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Comparative assessment of Ni and As(III) mediated alterations in diazotrophic cyanobacteria, *Anabaena doliolum* and *Anabaena* sp. PCC7120

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The comparative effects of nickel (Ni^{2+}) and arsenite (As(III)) on two diazotrophic cyanobacterial species were investigated in terms of photosynthetic attributes. Both metals demonstrated inhibitory effects on growth, pigments (chl a and phycocyanin) and photosystem II (PS II) photochemistry. However As(III) exerted severe effects as compared to Ni reflected by (1) reduced growth (2) significant inhibition of chl a and phycocyanin, (3) reduction in maximum photochemical efficiency of PSII and (4) depleted plastoquinone pool, thus suggesting it as more toxic. Moreover, comparative analysis of two species also demonstrated interspecies variation in terms of stress adaptive strategies reflected through higher sensitivity of *Anabaena doliolum* over *Anabaena* PCC7120. Thus the study recommends application of *A.* PCC7120 as biofertilizer in Ni and As(III) contaminated paddy fields.

Key words: *Anabaena* sp. PCC7120, *Anabaena doliolum*, Nickel, As(III), maximal photochemical yield (Fv/Fm).

INTRODUCTION

Anthropogenic activities have altered the global biogeochemistry due to release of metals in recent years (Bhagat et al., 2016). Not only aquatic ecosystem but soil organisms are also negatively affected by metal contamination. Effect of elevated metal input on soil organism is reflected in form of reduced species diversity, abundance and biomass and changes in microbe mediated processes (Bengtsson and Tranvik, 1989; Giller et al., 1998; Vig et al., 2003). Although few metals hold prime importance for all living organisms due to their key role in basic life processes like photosynthesis and respiration, their elevated concentration in cells causes either their inappropriate binding to metal binding sites of enzymes or undesirable redox reactions thus causing lethal effects (Waldron et al., 2009a, b, 2010).

Nickel is one such metal that plays a vital role in the cellular physiology of living organism (Poonkothai and Vijaywathi, 2012). It is coordinated by proteins either

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directly or through tetrapyrrole ring of coenzyme F_{430} which coordinates a nickel atom in methyl-coenzyme M reductase (Ragsdale, 2003). Statistical data revealed that nickel emission from natural and anthropogenic sources are 2.9-56.8×10^3 and 33.1-194.2×10^3 t $year^{-1}$, respectively (Tercier-Waeber and Taillefert, 2008). Some anthropogenic sources that causes elevated Ni level into environment are energy supplying power stations (coal burning power plants, petroleum combustion and nuclear power stations), mining and associated activities, disposal of NiCd batteries, chemical industries (planting, metal finishing, pigment production, cement manufacturing) (Poonkothai and Vijaywathi, 2012; Nnorom and Osibanjo, 2009).

Apart from them, heavy metals are the non-degradable elements that occur naturally in biosphere. In past few years, their accumulation in environment as a result of their increased utilization in industrial activities such as in mining processes has raised a global concern (Huertas et al., 2014). Arsenic is a toxic metalloid and present in two biologically active forms arsenate (As^V) and arsenite (As^{III}). Arsenate is analogous to phosphate thus replaces phosphate from essential biochemical reactions such as glycolysis and oxidative phosphorylation causing toxic effects (Tawfik and Viola, 2011; Nriagu and Jerome, 2000). However arsenite is reported to bind dithiols, forming dithiols thus disrupting protein functions and producing reactive oxygen species (ROS) (Liu et al., 2002; Meng et al., 2004; Wysocki et al., 2001). Use of arsenic as herbicides, insecticides, rodenticides, food preservatives and byproduct of used fosil fuel are major anthropogenic activities that are challenging the environment (Flora et al., 1995).

Diazotrophic cyanobacteria are the only group of prokaryotes proficient in performing oxygenic photosynthesis and N_2-fixation, thus contributing significantly to global photosynthetic biomass production and biofertilizer (Dadheech, 2010). Being an essential component of cyanobacterial ureases and hydrogenases Ni is required at low concentration (Huertas et al., 2014), however at higher concentrations it causes inhibition of pigments (chlorophyll, phycocyanin and carotenoids), enzyme activities (nitrate reductase and glutamine synthatase) and loss of electrolyte (Na^+ and K^+) (Rai et al., 1985, 1986, 1990; Martínez-Ruiz and Martínez-Jerónimo, 2015).

