Chapter 4

Evaluation of metal-induced molecular modulations in protein, DNA and lipid in the survival of N. muscorum
4.1 INTRODUCTION

In the last two decades there has been an explosive interest in the role of reactive oxygen species (ROS) in mediating oxidative damage to cellular components. The ROS generated as a result of stress gain biological and toxicological importance, because of its potent oxidative potential and indiscriminate reactivity with cellular components, such as lipids of biological membranes, proteins of enzymes, and DNA (Jackson and Loeb, 2001; Stadtman and Levine, 2000; Richter, 1987)

Membrane phospholipids of aerobic organisms including cyanobacteria are continually subjected to oxidant challenges from endogenous and exogenous sources. Metals and their chelate complexes, such as copper, chromium, nickel, and cadmium, are implicated in lipid peroxidation which is initiated mainly by hydroxyl radicals, especially in transition metal-catalyzed reactions and subsequently in the promotion of cell death (Sole et al., 1990; Kasprzak, 1995). DNA is another key cellular component that is particularly susceptible to oxidative damage by ROS (Cerutti, 1985). The polyanionic nature of DNA provides a useful substrate for infiltration through membranes and adherence of metal cations, thus facilitating the formation of HO• adjacent to these critical biological targets (Halliwell and Aruoma, 1991). Additionally, the heterogeneity of DNA molecules allows for HO• attacks, including the nucleobases and the sugar–phosphate backbone (Buxton et al., 1988) resulting in fragmentation of deoxyribose with single-strand breaks, and oxidation of the sugar moiety leading to programmed cell death (PCD) (Dizdaroglu et al., 1975; Breen and Murphy, 1995).
PCD is a mode of cell death that most often occurs during metamorphosis, tissue homeostasis, the elimination of damaged or abnormal cells and defense against adverse stimuli, alongside more familiar processes of cell growth and differentiation (Bergmann et al., 1998; Jones, 2001). PCD-like cell death is well established in all eukaryotes ranging from unicellular organisms such as amoebae, fungi and yeast (Matsuyama et al., 1999; Frohlich and Madeo, 2000), invertebrates (Bergmann et al., 1998), to higher animals and plants. The physiological cell death processes occurring in many prokaryotic microorganisms including bacteria (Ameisen. 1996; Yarmolinsky, 1995; Chaloupka and Vinter 1996; Samuilov et al., 2000) and the phytoplankton cyanophyceae (Lee and Rhe, 1997) are also considered types of PCD (Ameisen, 1996; Lee and Rhe, 1997, 1999; Samuilov et al., 2000). The earlier prediction that PCD occurs in Anabaena (Hochman 1997; Lee and Rhe, 1999) was later on proved by Ning et al., 2002 whose study demonstrated salinity induced PCD in Anabaena. Till date no data is available regarding metal induced PCD in cyanobacteria. As generation of ROS is an unifying factor in all kind of stresses, it can be predicted that PCD can also be induced due to metal stress.

Furthermore, proteins are another important target of ROS. Metal-catalyzed damage to proteins involves oxidative scission, loss of histidine residues, bityrosine crosslinks, the introduction of carbonyl groups, and the formation of protein-centred alkyl, R•, alkoxyl, RO•, and alkylperoxyl, ROO•, radicals. (Davis,1987 and Stadtman, 2000); oxidative modifications of amino acid side chains, reactive oxygen- species-mediated peptide cleavage, reactions of peptides with lipids and carbohydrate oxidation products.
Alterations in the expression and activities of proteins act as the most important molecular biomarker of environmental stress. To face stress, the functioning of some proteins is inhibited or lost and that of others are enhanced or induced. Low molecular weight proteins like Metallothioneins (MT) are induced in a variety of species (mammals, plants, and microorganisms) and several marine invertebrates following metal exposure. The metal induced peptides act as metal-chelating agents for the excess of toxic metals in the cells, thus playing important roles in metal metabolism in aquatic organisms and particularly in the detoxification mechanisms, through oxygen free radical scavenging actions and metal binding (Bebianno and Serafim, 1998; Kalpaxis et al., 2003; Cavalento et al., 2002).

In view of some gaps in elucidating the underlying mechanism(s) of Al-induced protection in *N. muscorum* as mentioned below: (i) Al effects has never been addressed in N₂-fixing cyanobacteria, *N. muscorum* employing protein profiling along with physiological, and biochemical variables, (ii) complete lack of data on the effect of Cu, Cd and Al on the DNA content and their role in mitigating PCD. Further, based on our observations of the previous chapter that *N. muscorum* showed a better survival in the presence of higher concentration of Al, probably due to modulation of some physiological and biochemical processes leading towards protection of *N.muscorum* from its deleterious effects, the present study, dealing with selected stress responsive biomolecules therefore, combines protein analysis by SDS-PAGE in tandem with DNA fragmentation studies and lipid profiling to decipher the molecular mechanism of Al toxicity in diazotrophic
cyanobacterium *N. muscorum*. Our results show that Al up-regulated a distinct pattern of proteins which might have protected the DNA from the deleterious effects of certain ROS and subsequently inhibited the PCD which in turn resulted in better survival.
4.2 Material and Methods

4.2.1 Analysis of protein by SDS-PAGE

**Preparation of crude extract**

The total crude protein was extracted from cyanobacterial biomass using modified method of Ivleva and Golden, 2008

