Chapter 19
Bioremediation of Toxic Metals Using Algae

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19.1 Introduction

The rapidly growing population and expanding technological activities have accelerated the rate of addition of numerous poisonous pollutants especially the metal ions to the surrounding environment. These pollutants become deleterious due to their mobilization, transport and deposition in the various aquatic as well as terrestrial ecosystems. The cyanobacteria and algae (commonly called together 'Algae') constitute the most ancient groups of autotrophic microorganisms and are invariably affected by the presence of metal ions in the environment (Whitton, 1970). Algae are the organisms which can resist the metal toxicity by biochemical, chemical and physical mechanisms resulting in cell surface adsorption, metabolism dependent accumulation and precipitation (Gadd, 1988). They instantly interact with metal pollutants differently at cellular level showing different responses and tolerance mechanisms, termed as 'algae-metal interactions'—which is the basis of phytoremediation process.

Metals and metalloids can be characterized based on their toxicity level towards biological organisms (Gadd, 1993). One of the most common toxic heavy metal is lead, causing severe damage to living organisms and arsenic, another toxic metalloid, ranking 28th in abundance on the earth's crust, is widely encountered in the environment and severely damages metabolic pathways in different organisms from prokaryotes to human beings. Special emphasis would be given in this review on phycoremediation (removal of toxic metals by algae) of these two toxic elements and their probable mechanisms.

To understand the phycoremediation process by algae, it is important to investigate the responses of particular alga to particular metal and its tolerance or sensitivity,

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together with the metal uptake capacity. The metal ions are either actively accumulated by living cells or adsorbed by dead biomass of algae, may also be chelated with the extracted metabolites, polysaccharides or other constituents of the cell surfaces. Overall the metal uptake by the algal biomass is considered to be quite complex phenomenon, influenced by several physico-chemical processes of the living or dead cells together with the external factors (Gadd, 1992). Overall there are two processes: (i) more rapid metabolism independent process or adsorption and, (ii) comparatively slower metabolism dependent process or uptake. In the first category, the metal ions remain adsorbed on the cell surface ligands and in other process or active absorption, transportation of metal ions through the cell membrane into the cytoplasm occurred (Bates et al., 1982; Mehta and Gaur, 2005). Thus the ability of cyanobacteria and microalgae to sorb metal pollutants from the surrounding environment at a higher concentration is a fascinating phenomenon which may easily be compared to that of other chemical sorbents. Therefore, they are highly suitable for use in the treatment of metal contaminated industrial or other effluents.

Almost countless reports are there regarding toxic metal removal by algae, few are mentioned here. As much as 98–100 % metal (Cd, Pb, Mn) removal efficiency have been recorded by many marine and fresh water algal genera (Esteves et al., 2000; Ele-Sheekh et al., 2005; Cervantes et al., 2001) and it seems clear that different species of algae accumulate metal in different degrees (Jordanova et al., 1999). On the other hand, in the natural ecosystem, biomagnification of toxic metals by algal genera may affect the entire food chain. Therefore, it is necessary to study the metal sorption capacity of algal genera for using them in bioremediation purpose on one hand and for pollution risk assessment in ecological niche, on the other. A number of reviews have been published by several authors giving detail account of metal sorption by algae, their tolerance mechanisms and biotechnology (Whitton et al., 1981; Genter, 1996; Mehta and Gaur, 2005). Whitton (1984) reviewed the metal accumulation by algae including biomonitoring of metals from natural population, assay of metal composition from algal population in laboratory condition, algal adaptation to elevated level of metals and effect of different metals on species and community composition. Rai et al. (1981) reviewed in detail the tolerance mechanisms of different algal genera giving special emphasis on metal binding on cell surface, exudation of metal complex ligands, efflux of metal ions and sequestration by phytochelatins and metallothioneins intracellularly. Mehta and Gaur (2005) critically reviewed the metal accumulation process including data analysis, mode of action, factors affecting metal sorption, regulation and reuse of metals in detail. A comprehensive review on microbial arsenic resistance systems have been elaborately illustrated by Mukhopadhyay et al. (2002), which stated about the global geocycling of arsenic, nature of arsenic resistant genes, arsenate reductase families, arsenite oxidation and methylation processes by microbes.