Similarly, arsenic is also reported to inhibit chlorophyll biosynthesis, photosynthetic pigments and Rubisco and generates oxidative stress through ROS generation thus damaging lipids, proteins and nucleic acids (Tantry et al., 2015; Srivastava et al., 2009; Pandey et al., 2012). Effect of arsenite and nickel on *Anabaena* spp. have been studied however no reports exists regarding comparative study of arsenite and Ni on different cyanobacterial strains. *Anabaena* spp. commonly found in tropical conditions have different geographical isolates (sps.) and displays niche specificity. This study is the first to provide comparative effect of Ni and As(III) on *Anabaena* sps. (*Anabaena doliolum* and *Anabaena* sp. PCC7120) in terms of (1) growth behavior, (2) photosynthetic pigments, (3) and chlorophyll fluorescence. Attempts have been made to verify these results statistically. Present study is important in the sense that the results would provide important information regarding the cyanobacteria's ability to tolerate arsenic and nickel.

MATERIALS AND METHODS

Organism and growth condition

Anabaena spp., *Anabaena* PCC7120 and *A. doliolum* were cultivated photoautotrophically under sterile condition in BG-11 medium (Supplementary Table 1) (N_2-fixing condition) buffered with Tris/HCl at 25 ± 2°C under day light fluorescent tubes emitting 72 µmol photon m^{-2} s^{-1} PAR (photosynthetically active radiation) light intensity with a photoperiod of 14:10 h at pH 7.5. The cultures were shaken manually 2 to 3 times daily for aeration.

Mode and source of stress application

Nickel stress was applied as $NiCl_2$ at concentrations 0 to 32 µM and arsenite stress was applied as sodium meta arsenite at concentrations 0 to 80 mM. Sodium meta arsenite and nickel chloride autoclaved separately and calculated amount were added directly into the sterilized medium to achieve the desired concentration and working standards were obtained by further dilutions.

Measurement of survival

Exponentially growing cells of *Anabaena* PCC7120 and *A. doliolum* treated with their respective concentrations were collected at four time points (1, 7, 10 and 15 days). Cells never exposed to nickel and arsenite were used as control. Growth was estimated by measuring the OD (optical density) of the culture at 750 nm in a UV–VIS spectrophotometer (Systronics, India) up to 16th day.

Pigments

Chlorophyll a, carotenoid and phycocyanin were measured as per the method of Bennett and Bogorad (Bennett and Bogorad, 1973), by taking the absorbance at 663, 480 and 645 nm respectively. The extinction coefficient of chl a at 665 nm in absolute methanol is 74 .5 ml/mg-cm (Mackinney, 1941).

Measurement of chlorophyll fluorescence

Chl fluorescence in dark- and light-adapted control as well as treated cultures was measured using a PAM 2500 Chl fluorometer (WALZ GmbH, Effeltrich, Germany). The fluorometer was connected to a computer by the data acquisition system (PAMWIN, Walz, Germany). Prior to each measurement, the culture was dark-adapted for 30 min (Guo et al., 2006). The minimal fluorescence yield of the dark-adapted state (F_0) was measured by the modulated light which was too low to induce significant