**Reagents**

a) Glass beads: diameter 0.2–0.3 mm (Sigma), acid-washed.

b) Phosphate-buffered saline (PBS) buffer (pH to 7.4)

136 mM NaCl,

2.6 mM KCl,

10 mM Na$_2$HPO$_4$,

1.76 mM KH$_2$PO$_4$;

c) PMSF 0.01M

0.01M stock solution of PMSF was prepared by dissolving 17.42 mg in 10 ml isopropanol and stored at -20°C

**Procedure**

A 100-mL of logarithmic phase culture of *N.muscorum* optical density 0.5 at 750 nm was taken and the cells were spun down for 10 min at 1500g. The pellets were washed by resuspending in 2 mL of PBS buffer and repeating the spin. The pellets
were again resuspended in 200 µL of PBS. The cells were frozen at –80°C and then quickly thawed at 37°C to allow partial cell breakage. For the remainder of the procedure, the samples were kept on ice and in the presence of protease inhibitors. 100 µL of PBS buffer was added and suspension was spun at 1000g. The supernatant fraction was collected and again spun at 1000g for 10s to pellet residual glass beads. The resulting green supernatant fraction was a crude cell extract.

The protein concentration was measured using Lowry method using BSA as standard. The cell extract was used for SDS-PAGE analysis

*Estimation of protein*

Protein was measured by the method developed by Lowry *et al.* (1951) and modified by Herbert *et al.* (1971). (Described in sec 2.2.15)

*Sodium Dodecyl-sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE) of the Crude protein extract*

SDS-PAGE of cyanobacterial protein was performed on 12% polyacrylamide resolving gel after seven days of metal treatment and carried out in a vertical system (Bangalore Geni) according to the method of Laemmli (1970)

SDS-PAGE is used for analyzing mixtures of proteins. In this technique, proteins are made to react with an anionic reagent, Sodium Dodecyl-sulfate (SDS), to form negatively charged complexes. The amount of SDS bound by protein, and so the charge on the complex, is proportional to its size. The proteins are denatured and
solubilized due to their binding with SDS. Thus, proteins of either acidic or basic pI form negatively charged complexes that can be separated on the basis of differences in charge and size by electrophoresis through a sieve like matrix of polyacrylamide gel.

**Reagents for SDS Gel Electrophoresis**

**Gel Preparation**

**Reagents**

**Resolving gel (5ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>Acrylamide mix</td>
<td>0.33 ml  (30% W/V)</td>
</tr>
<tr>
<td>Tris (pH 8.8)</td>
<td>1.3 ml   (1.5 M)</td>
</tr>
<tr>
<td>SDS</td>
<td>50 µl    (10% W/V)</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>50 µl    (10% W/V)</td>
</tr>
<tr>
<td>TEMED</td>
<td>3 µl</td>
</tr>
</tbody>
</table>

(N, N, N, N–Tetramethylethylene diamine)

**Stacking gel**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>1.4 ml</td>
</tr>
<tr>
<td>Acrylamide mix</td>
<td>500 µl   (30% W/V)</td>
</tr>
</tbody>
</table>
Tris (pH 6.8) — 250 µl (1 M)
SDS — 20 µl (10%)
(Sodium dodecyl sulphate)
Ammonium persulphate — 20 µl (10%)
TEMED — 2 µl
(N, N, N, N, Tetramethyl ethylene diamine)

Procedure

Thoroughly cleaned, dried glass plates and three spacers were taken and assembled properly. The spacers were set with 1 mm distance from the edges of the glass plates. The construction was held together with bull-dog clips. The chamber was clamped in an upright, level position. A sufficient volume of separating gel mixture (5ml) was prepared. The freshly mixed solution was carefully pipetted or poured into the chamber without generating air bubbles. It was poured to a level about 1 cm below, where the bottom of the well-forming comb would rest when it would be in position. A thin layer of water or methanol was poured to prevent oxidation of resolving gel. The layer was discarded when resolving gel was polymerized. The mixture was left to stand until it was set.

Stacking gel (2 ml) was prepared properly. The solution was pipetted into the chamber. The comb was inserted and the gel was allowed to stand until set. When the stacking gel was set, the comb was removed without distorting the shape of the wells. The clips, holding the plates together, were removed and gel was
installed in the apparatus. The apparatus was filled with reservoir buffer (5 X Tris glycine electrophoresis buffer: (stock solution) (500 ml) is prepared by adding 7.55g Tris Base and 47g Glycine (pH 8.3 ) in 400 ml of dH₂O. Then 25 ml of a 10% (w/v) stock solution of SDS was added and adjusted the volume to 500 ml)

Samples were mixed with loading dye (100 mM DTT (Dithiothreitol), 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% (v/v) glycerol, 0.1% bromphenol blue dye) while the gel was setting/polymerizing. The sample solution was heated in boiling water for 2 min and cooled before loading. The gel was finally loaded. Required volume of sample solution was taken up in a pipette and carefully injected into a sample well through the reservoir buffer.

After loading all the samples, electrophoresis was started by turning on power (DC) on a gel of about 1mm thickness and about 14 cm length, with an applied voltage of about 80 V when samples are in stacking gel then on 120 V through running gel. At the end of electrophoresis (when the dye front reached the bottom of the gel), protein bands in the gel are visualized by staining.