Many algae exhibit tolerance to high concentration of toxic metals showing higher level of biosorption (De Filippis and Pallaghy, 1994). The tolerance limit differs for different metals for same or different algal genera (Whitton, 1970; Foster, 1982; Takamura et al., 1989; Agrawal and Kumar, 1975; Harding and Whitton, 1976; Say et al., 1977; Whitton, 1980). Metal tolerance of algae is the result of

different physiological activities like cell surface adsorption, secretion of extracellular ligands for metal complexation, exclusion of metals and sequestration by phytochelatins, sequestration of ROS by stress enzymes and other chelators etc. (Rai et al., 1981; Whitton, 1970; Mehta and Gaur, 2005). Among the metals used for these studies, most commonly used metals are cadmium, chromium (Cr II, III and VI), copper, nickel and zinc (De Carvalho et al., 1995; Chong and Volesky, 1996; Sandau et al., 1996; Roux, 1998; Singh et al., 1998; Zhou et al., 1998; Lau et al., 1999; Chong et al., 2000; Mehta and Gaur, 2001a, b, c; Yin et al., 2001; Cossich et al., 2002; Mehta et al., 2002a, b; Chaisuksant, 2003; Chojnacka et al., 2004; Feng and Aldrich, 2004; Hashim and Chu, 2004; Lee et al., 2004; Sheng et al., 2004b; Chojnacka et al., 2005; Gardea-Torresdey et al., 2005; Vijayaraghavan et al., 2005). Other metals tested are aluminium, cobalt, iron, mercury and lead (Ting et al., 1995; Matheickal and Yu, 1996; Sandau et al., 1996; Gardea-Torresdey et al., 1998; Ozer et al., 1999; Carrilho and Gilbert, 2000; Klimmek et al., 2001; Feng and Aldrich, 2004; Lee et al., 2004; Chojnacka et al., 2004; Prasher et al., 2004; Mahapatra and Gupta, 2005; Vijayaraghavan et al., 2005). Among the precious metals Au and Ag have been tried by a few authors for bioaccumulation study and to estimate the safe and toxic concentration also (Steele and Thursby, 1983; Green et al. 1986; Ting et al., 1995; Niu and Volesky, 2000; Lengke et al. 2006a, b). Our laboratory has published a series of papers on gold accumulation and recovery by algal genera of different groups like cyanobacteria, chlorophyta, diatoms, etc., associated with nanogold production or reduction of Au^{3+} to Au^{0} (Nayak et al., 2006; Chakraborty et al., 2006; 2009; Parial et al., 2012).

Cyanobacterial members are quite efficient in metal removal process. Therefore, many authors used several cyanobacterial strains for bioaccumulation studies. Most successfully used taxa are *Oscillatoria*, *Anabaena*, *Spirulina*, *Lyngbya*, *Synechococcus* PCC 7942, *Synechocystis*, *Microcystis* etc. (Sandau et al., 1996; Gardea-Torresdey et al., 1998; Pradhan et al., 1998; Singh et al., 1998; Ahuza et al., 1999; Donmez et al., 1999; Klimmek et al., 2001; Chojnacka et al., 2004; Chojnacka et al., 2005; Mahapatra and Gupta, 2005). Among the chlorophycean members Chlorella vulgaris and a few other species like *Scenedesmus*, *Selenastrum* and *Cladophora* are most commonly used for bioaccumulation studies for Cd, Cu, Ni, Zn and Au (Keeney et al., 1976; Sandau et al., 1996; Donmez et al., 1999; Lau et al., 1999; Chong et al., 2000; Mehta and Gaur, 2001a, b, c; Mehta et al., 2002a, b). A large number of seaweeds have been employed for metal removal process like *Laminaria*, *Sargassum*, *Ulva*, *Ceramium*, *Ecklonia*, *Fucus*, *Gigartina*, *Padina*, *Ascophyllum* and *Palmaria* for Au, Co, Cd, Cu and Pb accumulation study (De Carvalho et al., 1995; Sandau et al., 1996; Yu and Kaewsarn, 1999; Niu and Volesky, 2000; Yin et al., 2001; Ofer et al., 2003; Sheng et al., 2004b; Feng and Aldrich, 2004; Hashim and Chu, 2004; Lee et al., 2004; Prasher et al., 2004; Vijayaraghavan et al., 2005). Zinc accumulation by Macrocystis (999.50 mg g^{-1}) indicated almost 100 % accumulation (Pradhan et al., 1998). For lead, maximum accumulation observed was 349.09 mg g^{-1} by *Laminaria japonica* (Lee et al., 2004) and that of nickel was 437.98 mg g^{-1} by *Chlorella vulgaris* (Mehta et al., 2002a, b). The seaweed genus *Ascophyllum* accumulated 129.9 mg g^{-1} Cr (III) (Kratochvil and