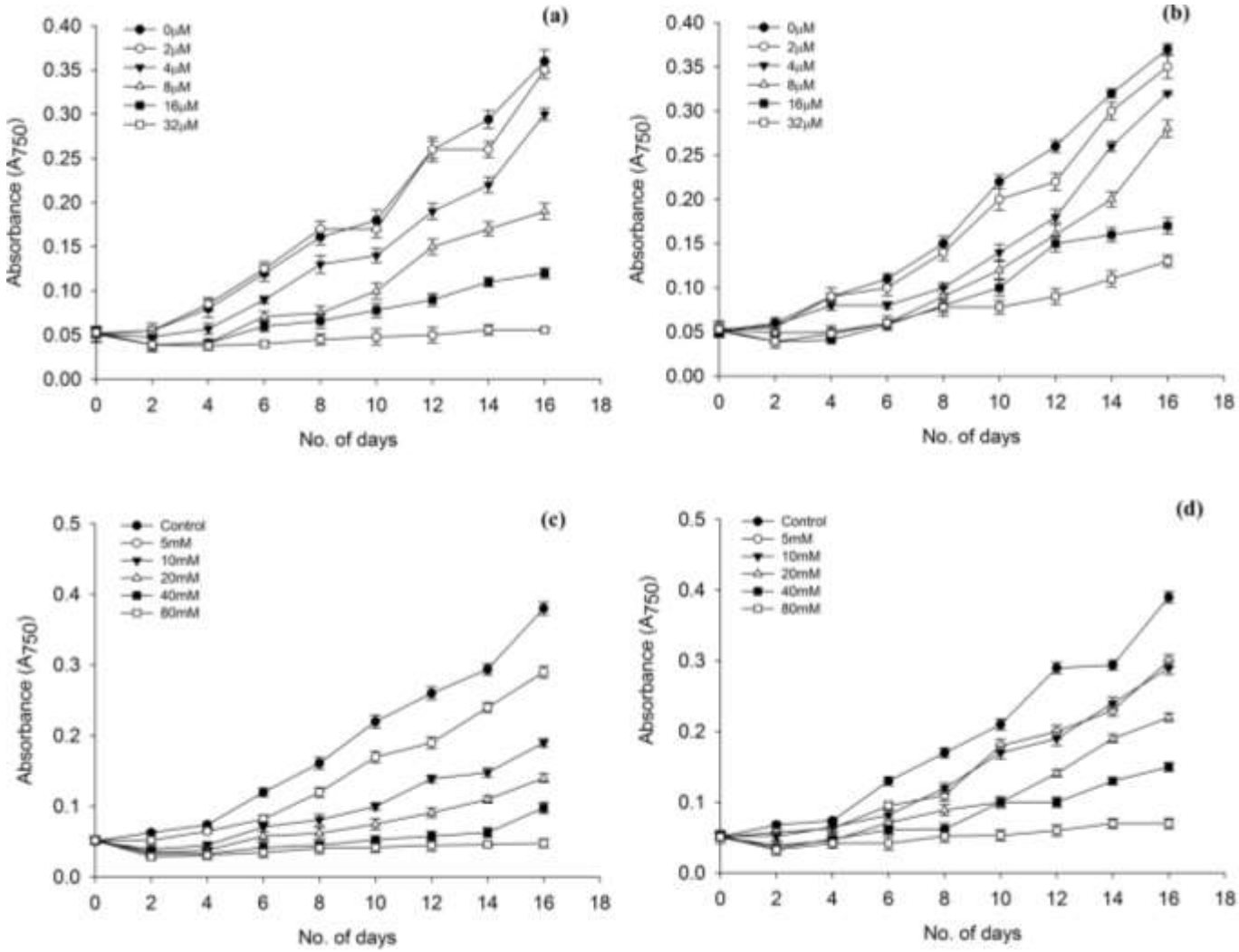

Figure 1. Population growth curves of (a) *A. doliolum* (b) *Anabaena* sp. PCC7120 exposed to different concentrations of Ni^{2+} (c) *A. doliolum* (d) *A.* sp. PCC7120 exposed to different concentrations of As(III). Mean values for three bioassays with three replicates ± standard deviation bars.

physiological changes in the plant, and was recorded after dark adaptation. Subsequently, a saturating pulse was given to measure the maximal fluorescence yield of the dark-adapted state (Fm) (Qin et al., 2006). The maximal photochemical quantum efficiency of PSII (Fv/Fm) was determined after a 20-min dark acclimation period in selected cultures. Other calculated fluorescence parameters was the pastoquinone pool (Fv/2) (Bolhar-Nordenkampf et al., 1989).

Statistical analysis

Each treatment consisted of three replicates; the results presented are mean values. Each experiment was repeated five or six time; results from a representative experiment are presented. The results were statistically analyzed by one-way ANOVA and the Duncan's new multiple range test (DMRT) to determine the significant difference among group means. A p value ≤0.05 was considered statistically significant (SPSS for Windows, version 20.0).

RESULTS

Measurement of growth and survival

The present study deals with assessment of comparative toxic effects of Ni and arsenite over two strains of *Anabaena* viz. *A. doliolum* and *Anabaena* sp. PCC7120. Being a vital component of paddy fields diazotrophic cyanobacteria have always fascinated researchers from all over the world. Figure 1a and b shows the growth trends for *A. doliolum* and *Anabaena* sp. PCC7120 respectively exposed to various concentrations of Ni; as displayed in the figure the cell density was inhibited significantly by all of the tested Ni concentrations except Ni (2 µM). Similarly, Figure 1c and d represents growth