The gel was removed from the glass plates and immersed in the protein stain immediately. The gel was left there with gentle agitation until the dye had penetrated the gel. Dye that was not bound to the protein was removed by transferring the gel to a destaining solution. After about 24 hr, with gentle agitation and several changes of destaining solution, the gel background became colorless and protein bands of blue, purple color is visible.
Finally, the whole cell protein profiles of the samples were visualized under Trans white light and captured using Gel Doc (BIO-RAD) All experiments were performed in three independent replicates and only those bands present in at least two gels of the independent set were taken for analysis.

4.2.2 DNA extraction and analysis

DNA isolation

Reagents

Lysis buffer

1M Tris HCl (pH 7.2)

0.5M EDTA (pH 8.0)

5M NaCl

SDS 20%

Proteinase K 20 mg/ml

Chloroform: Isoamyl alcohol 24:1

RNAse A (1mg/ml)

Isopropanol

Ethanol 70%

Procedure

A 50-mL of logarithmic phase culture of untreated and metal treated (Al, Cu and Cd; 20 µM for 48 hrs) N.muscorum optical density 0.5 at 750 nm was taken and
DNA was extracted by proteinase K digestion method (Sambrook and Russell, 2001 modified by Arif et al., unpublished). Cells were resuspended in 567 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) by repeated pipetting. Lysis of the cells was performed using 30 µl of 10 % SDS and 3 µl of 20 mg/ml proteinase K. The mix was subsequently incubated for 1 h at 37 °C. 100 µl of 5 M NaCl was added. The samples were gently mixed by inversion and incubated for 10 min at 65 °C. Nucleic acids were thereafter isolated by a chloroform:isoamyl alcohol (24:1) separation. DNA was finally recovered by precipitation using 0.6 volume of isopropanol and centrifugation (5 min, 4 °C, 15000 rpm). The DNA pellets were washed with 1 ml of cold 70 % ethanol. Finally, the tubes were centrifuged one last time for 5 min (4 °C, 15000 rpm), the supernatant was discarded, and each pellet dried before being resuspended in 100 µl TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

**DNA Concentration, yield and purity**

Tris -10 mM

EDTA- 1 mM

Spectrophotometric analysis and agarose gel electrophoresis will reveal the concentration and the purity of the genomic DNA. The samples were diluted with TE buffer and the absorbance was taken at 260 nm and 280 nm using a quartz cuvette. An absorbance of 1.0 at 260 nm corresponds to 50 µg/ml of DNA. Purity
of DNA is determined by measuring the ratio of absorbance at 260 nm and 280 nm.

Concentration of DNA sample ($\mu$g/ml) = 50 * $A_{260}$ * dilution factor

**Gel preparation**

Agarose 1% in TAE buffer (40mM Tris-HCL, pH 8.0 containing 2 mM EDTA) was dissolved by heating. The solution was cooled to about 60°C, ethidium bromide (0.5 $\mu$g/ml) was added and then poured into the gel tray and allowed to solidify at room temperature.

**Agarose gel electrophoresis**

Untreated and metal treated DNA were mixed with the sample buffer (0.125% bromophenol blue, 30% glycerol) in 3:1 ratio and loaded in the wells of 1% agarose gels. Electrophoresis was done for 2 hr at 50V and the gels were viewed under UV light.

**Fluorescence emission profile of isolated DNA**

Fluorescence emission spectral analysis of isolated DNA (5 $\mu$g/ml) of untreated and metal treated (Al, Cu and Cd) *N. muscorum* was undertaken in the wavelength range of 500-700 nm using quartz cuvettes on a Shimadzu RF-5301 spectrofluorometer at an excitation wavelength of 325 nm. Ethidium bromide (2.5 $\mu$g/ml) was used as an external chromophore for the process.
4.2.3 Lipid Profiling

**Extraction and estimation of total lipids**

The total lipids were extracted from treated and untreated cyanobacterial biomass using modified method of Bligh and Dyer, (1959). The lipids were extracted from dried biomass of the cyanobacterium with chloroform-methanol (2:1, v/v). The extract was then subjected to the separating funnel for the separation into chloroform and aqueous methanol layers by the addition of methanol and water to give a final solvent ratio of chloroform: methanol: water of 1:1:0.9. The chloroform layer was washed with 20 ml of 5% NaCl solution, and evaporated to dryness.

**Partial purification of Total lipids**

The extracted total lipids thus obtained from the above process were again separated by Thin layer chromatography (TLC).

**Thin layer chromatography**

TLC was carried out on silica gel F_{254} plates (thickness = 0.25 mm; Merck, Darmstadt, Germany) following the method of Nichols and Wood (1968) and Walsby and Nichols (1969). A known volume of the lipid extract was spotted on to the plates. The plates were activated at 60 °C for 2 h before spotting and then kept in chromatographic tank, saturated with solvent system and various lipids were separated. The solvents used were chloroform: methanol: glacial acetic acid...
and water (85: 15: 10: 3.7). After the development, plates were removed and dried at room temperature for complete evaporation.

**Methods for visualization of lipids on TLC Plate**

Visualization of the plate was performed by Iodine vapour method (Randerath, 1964). The plates were exposed to the iodine vapour for 10 min. Unsaturated lipid appeared as yellow, grey, blue and pink colour. Colours depending on their nature are also given by saturated lipids containing nitrogen and esters of fatty acids.
4.3 Results

4.3.1 Alterations in Protein Profile of *N. muscorum* under metal stress

To analyze the alterations in protein profile under metal stress, equal amounts of a soluble protein fraction from control, and metal-treated (Al, Cu and Cd) *N. muscorum* were subjected to SDS-PAGE. Protein samples were run on 12% polyacrylamide gel. The CBB-stained, one-dimensional protein pattern shown in Figure 4.1-4.3 reveals the first differences between the control and treatments.