Volesky, 1998), whereas 146.12 mg g^{-1} cadmium sorption was noticed by *Laminaria japonica* (Yin et al., 2001). A few authors studied the role of alginate and fucoidan present in the cell wall of brown algae in metal binding process (Davis et al., 2003). Haug (1967) reported different degrees of binding capacity of various metals by alginic acid extracted from *Laminaia digitata* in a descending series: Pb^{2+} -Cu^{2+} -Cd^{2+} -Ba^{2+} -Sr^{2+} -Ca^{2+} -Co^{2+} -Ni^{2+} -Mn^{2+} -Mg^{2+}.

Availability of lead on the earth's surface is quite high (5–25 mg kg^{-1}), evolving from rocks, being released into the environment as gases during volcanic activity and associated with natural mobilisation into the environment (Goldberg and Gross, 1971). Chow (1968) reported that lead content of lakes and rivers varies between 1 and 10 μg L^{-1}. As lead is one of the major heavy metals and is a potent environmental pollutant, it gained considerable importance in environmental research.

In contrast to higher plants comparatively less data are available regarding Pb uptake, transport and detoxification process in algal systems. A few reports are available regarding bioaccumulation of Pb by different algal genera. Lead content of red snow alga *Chlamydomonas* from Greenland and Spitspergen (Fjerdingstad et al., 1974) were estimated employing proton induced X-ray spectrometry and found higher Pb content in Spitspergen (42.1 μg g^{-1}) sample than that of Greenland (13.2 μg g^{-1}). The high content of Pb in *Chlamydomonas* indicates the Pb contaminated environment due to more industrialisation in Spitspergen. Harding and Whitton (1978) reported the Zn and Pb content of *Nitella flexilis* from Pb contaminated reservoir polluted by mining activities. The seaweed genus *Laminaria japonica* and cyanobacterial member *Lyngbya taylori* showed comparatively high amount of Pb accumulation, which was 349.09 mg g^{-1} and 304.56 mg g^{-1} (DW) respectively (Lee et al., 2004; Klimmek et al., 2001). Another seaweed genus *Ecklonia* accumulated 243 to 281 mg g^{-1} of lead (Matheickal and Yu, 1996; Feng and Aldrich 2004). But other genera like *Spirulina*, *Schizomeris*, *Synechococcus*, *Chlorella* and *Palmaria* showed 0.01 to 65.47 mg g^{-1} of Pb accumulation (Chojnacka et al., 2004; Sandau et al., 1996; Ozer et al., 1999; Prasher et al., 2004). Holan and Volesky (1994) reported Pb accumulation as much as 1.1 to 1.3 mmol g^{-1} in phaeophycean genera like *Ascophyllum*, *Sargassum* and *Fucus*.

Arsenic, the other toxic metalloid, is widely spread in different layers of earth's crust, with a concentration range from 0.1 to more than 1000 ppm (mg kg^{-1}) in soil, 50–400 ppm in atmospheric dust, up to 2.6 ppb in seawater, and up to 0.4 ppb in fresh water (Mukhopadhyay et al., 2002). In many countries, arsenic contamination in ground water have been reported like Bangladesh, India, China, Taiwan etc. and investigated by several authors (Dhar et al., 1997; Biswas et al. 1998; Mandal et al., 1996, 1997; Liangfang and Jianghong, 1994; Chen et al., 1995; Tondel et al., 1999). The permissible limit for drinking water is only 0.01 mg L^{-1}, as designated by the World Health Organization (WHO). The national standard of arsenic concentration for drinking water in Bangladesh and India is 0.05 mg L^{-1}, which is much higher than the WHO standard limit. The highest arsenic concentration has been recorded as 0.9 mg L^{-1} in Nadia district of West Bengal, India which is 90 times than the WHO standard limit and almost 2 mg L^{-1} in Bangladesh (Chakraborti, 1999; Tondel et al., 1999).