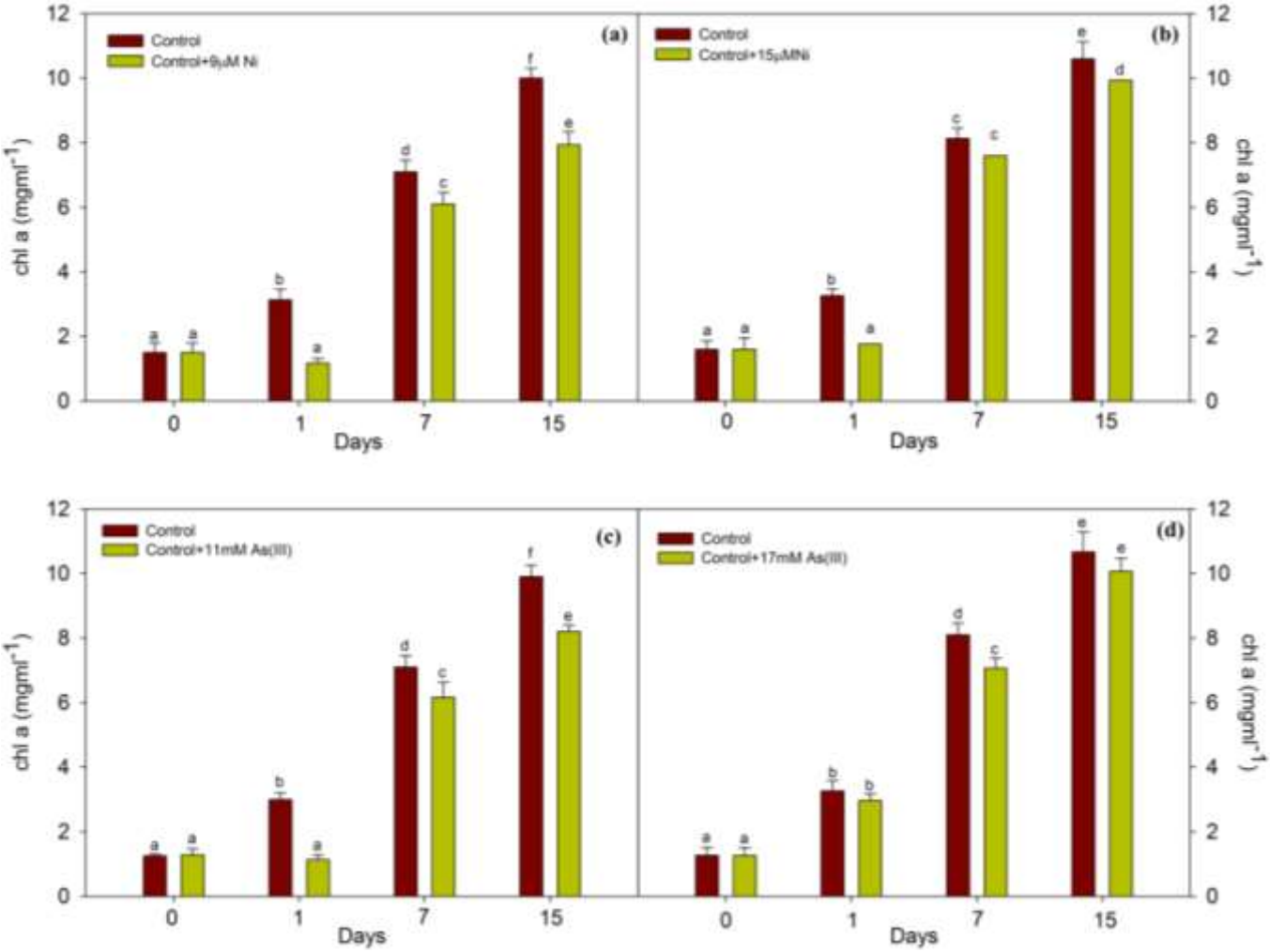

Figure 2. Effect on chlorophyll a content of (a) *A. doliolum* (b) *Anabaena* sp. PCC7120 exposed to different concentrations of Ni^{2+} (c) *A. doliolum* (d) *A.* sp. PCC7120 exposed to different concentrations of As(III). Mean values for three bioassays with three replicates ± standard deviation bars.

pattern for *A. doliolum* and *Anabaena* sp. PCC7120 respectively exposed to arsenite. However under arsenite treatment all the concentrations were inhibitory. Moreover *A. doliolum* appears to be more sensitive as compared to *Anabaena* sp. PCC7120 (Figure 1a to c). The average IC_{50} determined for Ni was 9 and 15 µM and for arsenite 11 and 17 mM respectively for *A. doliolum* and *Anabaena* sp. PCC7120.

Pigments

Figure 2a and b displays effect on chl a content following Ni stress in *A. doliolum* and *Anabaena* sp. PCC7120 respectively and Figure 2c and d shows chl a content following As(III) treatment in *A. doliolum* and *Anabaena* sp. PCC7120 respectively. It clearly demonstrates that both Ni and As caused more pronounced inhibition of chl a content in *A. doliolum* as compared to *Anabaena* sp. PCC7120. *A. doliolum* exhibited significant decrease in chl a content at all days of treatment however in *Anabaena* sp. PCC7120 significant decrease was observed only at 7th day of As(III) treatment and 1st and 15th day of Ni treatment as measured by Duncan's test (DMRT). Figure 3a and b demonstrates effect of Ni on carotenoid content in *A. doliolum* and *A.* PCC7120 respectively and Figure 3c and d displays As(III) mediated alterations on carotenoid content in *A. doliolum* and *A.* PCC7120 respectively. Significant increase was found in *A. doliolum* at 1, 7 days of Ni treatment and at 1 and 15 days of As(III) treatment, however in *A.* PCC7120 at 1st day of Ni treatment and 1 and 7 day of As(III) treatment, carotenoid content was significantly increased. Similar to chl a, significant decrease in phycocyanin

