medium along with other cellular contents (Table no. 3.4.17). This active plus passive uptake of Hg$^{2+}$ was collectively responsible for the observed leakages. The 2-way ANOVA (analysis of variance) suggested that the leakage of were more respondent to exposure time than the Hg$^{2+}$ concentrations (Fig. 3.4.19, $F_{\text{Hg}}^{2+} 5,35 = 26.41$, $p<0.025$; $F_{\text{hours}} 7,35 = 5.95$, $p<0.05$; $r = 0.98$, $p< 0.05$). A corollary of these findings is that the extent of intracellular Hg$^{2+}$ build-up (Table no. 3.4.2) is the ultimate crucial moiety responsible for making it unviable to retain the free phycocyanin and accumulated Hg$^{2+}$ ions intracellularly. The observed reductions in the effectiveness of Hg$^{2+}$ ions to induce the leakage of phycocyanin from *A. variabilis* cells in the presence of EDTA (Fig. 3.4.16) (Kaplan et al., 1987), have been attributed to the lesser bioavailability of Hg$^{2+}$ ions due to the formation of less mobile Hg$^{2+}$-EDTA-complex, imparting less chances for the Hg$^{2+}$ ions to get adsorbed on the cell surface for its follow-up uptake ($F_{\text{chelators}} 4, 28 = 28.89$, $p<0.025$; $F_{\text{hours}} 7,28 =
106.37, p<0.01; r= 0.86, p< 0.01) (Khalil 1991). This interpretation is based on the fact that the chelated toxic metal ions become less toxic than the corresponding free metal ions as a consequence of reduced mobility of metal-chelate-complex, resulted due to metal-chelate interactions (Hart 1981, Rai and Raizada 1985), which in turn is governed by the bonding preferences of metal ion (the donor preference) and the chelating agent (acceptor preference)(Jones 1984).

The observed decrease in the protein (Fig. 3.4.7, 3.4.8) and carbohydrate contents (Fig. 3.4.9, 3.4.10) in Hg-treated cells (Hutchins et al., 1986) suggests that mercury shares such inhibitory potentials with those of Cd, as reported for other cyanobacteria (Hofner et al., 1986). Almost similar pattern of metal-induced decrease in the respective amounts of protein (Table no. 3.4.8, 3.4.9) has also been observed in many other organisms (Singh et al., 1989, Verma and Singh 1990, Awasthi 2005). Such a situation has been suggested to arise mainly as a consequence of decreased metabolic activities under Hg-stress or alternatively, it can also be suggested that Hg-toxicity could eventually result in the depolymerization of the stored glycogen (Fig. 3.4.7, F_{Hg}^{2+},_{6,48}= 1.05, p<0.05; F_{days8,48}= 12.50, p<0.05; r= 0.094, p< 0.01) (Allen and Smith 1969). However, the present data accounts for only the total carbohydrate content (Fig. 3.4.9, F_{Hg}^{2+},_{6, 48}= 23.35, p<0.01; F_{days8, 48}= 17.01, p<0.05; r= 0.84, p< 0.05) of the cyanobacterium (Table no. 3.4.10, 3.4.11).

After studying the deleterious impacts of Hg on the direct or indirect measurements of growth of A. variabilis have been carried out, experiments were directed towards the nutrient uptake studies. Among the various nutrients, only the nitrate and ammonium uptake studies were undertaken presently in order to assess the impact of Hg on such energy dependent metabolic events (Crist et al., 1981; 1988, Brouers and Hall 1986, Ernst et al., 2005). NO₃⁻ uptake in A. variabilis (Fig. 3.4.21) followed a similar pattern as described for
other cyanobacteria (Vitousek et al., 2002), and the process is known to be mediated through the ferrodoxin-nitrate Reductase-system (Guerrero et al., 1981). The uptake of this nitrogen source (Table no. 3.4.21, 3.4.22) is largely an energy dependent phenomenon, and the inducible enzyme nitrate reductase, operates at the expenses of electrons derived through photosynthesis (De Filippis et al., 1981, Fathi and El-Shahed 2000). The controlling mechanism of NO$_3^-$ utilization and its reduction is the regulation of synthesis and activity both, the uptake and reducing system of NO$_3^-$, apart from a more or less direct control exercised by other events like translocation, compartmentalization, substrate and reductant availability as well as the various environmental factors (Camm and Stain 1974, Whitton and Potts 2000). The displacement of an essential metal ion forming the central and functional part of the enzyme protein may be one of the reasons for inhibition of NR by heavy metals, and secondly, interference with sulphydryl (-SH) group which often determine the secondary and tertiary structures of proteins. Besides, a reduced energy supply due to inhibition of photosynthetic electron transport and an indirect inhibition of uptake of substrate (NO$_3^-$) of energy may be other important reason. In case, heavy metal cations damage the cell membrane, compete with essential nutrient ions, interact with sulphydryl groups or result in depletion of cellular ATP and reductant pools (Apte et al., 1998), would not only affect NO$_3^-$ utilization but also other physiological/biochemical reaction in cyanobacteria (Apte and Bhagwat 1989). The present investigation on uptake and reduction of NO$_3^-$ in A. variabilis (Table no. 3.4.21, 3.4.22, 3.4.23) revealed that the exposure of the algae to graded concentrations of Hg$^{2+}$ in a medium supplemented with saturating NO$_3^-$ concentrations (Fig. 3.4.21, 3.4.21), resulted in the inhibition of NO$_3^-$ uptake depending on the concentration of the metal used (Fig. 3.4.21, $F_{Hg}^{\ 2+\ 6,48}= 38.78, p<0.05$; $F_{days8,48}= 26.39, p<0.025$; $r= 0.94, p< 0.05$). NO$_3^-$ ions enter the cell via a transport system and the anion act as the inductor of nitrate
reductase (Flores et al., 1983). The impacts of Hg$^{2+}$ ions on NO$_3^-$ uptake was in non-competitive manner (Fig. 3.4.22, 3.4.23, F$_{\text{metals}}$ 5,40 = 20.34, p<0.01; F$_{\text{days 8,40}}$ = 3.48, p<0.01; r= 0.91, p< 0.05). So, an indirect route of Hg- inhibition of NO$_3^-$ uptake is suggested through the depletion of cellular energy and reductants (Jones 1984).