Comparing the selected metal (Al, Cu and Cd) treatment of *N. muscorum* for 7 days with its corresponding control, the appearance of several differentially expressed significant protein bands in all the three metal (Al, Cu and Cd) treated *N. muscorum* was visualized. In addition to several up regulated and down regulated proteins, newer protein bands also appeared in treated cultures when compared to the corresponding control.

4.3.1.1 Changes in polypeptide pattern of *N. muscorum* under Al stress

Al treated cells showed a very unique protein profile different (Figure 4.1). None of the polypeptides showed disappearance or even downregulation after being treated with 20 µM of Al for 7 days, thus higher concentrations of Al (40 and 80 µM) was also used (Table 4.2 – 4.4). A total of eight polypeptides showed upregulation out of which the 37.84, 32.45 and 54.23KDa showed significant enhancement in intensity. The densitometric analysis (Table 4.1) showed that the intensities of the above bands increased which was directly proportional to the concentration of the metal used.
Fig. 4.1 Protein profiles of *N. muscorum* in the presence of different concentrations of Al (Lane-1, Marker (M); Lane 2, Control (C); Lane 3, 20 µM Al; Lane 4, 40 µM Al; Lane 5, 80 µM Al) The polypeptide bands which became enhanced in response to Al in a dose dependent manner are indicated by red arrows. Mol wts are expressed in KDa.
Table 4.1 Comparative densitometric analysis of the protein bands in the concentration dependent Al-treated *N. muscorum*.

<table>
<thead>
<tr>
<th>Mol wt of Band enhanced(Kda)</th>
<th>Intensity ( INT/mm² )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20µM Al</td>
</tr>
<tr>
<td>54.23</td>
<td>4749.2</td>
</tr>
<tr>
<td>37.84</td>
<td>2652.35</td>
</tr>
<tr>
<td>32.45</td>
<td>3784.85</td>
</tr>
</tbody>
</table>

4.3.1.2 Changes in polypeptide pattern of *N.muscorum* under Cu stress

The electrophoretic pattern of crude extract of protein isolated from the control and Cu-treated cyanobacterial cells after seven days of treatment are shown in Figure 4.2. The number of polypeptides bands which showed disappearance were four having mol wt of 15.78, 37.84, 42.78 and 69.63KDa. A total of five bands corresponding to 16.83, 32, 48.15, 54.23 and 78 KDa registered downregulation whereas some polypeptides showed upregulation which were of 23.24, 25.96, 29.15 and 45.64 kDa (Table 4.2 - 4.4). Among these the intensity of polypeptide band of ~ 25.96 KDa showed remarkable upregulation as compared to control.
Fig 4.2 Protein profiles of *N. muscorum* in the presence of different concentrations of Cu (Lane-1, Marker; Lane 2, Control; Lane 3, 20 µM Cu). The polypeptide bands which became enhanced in response to Cu are indicated by red arrows. Mol wts are expressed in KDa.
4.3.1.3 Changes in polypeptide pattern of *N.muscorum* under Cd stress

The electrophoretic pattern of crude extract of protein isolated from the control and Cd-treated cyanobacterial cells after seven days of treatment are shown in Figure 4.3. The bands resolved were classified according to their molecular weight markers. The Lane 2 and Lane 3 showed the protein profiles in control and Cd-treated *N. muscorum*, respectively. Out of the several polypeptide bands three bands completely disappeared, seven showed downregulation and two polypeptides were upregulated. (Table 4.2-4.4) The selected dose of Cd (20 μM) showed the induction of new polypeptide of ~23.24 KDa, enhancement in the polypeptide corresponding to 97.4 KDa and the complete elimination of polypeptide band of 27.19 KDa, 37.84 KDa and 69.63 KDa whereas the remaining bands were inhibited as compared to control. Most of the bands which showed downregulation were of low mol wt. corresponding to 16.83 KDa, 32.45 KDa, 42.78 KDa, 48.15 KDa and 54.23 KDa. Among the high mol wt polypeptides, bands corresponding to 78 KDa and 95.6 KDa showed downregulation.
Fig 4.3 Protein profiles of *N. muscorum* in the presence of different concentrations of Cd (Lane-1, Marker; Lane 2, Control; Lane 3, 20 µM Cd). The polypeptide bands which became enhanced or appeared in response to Cd are indicated by red arrows. Mol wts are expressed in KDa.
**Table 4.2** Comparative analysis of the no. of protein bands which showed complete disappearance in different mol wt ranges in metal-treated *N. muscorum*.

<table>
<thead>
<tr>
<th>Metal (20 µM)</th>
<th>Range of mol wt marker and no. of bands which showed complete disappearance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14-29</td>
</tr>
<tr>
<td>Al</td>
<td>0</td>
</tr>
<tr>
<td>Cu</td>
<td>1</td>
</tr>
<tr>
<td>Cd</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 4.3** Comparative analysis of the no. of protein bands which showed decrease in intensity in different mol wt ranges in metal-treated *N. muscorum*.