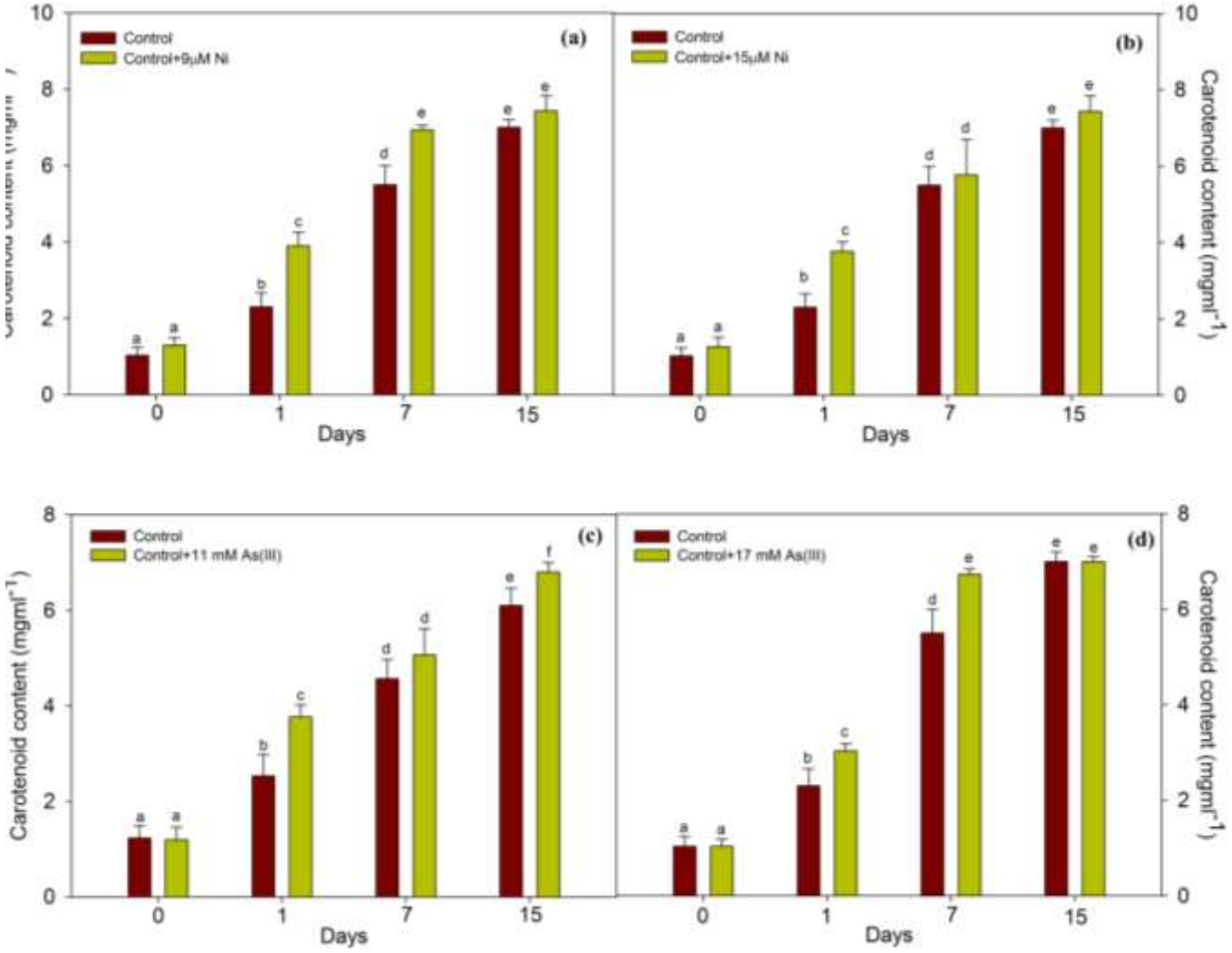

Figure 3. Effect on carotenoid content of (a) *A. doliolum* (b) *Anabaena* sp. PCC7120 exposed to different concentrations of Ni^{2+} (c) *A. doliolum* (d) *Anabaena* sp. PCC7120 exposed to different concentrations of As(III). Mean values for three bioassays with three replicates ± standard deviation bars.

content was noticed at all days of treatment under both Ni and As(III) stress in both species, however among both stresses As(III) caused more pronounced inhibition in both species (Figure 4a to d).

Chlorophyll fluorescence

The test metals were found to reduce maximal quantum yield in a concentration-dependent manner, which was more pronounced in *A. doliolum* following As(III) treatment (Figure 5). Figure 6 presents the impact of the test metals on plastoquinone pool (Fv/2) of *A. doliolum* and *A.* sp. PCC7120 after 24 h of Ni and As (III) treatment.