Different valency states of arsenic like, −3, 0, +3 and +5 are present in nature. Among them, arsenite [As(III)] is the dominant form under reducing conditions whereas in oxygenated environments, arsenate [As(V)] is the stable form. As is the predominant form in soil and in groundwater and in submerged soil condition, the predominant form is arsenite. Methylated As are present in agricultural land, where microorganisms based conversion from inorganic arsenic to organic forms are reported including different forms of arsenic, like momomethyl arsinic acid (MMAA) and dimethyl arsinic acid (DMMA) (Takamatsu et al., 1982). However, the main source of arsenic on the Earth's surface is the igneous activity, i.e., formed during volcanic eruption. A schematic diagram for conversion is given in Fig. 19.1.

In marine ecosystem, the flora and fauna like phytoplanktons, macroalgae, crustaceans, mollusks and larger fishes, being continuously exposed to arsenic pollution, convert arsenate to MMA, DMA or other forms of organic storage. They either store these compounds or secret into the environment (Knowles and Benson, 1983; Frankenberger, 2001). Many algal species are reported to accumulate organoarseni-

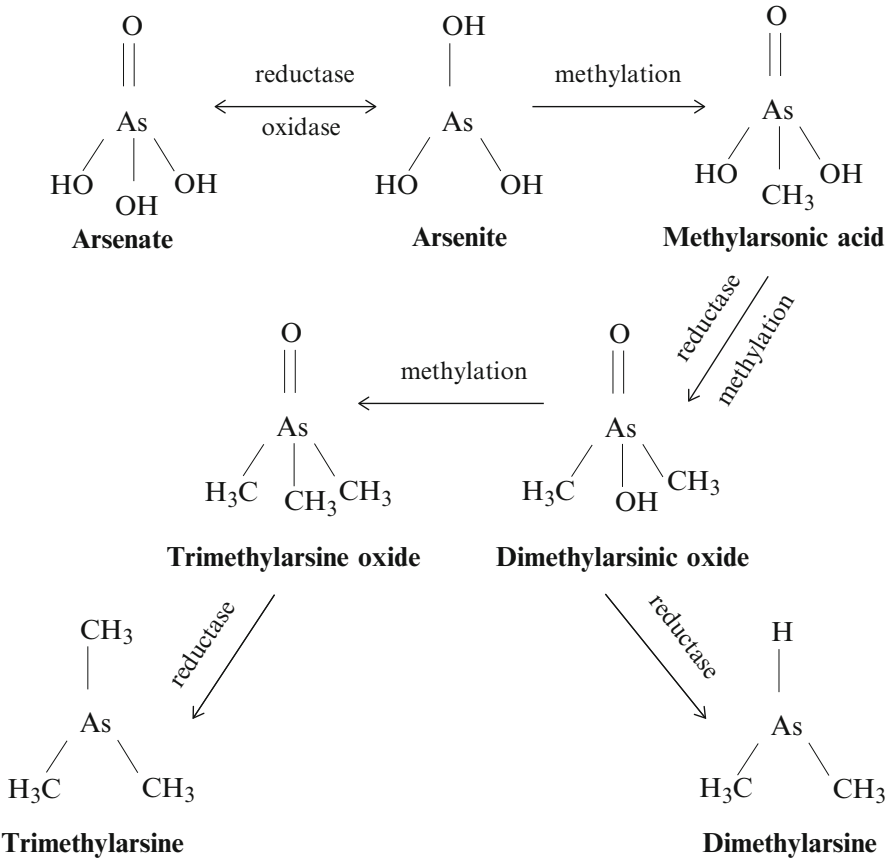

Fig. 19.1 Schematic diagram showing different forms of arsenic.

cal compounds like water-soluble arsenosugars (i.e. dimethylarsenosugars) and lipid-soluble compounds (arsenolipids). Fish and marine invertebrates store 99 % of accumulated arsenic in the form of arsenobetaine, which are passed through the food chain by phytoplanktons (Klumpp 1980). Arsenobetaine in turn is degraded by microbial metabolism in coastal seawater sediments to methylarsonic acid and inorganic arsenic. This way biological cycling of arsenic occurs in the marine system (Mukhopadhyay et al., 2002). Arsenic is metabolized by the human body quite differently from food and water depending on the chemical species administered (National Research Council, 1999; Eisler, 1994) and also in the animal species (Aposhian, 1997; Vahter, 2000; Mitchell et al., 2000). As a result of accumulation and bio-transformation processes, the organic arsenic concentration varies from 1–100 mg kg^{-1} in algae and marine animals (Cullen and Nelson 1993). Among the nontoxic organic forms arsenocholine (AsC), arsenobetaine (AsB) and arsenosugars are the major biosynthetic products in marine animals (Gailer et al., 1995; Larsen, 1995) and as a result trace amount of MMA and DMA are sometimes detected in seafood products.