After studying the pattern of NO$_3^-$ uptake in the experimental organism, the another nutrient, NH$_4^+$ ion was also explored for its uptake by the selected cyanobacterium (Musgrave et al., 1982, Jeanfils and Loudeche 1986, Berman and Bronk 2003). The NH$_4^+$ uptake pattern in A. variabilis showed saturation at 1.0 mM NH$_4$Cl (Table no. 3.4.24, Fig. 3.4.24). The simultaneous addition of Hg (0.5-1.0 µM) showed that the uptake process was most sensitive to the metal ions even at low concentrations of Hg (Fig. 3.4.25). The most possible explanation offered to such observations would be that the entry of nutrient ions from the nutrient solution depends on the interaction/encounter with other ions also (Table no. 3.4.26). Subsequent experiments on NH$_4^+$ uptake showed that the nature of Hg-inhibition was similar to those of NO$_3^-$ uptake. The data (Table no. 3.4.25) also establish a correlation between metal concentration and exposure time (F$_{\text{Hg}}$ 6,48 = 26.0, p<0.01; F$_{\text{days 8,48}}$ = 24.0, p<0.01; r= 0.95, p< 0.05), and suggest that in such cases, the concentration of a metal required is inversely proportional to the exposure time of the selected blue green alga. This statement is equally applicable to interacting metals (Fig. 3.4.26, F$_{\text{metals}}$ 5,40 = 32.22, p<0.05; F$_{\text{days 8,40}}$ = 14.44, p<0.025; r= 0.91, p< 0.05).

In plants, nitrate reductase (NR) and nitric oxide synthase (NOS) have been proposed as two enzymatic systems responsible for NO production (Berges 1997, Bartha et al., 2005). NO is involved in alleviation of heavy metals induced toxicity by directly regulating accumulation and translocation of heavy metals in plants (Chen and Yang 2012). NO have been demonstrated to involve the regulation of Hg-induced oxidative stress and plant tolerance to
algae (Cho and Park 2000, Cargnelutti et al., 2006). NO is shown to depress the
 generation of hydrogen peroxide (H₂O₂) and alleviate phytotoxicity by
 enhancing antioxidative capability (Park et al., 1991, Prabaharan and
 the generation of endogenous NO (Bartha et al., 2005, Chen and Yang 2012)
 which is closely associated with the intrinsically physiological processes in
 plants. Any stimulation in the ATP pool and availability of NADPH may
 stimulate ATP dependent processes like NR activity (Awasthi 2005). Thus,
 enhanced photosynthetic activity indicates towards the increase of the
 enzymatic activities. The ability of metal ions to enter into a chemical reaction
 as a positively charged ion and their capacity to bind to the enzyme prosthetic
 group is an important reason behind the mechanism of enzyme Nitrate
 Reductase activity inhibition (Table no. 3.4.29, Fig.3.4.28, 3.4.29). Metals like
 Zn²⁺, Ni²⁺ and Cd²⁺ had interacted negatively (Fig.3.4.29, F_{metals 5,40}= 72.95,
p<0.05; F_{days 8,40}= 13.87, p<0.01; r= 0.92, p< 0.05) with the NR activity of blue
 green algae, Anacystis nidulans and a green algae, Chlorella vulgaris in both
 free and immobilized states (Awasthi 2005, Awasthi and Rai 2005). The
 sensitive histochemical based detection of Hg-triggered accumulation of ROS
 and oxidative injury can be used as biomarkers to indicate the toxicity in plants
 (Ge et al., 2009, Chen and Yang 2012).

 NR (Nitrate Reductase) is the enzyme that is being used to help clean up
 the environment (Berges 1997, Bier 2002), and has great potential to be part of
 the solution to the global problem of excess nitrate and related nitrogen-
 nutrients in water sources (Campbell and Campbell 1998). NR activity is the
 limiting factor when considering the growth and protein production of algae
 (Lau et al., 1998). Each organism has different potential to accumulate NO₃⁻
 from the environment due to variation in its NR characteristics (Berges 1997).
 Since heavy metals and nitrate, both are harmful pollutants and often occur
together, it is necessary to know the effects of heavy metals on the enzyme activity more so that the NR in algae can be used to treat the nutrient pollution. Nitrate reduction processes are perhaps most significant in maintaining the water quality by alteration of nitrate to nitrite (Gendel and Nohr 1989, Bier 2002, Awasthi and Rai 2005).

It has also been reported that the metal uptake capacities of certain algae are much higher than activated carbon, natural zeolite and synthetic ion exchange resins. The work of Volesky (Volesky 1992; 2001, Volesky and Holan 1995) has demonstrated the use of marine algae to take metal ions. Algae, a renewable natural biomass exhibit different affinities towards different metals and therefore, are a very important candidate to be employed as a biosorbent material. The use of both marine and river algae for adsorption and elution of gold, silver and cobalt has been reported (Volesky 2001). They have developed natural methods and resistance towards heavy metals and their utilization for treatment of industrial effluents and metal recovery has been also proved successful (Wehrlein and Wettern 1994, Kiran and Kaushik 2012).