DISCUSSION

Growth behavior studies suggested sensitivity of *A. doliolum* over *A.* sp. PCC7120. This finds support from the studies of Singh et al. (2015), they found that *A. doliolum* is more sensitive as compared to *Anabaena* sp. PCC7120 under cadmium stress. Similarly, Agrawal et al. (2014) found following trend of tolerant behavior *A.* L31> *Anabaena* sp. PCC7120>*A. doliolum* under butachlor stress among three closely related species of *Anabaena*. This further attested the tolerant behavior *A. doliolum* over *Anabaena* sp. PCC7120 thus suggesting the presence of separate strategies to combat stress even within species. Further requirement of high concentration of As(III) as compared to Ni may be attributed to ability of

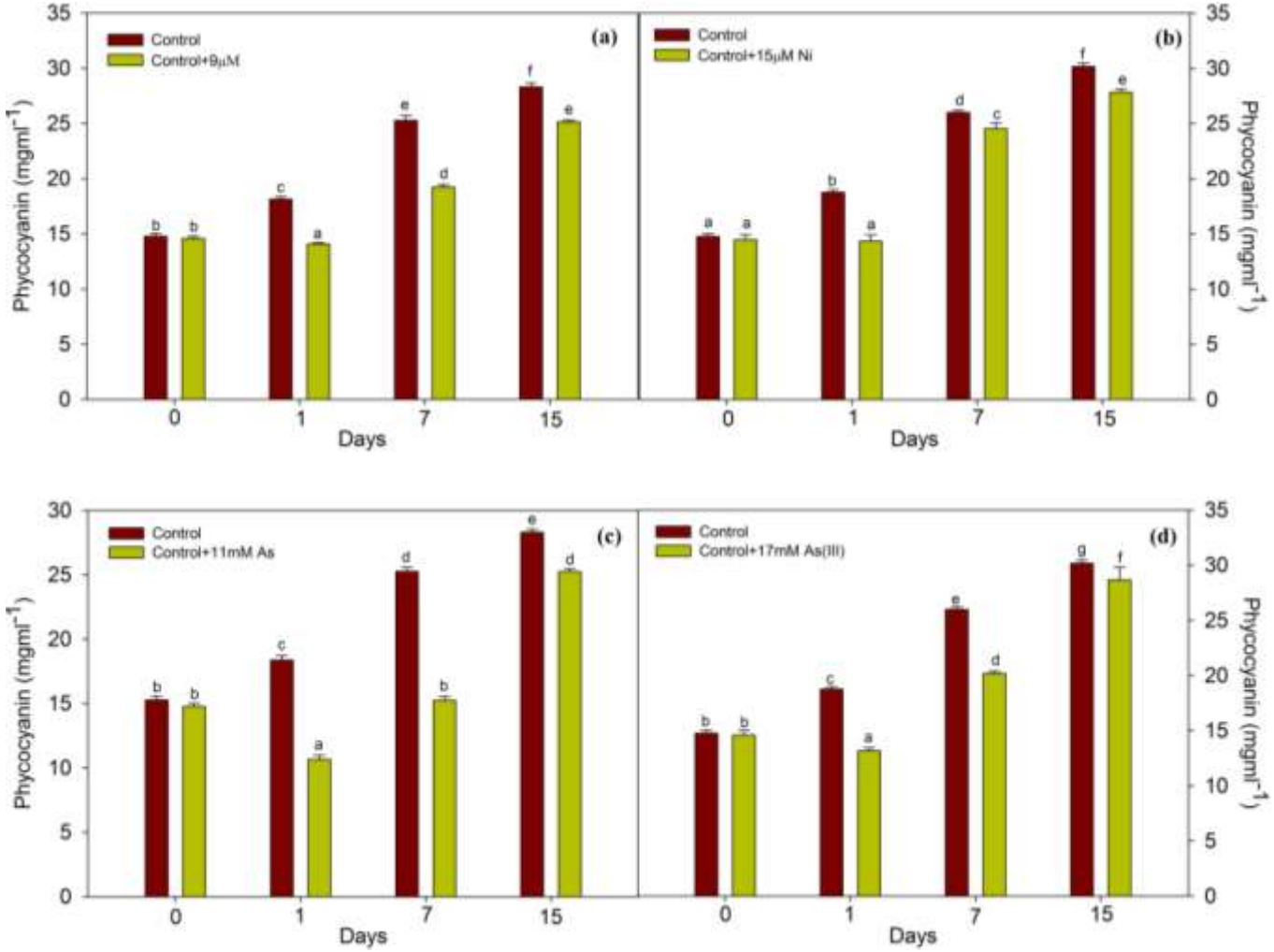

Figure 4. Effect on phycocyanin content of (a) *A. doliolum* (b) *Anabaena* sp. PCC7120 exposed to different concentrations of Ni^{2+} (c) *A. doliolum* (d) *Anabaena* sp. PCC7120 exposed to different concentrations of As(III). Mean values for three bioassays with three replicates ± standard deviation bars.