Arsenic toxicity in algae and their tolerance limits have been reported by many authors. Algae accumulate and transform arsenate because of its similarity (analogous) to the essential and often growth-limiting nutrient PO_4 [$(PO\text{-}(OH)_3$]. Arsenic induced growth inhibition tests were performed in *Chlamydomonas reinhardii* with 100 mg L^{-1} As(V) (Jurewicz and Buikema, 1980). But no significant effect on growth of the freshwater diatom *Asterionella formosa* was recorded, when exposed to 160 µg L^{-1} As(V) (Conway, 1978). Sanders (1979b) exposed the diatom Skeletonema costatum to organic and inorganic arsenic and found that DMA had no significant effect on carbon uptake, and additions of phosphate to the media reduced the arsenate toxicity. In case of other taxa also growth were not affected in high concentrations of arsenite or arsenate, like *Tetraselmis chui* and *Hymenomonas carterae* (Bottino et al., 1978), Dunaliella sp. (Yamaoka et al., 1988), *Chlorella vulgaris* (Maeda et al., 1985) in 100 to 2000 mg L^{-1} As(V). Therefore, it can be inferred that arsenic does not affect the growth of some algae and cyanobacteria at even high concentrations. The pH level of experimental media also affects the toxicity of arsenate as observed by Michnowicz and Weaks (1984) in *Selenastrum capricornutum*, where growth enhancement is recorded at a higher pH. There are many reports regarding arsenic toxicity in diatoms. Hollibaugh et al. (1980) studied the toxicity of arsenic to *Thalassiosira aestevalis*.

Different studies have been done from time to time to understand the arsenic accumulating capacity of different cyanobacterial and algal population in natural and experimental conditions (Imamul Huq et al., 2005; Shamsuddoha et al., 2006). Maeda et al. (1987) exposed the cyanobacterium (*Nostoc* sp.) to 1 and 10 mg As(V) L^{-1} for 32 d and found 32 and 77 mg As kg^{-1} of dry cell weight with no significant effect on growth. Methylation and excretion of As by arsenic resistant genus Phormidium has been reported by Maeda et al. (2004). They also reported increased growth rate of algal biomass up to 100 mg g^{-1} As in growth medium. *Skeletonema costatum* was found to increase their arsenic concentrations by 40 % (Sanders and Windom, 1980) and other phytoplanktons accumulate arsenic from 5.7 to

17.7 mg kg^{-1} (dry weight) when cultured for 48–96 h at 25 μg L^{-1} As(V) (Sanders et al., 1989). A study by Maeda et al. (1992) found that arsenate was actively accumulated when the cells were exposed during the early exponential phase of *Chlorella*. The other unicellular green alga *Dunaliella salina* accumulated more arsenic at higher nitrogen concentrations (Yamaoka et al., 1992). Reuther (1992) observed that when arsenate was added to a freshwater model ecosystem, it was readily accumulated by plankton with arsenic residues of 37–47 mg kg^{-1} (dry weight) at 5 μg L^{-1} As(V) exposure and >200 mg kg^{-1} at 50 μg L^{-1} As(V) after 65 d exposures. Arsenic also induced changes in cellular metabolites. Accumulation of inorganic As increased the beta carotene and fatty acids (C18:1 and C18:3) and water extractable carbohydrate content in the cells of *D. salina* (Yamaoka et al. 1992).