<table>
<thead>
<tr>
<th>Metal (20 µM)</th>
<th>Range of mol wt marker and no. of bands which showed decrease in intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14-29</td>
</tr>
<tr>
<td>Al</td>
<td>0</td>
</tr>
<tr>
<td>Cu</td>
<td>1</td>
</tr>
<tr>
<td>Cd</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4.4 Comparative analysis of the no. of protein bands which showed increase in intensity in different mol wt ranges in metal-treated *N. muscorum*.

<table>
<thead>
<tr>
<th>Metal (20 µM)</th>
<th>Range of mol wt marker and no. of bands which showed increase in intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14-29</td>
</tr>
<tr>
<td>Al</td>
<td>1</td>
</tr>
<tr>
<td>Cu</td>
<td>2</td>
</tr>
<tr>
<td>Cd</td>
<td>0</td>
</tr>
</tbody>
</table>

4.3.2 DNA yield from *N.muscorum* treated with Al, Cu and Cd

After exposure of cells of *N.muscorum* to 20 µM of metals (Al, Cu and Cd) for 48 hours, DNA was isolated as described in materials and methods. The ratio of absorbance at 260 and 280nm for DNA was 1.7 indicating a good quality of DNA and was used for fragmentation studies. DNA yield was calculated using the formula mentioned in Sec 4.2.3. Al showed insignificant change in the DNA yield whereas both Cd and Cu significantly decreased the DNA yield. As shown in Figure 4.4 Cu appeared to be more toxic than Cd causing ~40% reduction in the DNA yield as compared to control.
4.3.3 DNA fragmentation analysis using agarose gel electrophoresis and fluorescence spectral studies under metal stress

When the DNA was extracted from treated cells and examined by 1.0 % agarose gel electrophoresis, DNA fragmentation was detected (Figure 4.5). As shown in Lane 1, genomic DNA was detected in the form of a single sharp band on the upper portion of the gel in the untreated sample showing no fragmentation of DNA. When *N.muscorum* was exposed to 20 µM Al for 48 hours, again a single band of DNA was observed at the position where genomic DNA was detected in the untreated *N.muscorum* indicating that the DNA in 20 µM Al treated *N.muscorum* was not fragmented.
Fig 4.5 DNA fragmentation pattern in presence of 20 µM Al (Lane- 1 DNA from untreated *N.muscorum*; Lane- 2 DNA from 20 µM Al-treated *N.muscorum*.

Fig 4.6 (Lane 2), clearly shows the fragmentation of DNA from Cu treated *N.muscorum*. In case of Cu, the fragmented DNA migrated rapidly in the agarose gel leaving a smear like pattern on the gel. DNA from Cd treated *N.muscorum* showed a similar pattern of DNA fragmentation. The DNA fragment of Cu treated sample moved further covering a greater distance indicating more pronounced fragmentation as compared to Cd (Fig 4.7, Lane 2).

The fragmentation caused in the treated DNA might be due to the generation of strand breaks. Therefore, in the light of above explanation fluorescence spectroscopy was employed to detect DNA strand breaks.
Fig 4.6 DNA fragmentation pattern in presence of 20 µM Cu (Lane 1 DNA from untreated *N.muscorum*; Lane 2 DNA from 20 µM Cu-treated *N.muscorum*).

Fig 4.7 DNA fragmentation pattern in presence of 20 µM Cd (Lane 1 DNA from untreated *N.muscorum*; Lane 2 DNA from 20 µM Cd-treated *N.muscorum*).
Fluorescence spectral analysis

Fluorescence spectra of isolated DNA (5 µg/ml) of untreated and metal treated (Al, Cu and Cd) *N. muscorum* was taken using ethidium bromide as an external chromophore (Figure 4.8). A decrease in the fluorescence intensity was seen in case of DNA isolated from metal treated *N. muscorum* as compared to untreated one. DNA isolated from Cu treated *N. muscorum* exhibited maximum decrease in fluorescence intensity as compared to the control. This decrease in fluorescence intensity was recorded to be 43.2%. Furthermore, DNA isolated from Cd treated cyanobacterium also showed decrease in fluorescence intensity but less than that of Cu which was found to be 31.3%. This may be attributed to the damage of DNA and strand breaks due to modification by the metals. However, Al treated cyanobacterium did not show any significant decrease in fluorescence intensity (7.1%).
**Fig 4.8** Fluorescence emission spectra of isolated DNA from untreated and metal treated (Al, Cu and Cd) *N.muscorum*.

### 4.3.3. Comparative Lipid profile using TLC

Lipid chromatography of *N.muscorum* following 20 µM metal (Al, Cd and Cu) exposure for 3 days showed 7 bands of lipid after iodine vapourisation (Figure 4.8 and 4.9) and 3 of them were relatively more prominent. Rf value: 0.36 corresponds to DG/PG (Digalactosyl diglyceride, phosphatidyl glycerol), Rf value: 0.47 corresponds to GL (Glycolipid) and Rf value: 0.57 corresponds to unidentified glycoside. The lipid profile pattern after the treatment with Al showed negligible decrease in the intensities of the bands (Rf value: 0.36, 0.47 and 0.57) whereas Cd and Cu treatments showed prominent decrease in band intensities.
Fig 4.8 Thin-layer chromatogram of lipid extracts from cell preparations of *N. muscorum* (Lane 1 Total lipids from untreated *N. muscorum*; Lane 2 Total lipids from 20 µM Al treated *N. muscorum*.
**Fig 4.9** Thin-layer chromatogram of lipid extracts from cell preparations of *N.muscorum* (Lane 1 Total lipids from 20 µM Cd treated *N.muscorum*; Lane2 Total lipids from untreated *N.muscorum* Lane 3 Total lipids from 20 µM Cu treated *N.muscorum*.}
4.4 DISCUSSION

The uninterrupted efflux of ROS due to metal stress results in continuous and accumulative oxidative damage to cellular components and alters many cellular functions. Among the biological targets most vulnerable to oxidative damage are proteinaceous enzymes, membrane lipids and DNA. The results of the present chapter demonstrate the metal-induced damage to major biomolecules i.e. Proteins, lipids and DNA. Amongst the three metals Cu showed maximum toxicity followed by Cd and Al.