Anabaena to accumulate high concentrations of As(III). Significant reductions in the photosynthetic pigments chl a and phycocyanin whereas significant increment in carotenoid content was found in both the species. Ni is known to affect the active site of O_2-evolving complex to which it interacts, thus causing depletion of 2 extrinsic polypeptides resulting in diminished e^- transport activity (Boisvert et al., 2007). However As(III) mediated chl a content inhibition may be attributed to inhibition of ϭ-aminolevulinic acid dehydrogenase, a key enzyme of chlorophyll biosynthetic pathway (Shrivastava et al., 2009). Other metals are also known to produce similar decrease in chl a content. For example, Carfagna et al. (2013), found decrease in chl a in a green alga, *Chlorella sorokiniana* under Cd/Pb stress.

Carotenoid content was significantly increased in *A. doliolum* at 1 and 7 days of Ni treatment and at 1 and 15 days of As(III) treatment, however in *A.* PCC7120 at 1st day of Ni treatment and 1 and 7 day of As(III) treatment. Carotenoids are known to be major players of antioxidant response against ROS and found to be increased under metal stress (Yu et al., 2015). Significant decrement in phycocyanin content was noticed at all days of treatment under both Ni and As(III) stress in both species. Phycocyanin is located on exterior side of thylakoid membrane and thus possibly toxicant exposure is prolonged causing severe inhibition as compared to chl a. This observation finds support from work of Pandey et al. (2012) they observed significant reduction in phycocyanin content under As(V) stress.

Maximum photochemical efficiency of PSII (efficiency at which light absorbed by PSII is used for

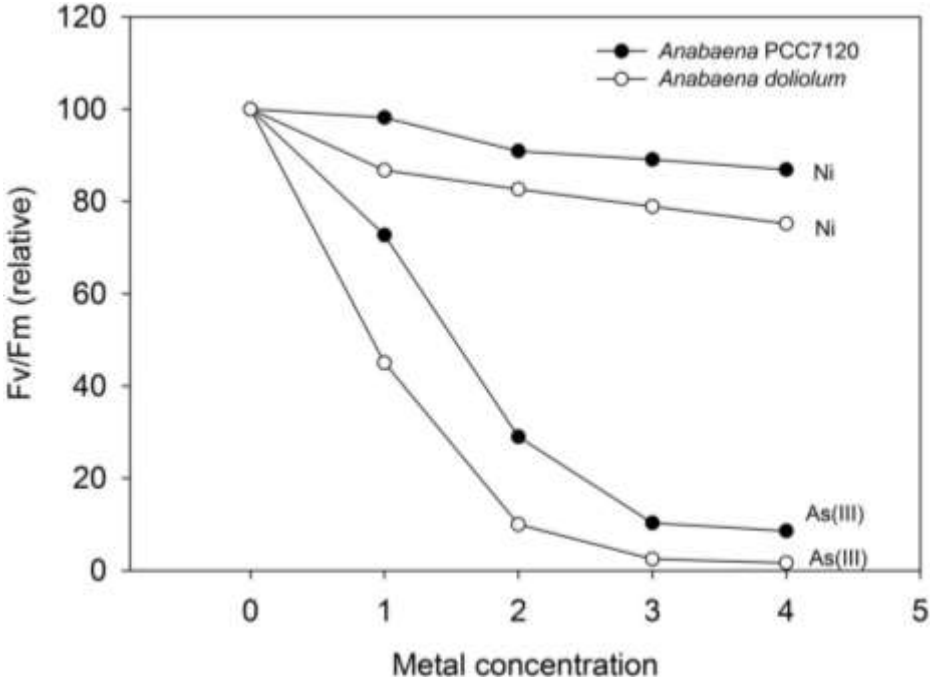

Figure 5. Ni and As (III) induced reduction in maximum quantum yield of *A. doliolum* and Anabaena sp. PCC7120 24 h of treatment, Fv/Fm (100%) for *A. doliolum* = 0.242±0.0003, Fv/Fm (100%) for *Anabaena* sp. PCC7120=0.275±0.0007. On the X-axis, metal concentrations (0, 1, 2, 3, 4, 5 and 6) represent, respectively, 0, 2, 4, 8, 16 and 32 µM for Ni and 0, 5, 10, 20, 40 and 80 mM for As(III).