Marine macro-algae like *Ascophyllum* and *Fucus* are known to accumulate As and selenium showing concentration factors of 1000 to 10000, compared to their environment (Lunde, 1970; Klumpp and Peterson, 1979). Several authors detected arseno-sugars using anion exchange HPLC or ICP-MS or by other methods from different genera of sea weeds like *Porphyra*, *Fucus*, *Sargassum*, *Ceramuim*, *Padina*, *Enteromorpha*, *Ulva*, *Eichlonia* etc. (McSheehy and Szpunar, 2000; Šlejkovec et al., 2006; Edmonds and Francesconi, 1981; Madsen et al., 2000). Many reports have illustrated the extraction and separation of arsenosugar species from marine algae. *Laminaria japonica* (brown algae), *Fucus serratus* and *Porphyra* (red algae) were found to contain four arsenosugars and methylarsonicals (Karthikeyan and Hirata, 2003). In environmental waters several algae influence the speciation of arsenic also (Bottino et al., 1978; Conway, 1978). Green alga *Chlorella* has been reported to reduce arsenate to arsenite (Knauer and Hemond, 2000). Garcia-Salgado et al. (2006) identified As(V) in *Hizikia* (46 ± 2 mg g^{-1}), *Sargassum* (38 ± 2 mg g^{-1}) and *Chlorella* (9 ± 1 mg g^{-1}) samples and DMA in *Chlorella* (13 ± 1 mg g^{-1}). Several authors have reported different species of arsenic like As(V), As(III), arsenobetaine, arsenocholine, arsenosugars, tri-MeOH-ribose, glycerol triethylated arsenoriboside; DMAE, dimethylarsenoyl ethanol; MA, methyl arsonate; DMA, dimethyl arsinate; TETRA, tetramethylarsonium ion; TMAO, trimethylarsine oxide in marine macroalgae, *Laminaria*, *Sargassum*, *Undaria*, *Hizika*, *Pelvetia*, *Myelophycus*, *Ceramium*, *Gelidium*, *Cystoseira*, *Enteromorpha*, *Fucus*, *Padina*, *Polisyphonia*, *Ulva*, *Chladophora*, *Chlorella*, *Eucheuma* etc. (Meier et al., 2005; Garcia-Salgado et al., 2006; Hirata and Toshimitsu, 2007; Šlejkovec et al., 2006; Rubio et al., 2010).

19.2 Mechanism of Phycoremediation

Algal cell walls from different groups with varied chemical nature have played important role in metal sorption process. Generally carboxylic group of cell wall polysaccharide play a predominant role in metal uptake by cyanobacteria and eukaryotic algae (Chojnacka et al., 2005). The other functional group like sulphonate, amino and hydroxyl groups in adsorption of various metal ions have also been

reported (Mehta and Gaur, 2005). Thiol group also plays an important role in sorption of metals like Cd at lower pH (Sheng et al., 2004a). Metal sorption by brown algae like *Ascophyllum*, *Sargassum* etc. is high due to their alginate content (Davis et al., 2004).

A number of metals are reported to bind to intracellular polyphosphate granules of algal cells. Therefore, the metals remain in bound form in presence of high concentration of PO_4^{3-}. Reports are available regarding Zn and Cd binding to polyphosphate granules (Bates et al., 1985; Walsh and Hunter, 1992). X-ray microanalysis revealed that polyphosphate bodies in *Chlorella* treated with Al, Fe, Cu and Zn (Wong et al., 1994) and in *Anabaena cylindrica* exposed to lead (Swift and Forciniti, 1997).

Algal enzyme systems are active to combat the metal stress. Some enzymes take active part in metal detoxification process by reducing the toxic metal to non-toxic form or by quenching the ROS (Hassan and Scandalios, 1990; Rice-Evans et al., 1996; Fridovich, 1997; Asada, 1999). In *Chlorella*, mercuric chloride and phenyle mercuric acetate is reduced to metallic, volatile mercury by NADPH or NADH (Ben-Bassat and Mayer, 1977).

Algal extracellular polysaccharides (EPS) are potential compounds for metal removal process due to the presence of different metal chelating ligands and are used in different biotechnological purposes. Uronic acid and $COOH^-$ group present in EPS of cyanophycean algae and $COOH^-$ and SO_4^- of heteropolysaccharides of green algal genera serve as prime metal chelating components. Oxidation of Myo-inositol is the key step in the formation of plant extracellular polymeric substances like gum, mucilage, glycoprotein etc. (Loewus and Loewus, 1983). From this point of view excess production of algal EPS in metal stressed condition can be attributed to oxidizing capacity of the ROS produced within the cells.