The above results has a three-prong explanations: Cu being an essential micronutrient for normal growth and development specific transporters are available for the uptake of metal ions which results in quick uptake of this metal when the cells are exposed to elevated levels of Cu whereas since Cd has no role in physiologic processes in cyanobacteria, it lacks any specific transporter. (Sunda, 1990). Also, Surosz and Palinska (2004) found that Cu was found not only in cells but also in the isolated sheath (traces), which further supports our view of rapid Cu uptake. Existence of MT is known for detoxification of Cd ions in animals and fungi (Gekeler et al.,1988) and cyanobacteria (El-Enany and Issa,2000); On the other hand, Cu is a redox active metal, generating ROS formation by the Fenton reaction (Stohs and Bagchi 1995), thus the redox properties that make Cu an essential element also contribute to its inherent toxicity. Redox cycling between Cu$^{2+}$ and Cu$^{+}$ can catalyze the production of highly toxic hydroxyl radicals, with subsequent damage to DNA, Lipids, proteins and other biomolecules (Halliwell and Gutteridge, 1989).
Al is a redox inactive metal and its toxicity is due to phosphorous limitation and dehydration. The damage to all the biomolecules was minimum in case of Al, which in good agreement with our results in the previous chapter where we have shown that Al is the weakest ROS generator compared to Cu or Cd.

4.4.1 Alterations in Protein Profile of *N.muscorum* under metal stress

Metal stress stimulated and inhibited the biosynthesis of different polypeptides within the cells of cyanobacteria grown in batch cultures. In the present investigation, the treatment of *N.muscorum* with metals (Al, Cu, and Cd) showed distinctly different protein profiles indicating that the alterations are dependent on the chemical nature and redox potential of the metals used. To gain insight into the biological effects of these metals and to identify activities relevant to its detoxification, we have analyzed the proteome in response to the test metals. Proteins can undergo direct and indirect damages following interaction with ROS, including peroxidation, damage to specific amino-acid residues, changes in their tertiary structure, degradation, and fragmentation. The consequences of protein damage as a response mechanism to stress are loss of enzymatic activity, altered cellular functions such as energy production, interference with the creation of membrane potentials, and changes in the type and level of cellular proteins. Exposure to Cd, Cu and Al caused noticeable changes on protein profile of *N.muscorum* and demonstrated the appearance and disappearance of some protein bands in *N. muscorum* treated with Cd and Cu. Disappearance of protein bands could be due to the interference of metal ions at the level of transcription, post-
translational degradation and translational level (Hussain and Jamil, 1992; Rai et al., 1995). However, increased intensity of protein bands may be playing a possible role in metal tolerance but their precise role is yet to be explored. The changes in protein composition as revealed by SDS-PAGE support the conclusion that the response of *N. muscorum* to Cu and Cd is based on the *de novo* biosynthesis of specific proteins and peptides.

From the general picture of protein profiles of Cu and Cd treated *N. muscorum* emerging from this study the major differences that is noteworthy is that although the number of bands that disappeared or were downregulated was almost the same in case of both the metals except one band in the 29-43 and 66-97.4 KDa range. The number of bands that showed enhanced intensity was more in case of Cu demonstrating that the antioxidant potential is more stimulated in case of Cu. (Fig.4.2). These results are in agreement with the findings of Sultan et al., 2007; Surosz and Palinska, 2004)

The bands which showed up-regulation in Cu and Cd treated cells were mostly in the range of 23-29 KDA which is expected to be that of SOD. For instance, the amino acid range in cyanobacterial FeSOD is 199–229 residues with a molecular weight of 21–25 KDa, whereas in MnSOD, it is 200–316 amino acids with a molecular weight of 22–34 KDa as demonstrated by Priya et al. (2007). Cd showed enhancement in only one of the polypeptide in this range. The present data also confirms our results in the previous chapters (chap 3) of the effect of these two metals on the antioxidant enzymes. The enzymes showed significant increase in activity in case of Cu. This increase can be explained in the light of the
reports that Cu accelerates production of O$_2^*$ and SOD converts it into peroxide (Halliwell and Gutteridge, 1989). Since SOD has been reported to act as a metal chelator (Ciriolo et al., 1994) it may also regulate the intracellular Cu level. These results are supported by Okamoto et al. (2001b) and Babu et al. (2005) who clearly showed induction of Fe-SOD at the transcriptional level in the dinoflagellate Gonyaulax polyedra and MnSOD in Lemna gibba exposed to Cu.

Cu treated N.muscorum showed significant upregulation in the polypeptide corresponding to 45 Kda which is the GTPases translation elongation factors (Ekman et al., 2008) and are the workhorses of protein synthesis on the ribosome. This may be playing a role in the synthesis of antioxidant enzymes.