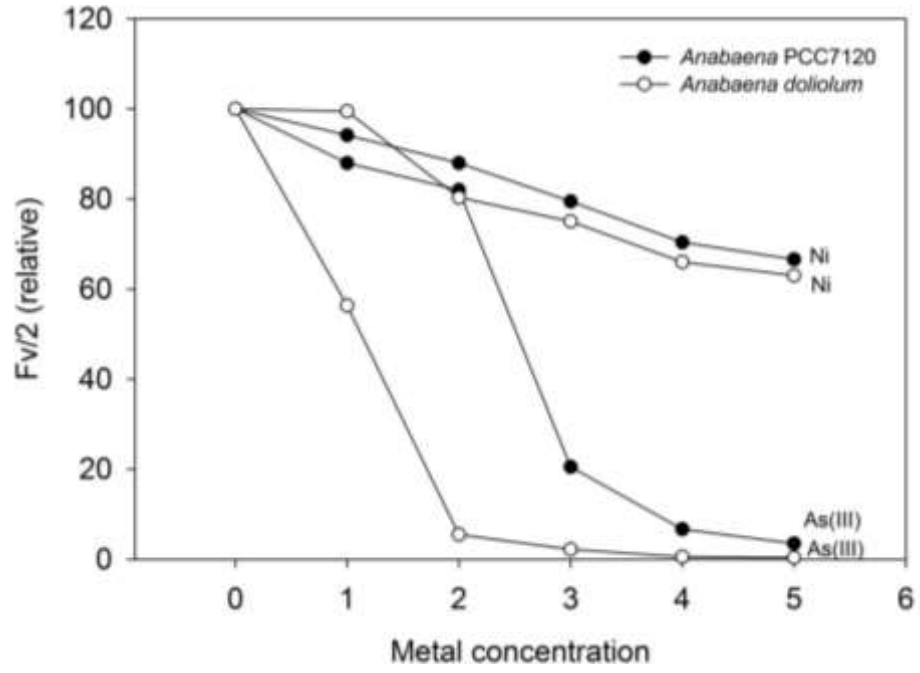

Figure 6. Effects of the test metals on the platoquinone pool of both cyanobacterium after 24 h of treatment. Fv/2 (100%) for *A. doliolum* = 0.181±0.0004, Fv/2(100%) for *Anabaena* sp. PCC7120 = 0.341±0.0003. On the X-axis, metal concentrations (0, 1, 2, 3, 4, 5 and 6) represent, respectively, 0, 2, 4, 8, 16 and 32 µM for Ni and 0, 5, 10, 20, 40 and 80 mM for As(III).

photochemistry when all reaction centers are open) of both test cyanobacteria following treatment with different concentrations of Ni and As(III) after 24 h was recorded. It was found to be affected significantly. The findings of our study are supported by Rahman et al. (2011). The ratio of Fv/Fm is considered as a stress indicator and designates the potential yield of the photochemical reaction (Björkman and Demmig, 1987). Fv/Fm remains high under control condition following irradiation because Q_A is in oxidized state due to transfer of electrons to NADP and finally to CO_2 via Q_B, the plastoquinone pool, and PSI. However under stress condition Fv/Fm may decrease because reoxidation of Q_A is restricted as a result of decrease or partial block of electron transport from PS II to PSI. A noteworthy decrease in the plastoquinone pool as represented by the Fv/2 ratio (Figure 6) could be one of the possible causes for the reduced quantum yield under metal stress.

In summary, among both test cyanobacterium *A. doliolum* appeared as a sensitive strain towards Ni as well as As(III) exposure at low concentrations which are toxicologically and environmen-tally relevant. Both metals significantly inhibited the population growth, pigment content (chl a, phycocyanin) and maximal photochemical efficiency of PSII, which was found to be more pronounced in *A. doliolum* (Figure 6). However increase in carotenoid content was found thus suggesting onset of defense mechanism. Thus present study suggests *Anabaena* sp. PCC7120 as more efficient candidate to be used as biofertilizer as compared to *A. doliolum* and needs to be further investigated. Further studies exploring effect on nitrogen fixing abilities and antioxidative defence system of both test cyanobacteria is ongoing so as to present a holistic view demonstrating integrative effect as well as help in unveiling the tolerance mechanism.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Table 1. Detailed composition of modified BG11 stock solution and 1X medium.

Macronutrient	**g/100 ml (Stock solution)**	**ml/litre**
$MgSO_4$	7.5	1.0
K_2HPO_4	4.0	1.0
$CaCl_2$	3.6	1.0
Citric acid with Ferrous ammonium citrate	0.6	1.0
EDTA	0.1	1.0
Na_2CO_3	2.0	1.0
Micronutrient	**Mg/100 ml**	**ml/litre**
H_3BO_3	286.0	1.0
$MnCl_2.4H_2O$	181.0	1.0
$ZnSO_4.7H_2O$	22.2	1.0
$Na_2MoO_4.5H_2O$	39.0	1.0
$CuSO_4.5H_2O$	7.9	1.0
$Co(NO_3)_2.6H_2O$	0.04	1.0

K_2HPO_4, EDTA and Ferrous ammonium citrate were autoclaved separately and added to the cold sterilized culture medium. The pH of the medium was maintained at 7.5. To avoid any alteration in pH, the medium was buffered with 0.5 g HEPES buffer.