Inhibition of growth, pigment content and PS II in Cu-treated control was observed in the previous chapters. These results are confirmed by electrophoresis results that exhibited complete disappearance of 15.78 KDa polypeptide that corresponds to photosystem I subunit II. These results also are supported from the reports of Rai et al. (1991) in A. doliolum and Kupper et al. (2003) in Scenedesmus quadricauda. Surosz and Palinska, 2004 also reported the loss of the low molecular–mass protein bands between 16 and 22 Kda, representing the phycocyanin (a,b monomers) and involved in photosystem II, after Cu treatment in Anabaena flos-aquae. A similar depletion of all PSII polypeptides such as 47, 33, 23 and 17 KDa polypeptides was observed by Inbaraj and Krishnaswamy, (2011) in Cowpea (Vigna unguiculata L. Walp. P152) under Zn stress.
Inhibition of protein accumulation induced by higher concentrations of heavy metals may be attributed to the toxic action of these heavy metals on the enzymatic reactions responsible for protein biosynthesis (Hart and Scaife, 1997; Kobbia et al., 1985; Rai et al., 1994; El-Naggar, 1993). A complete elimination of 37.84 KDa band corresponding to the enzyme Fructose 1,6-bisphosphatase II (Pandey et al., 2011) which has a role in carbon flux in the Calvin cycle was noticed in the protein profile of both Cd and Cu treated N. muscorum. The Calvin cycle is the primary pathway of carbon fixation in chloroplasts of C3 plants (Sharkey, 1985). Due to the inhibition of the enzyme Fructose 1, 6-bisphosphatase II, CO₂ fixation is reduced which affects the overall growth of the treated cells. Further, Phosphoglycerate kinase (PGK), a monomeric two domain enzyme also involved in the Calvin cycle and energy conserving phase of glycolysis showed downregulution in Cd treated cells whereas Cu caused the complete degradation of this protein. PGK's primary function is to generate ATP and increase the respiration rate under stressful conditions. Thus, the observed decline in PGK may be responsible for decreased ATP synthesis required for energy and maintenance of the Calvin cycle. One other polypeptide in both Cu and Cd of 54.23KDa identified as of ATP synthase subunit A was down-regulated (Pandey et al., 2011) which added to the overall decline in the energy status of the stressed N. muscorum.

The 69.63 KDa band which also showed disappearance corresponds to DnaK type molecular chaperone which plays a role in Protein synthesis and folding. Due to
the complete loss of this polypeptide the synthesis of almost all the proteins was inhibited.

A 78 KDa polypeptide was downregulated in both Cd and Cu treated *N. muscorum*. This band corresponds to Polyribonucleotide nucleotidylytransferase (polynucleotide phosphorylase) (Ran *et al.*, 2007) Polynucleotide Phosphorylase (PNPase) is a bifunctional enzyme with a phosphorolytic 3’ to 5’ exoribonuclease activity and a 3’-terminal oligonucleotide polymerase activity (Mohanty and Kushner, 2000). It is also involved on mRNA processing and degradation in both prokaryotes and eukaryotes. Thus its downregulation can cause an overall inhibition of all the cellular and molecular processes in the stressed cells, which is also observed in our study. On the other hand, the level of polypeptide aconitate hydratase having mol wt of 95 KDa (Ekman *et al.*, 2008) was not significantly changed in the Cd treated cells, which may indicate that glycolysis and the TCA cycle are active to some degree under the metal stress.

The protein profile in case of Al showed a very unique pattern absolutely different from that of Cd and Cu. There was no polypeptide band which was downregulated or showed complete disappearance. The proteins identified in the Al treated *N. muscorum* were restricted to those most highly expressed, and the majority of these proteins have functions related to fundamental processes in the cyanobacterial cell machinery, such as translation, stress response, ATP synthesis, ROS detoxification, and carbon and nitrogen metabolism
The 78 KDa polypeptide which was downregulated in both Cd and Cu treated *N. muscorum* showed upregulation in case of Al. This band which corresponds to Polyribonucleotide nucleotidyltransferase may be responsible for proper functioning of the proteins minimizing the overall toxicity of Al. A 42.78KDa protein may be correlated to dehydrin like proteins. Metal toxicity reduced water content even in plants growing hydroponically (Barcelo and Poschenrieder, 1990). The results of Tamas *et al.*, 2006 demonstrated that the expression of the dehydrin gene, which is the major drought-induced gene in barley, is strongly induced during Al stress (Fig. 4.3). Thus Al is known to cause dehydration stress as a result of which an osmotic stress protein ranging from 40-44 KDa may be expressed. The other polypeptides which were significantly upregulated corresponded to SOD and important enzymes of the metabolic pathways like oxidoreductases (32.45 KDa), ADP glucopyro phosphorylase (48.15 KDa) and ATP synthase subunit A (54.23 KDa). This differential response may reflect more elaborate adaptations present/ elicited in Al treated cells for either evading or tolerating the metal stress.

### 4.3.2 DNA fragmentation analysis under metal stress using agarose gel electrophoresis

Prokaryotic DNA is very sensitive to the action of metals especially the transition ones as they are not protected by a defined nuclear membrane making naked DNA more prone to attack by metal generated ROS, eventually leading to DNA fragmentation. ROS can cause several types of damage: modification of DNA
bases, single- and double-DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage, and damage to the DNA repair system (Kohen and Nyska, 2002).

Not all ROS can cause damage; most is attributable to hydroxyl radicals. For example, following exposure of DNA to hydroxyl radicals, like those induced by ionizing irradiation, a variety of adducts are formed. Transition metals like Fe and Cu that possess high-binding affinity to DNA sites can catalyze the production of OH• in close proximity to the DNA molecule, thus ensuring repeated attack upon the DNA by an efflux of hydroxyl radicals (Kasprzak, 2002; Ames, 2001; Poulsen, 2000; Halliwell, 1999).

Environmental toxicology tests carried on mussels stated that the ranking of genotoxicity potential for the metals was in the decreasing order Hg>Cu>Cd. Cu and Hg increased DNA single-strand breaks and micronuclei frequency, whereas Cd induced statistically significant increase of DNA damage (Bolognesi et al., 1999). Our study also revealed Cu as more cytotoxic than Cd. A similar pattern of DNA damage was observed by Bhandari and Sharma (2006) in freshwater (Nostoc spongiaeforme) and marine (Phormidium corium) cyanobacteria exposed to light stress. However, further work is needed to identify the actual cause and mechanism of degradation. Further, the levels of lesions in the untreated DNA were quite similar to that obtained with the Al-treated DNA indicating no DNA damage with Al.
It is a well known fact that Al induces dehydration stress and the relatively no DNA damage in the Al-treated *N.muscorum* could be attributed to its dehydrating ability as the DNA in dry state is less prone to the damage by ROS (Shirkey et al., 2003). In another study conducted by Achary and Panda (2010) on root cells of *Allium cepa*, it was shown that low concentrations of Al (1–10 µM) induced adaptive response conferring genomic protection which is again in good agreement with the induction of various proteins in the Al-treated *N.muscorum*.

Decrease in DNA content and DNA fragmentation can be attributed as hallmarks of PCD in *N. muscorum* exposed to metal stress. To our knowledge, only one publication has shown DNA degradation during KCl-induced PCD in *Anabaena* sp. (Ning et al., 2002). In the present study, it is demonstrated that the metal stress (Cu and Cd) causes DNA fragmentation in *N. muscorum*. This is in accordance with Huh et al., (2002) who presented evidence, that salt-induced PCD in plants and yeast is caused by ionic stress. Affenzeller et al., (2008) also demonstrated that salt stress-induced cell death in the unicellular green alga *Micrasterias denticulata*. Salt stress-induced DNA laddering as a hallmark of PCD has also been described in barley roots (Katsuhera and Kawasaki, 1996; Katsuhera, 1997), rice root tips (Li et al., 2007a, b) or tobacco protoplasts (Lin et al., 2005,2006).

Further the fluorescence emission spectral studies of DNA which were carried out in the presence of the three test metals showed the occurrence of metal induced strand breaks. Cu was found to be the most damaging followed by Cd and Al.
This may be attributed to the perturbation of electronic state of the bases by interaction of Cu$^{2+}$ ions in G-C pairs finally leading to strand breaks.

**Comparative Lipid profile using TLC**

Thin layer chromatographical analysis of lipid content in *N.muscorum* following the exposure of metal is shown in fig 4.5 and fig 4.6 In the present investigation when the plates were exposed to iodine vapour, many lipids spots appeared out of which 3 were more prominent and these are represented by arrows. Intensity of all the spots were found to diminished when *N. muscorum* was treated with the Cd for 2 days. Similar pattern of decrease in the intensity of lipid was found in Cu treated cells but the effect was higher as compared to that of Cd treated cells. Earlier worker reported the presence of 10 lipid spots in *Anabaena ambigua* (Wahal *et al.*, 1973 and Yadav, 1975) and 8 spots in *Mastigocladus laminosus* (Prasad, 1988). In present findings the Lipid spots showed similar Rf values (.36, .47 and .57) as reported by earlier workers suggesting the presence of sulphaquionovosyl diglyceride, Glycerol and digalactosyl diglyceride, monogalactosyl diglyceride. Polyunsaturated lipids are widely distributed in cellular membrane. The los of intensity of lipids bands by Cu and Cd might be due to the destruction of lipids caused by peroxidation (Kumar *et al.*, 2011; Certik *et al*. 2005; Barker and Bernheim, 1967). No change in the lipid profile in the Al-treated *N.muscorum* further strengthen our notion that the Al either produces relatively less level of deleterious ROS or they may be detoxified due to induction of various dehydrins like protein. The mechanism of relatively reduced toxicity
by Al is yet to be elucidated; however, proline accumulation with the Al exposure might have some conclusive role in the induction of dehydrin like protein which may confer its protection.

4.5 Conclusions

In conclusion, our results describe the molecular changes in the pattern of various macromolecules of *N. muscorum* exposed to metal stress like Al, Cu and Cd. Cu- and Cd-induced DNA fragmentation, compared to no fragmentation with Al, subsequently initiated the programmed cell death in *N. muscorum*. However, Al presented some unique results showing glimpses of rather different mechanism(s) of action of its toxicity or perhaps protection. The SDS-PAGE analysis showed induction of various proteins in Al-exposed *N. muscorum* resembling to the dehydrin-like proteins commonly known to get induced in higher plants under drought or dehydration stress. These results prompted me to further investigate the precise mechanism(s) of tolerance or defense in the Al-adapted *N. muscorum*. 