Several cyanophycean and chlorophycean algal species have been reported to produce copper-complexing polysaccharidic ligands. Pistocchi et al. (1997) reported higher extracellular carbohydrate production in *Cylindrotheca fusiformis* than *Gymnodinium* sp. with increased toxic Cu concentration of 0.2 to 0.5 ppm after 12 to 16 days of growth. McKnight and Morel (1979, 1980) detected strong copper complexing chelate in culture of cyanophytes at stationary growth phase. Results show that cyanobacteria complexes are generally stronger than those of eukaryotic ones. Nordi et al. (2005) investigated the potentiality of EPS produced by *Anabaena spiroides* in binding Mn(II), Cu(II), Pb(II) and Hg(II) and successfully used for bioremoval process. The algal cell may show the metal resistance employing different biochemical pathways as shown in Fig. 19.2.

19.3 Phytoremediation and Oxidative Stress

Nonessential metals in sub lethal concentrations trigger oxidative stress to the plants leading to the formation of persistent reactive oxygen species (ROS), which damages the cell organelles and disturbs the cellular metabolism. Enzymatic

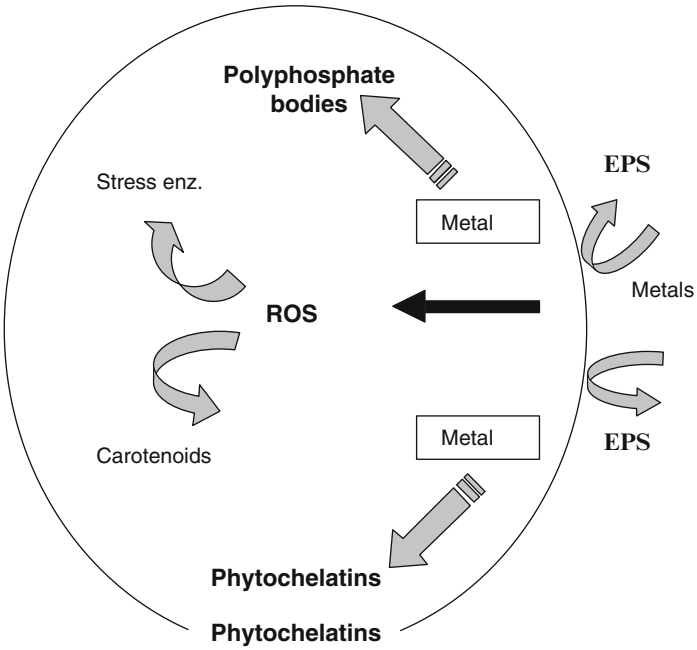

Fig. 19.2 Pathways of metal sequestration in the algal cell (arrows indicate metal sequestration pathways).

components like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) as well as non-enzymatic molecules such as ascorbic acid, cystein, reduced glutathione, α-tocopherol, hydroquinone, carotenoids and polyamines form the anti-oxidative systems of the cell suppressing the ROS level, therefore, reducing the metal toxicity. This is the main basis for phycoremediation of toxic metals. In metal exposure, oxidative stress results from reduced antioxidant enzymatic defense, or low molecular mass like glutathione, α-tocopherol, ascorbate and/or an increase in production of reactive oxygen species (ROS) (Mallick and Mohan, 2000; Okamoto and Colepicolo, 2001; Pinto et al., 2003). The internal ROS level exceeds the tolerance level for inducing oxidation of lipids, proteins and nucleic acids toxicity results (Halliwell and Gulleridge, 1999). Under severe metal pollution, not only excessive oxidation occurs, but the efficiency of anti-oxidative defence is greatly altered. In non-resistant plants these mechanisms are weak. Algae generally respond at molecular level quickly to combat the toxicity (Reed and Gadd, 1990; Rodriguez-Ariza et al., 1991; Holovská et al., 1996; Okamoto et al., 1996; Pinto et al., 2003).

One important major non-protein thiol-glutathione plays main role in scheming the organism's antioxidant defense mechanism; especially the reduced thiols are reported to recycle the antioxidants, vitamin E and vitamin C (Constantinescu et al., 1993). It also acts as a reductant in the highly oxidizing environment of photosyn-

thetic cells in plants (Alscher, 1989; Noctor and Foyer, 1998). The SH group of GSH can be used to reduce peroxides derived as by-products of metabolism which in turn may lead to enhance the peroxidation of membrane lipids and loss of cell viability (De Vos, 1992; Pinto et al., 2003). Therefore, consumption of cellular GSH in scavenging reactive species and free radicals produced in metal stressed condition is obvious. Reduction in GSH amount can also be seen in case of enhanced activity of H_2O_2 removing ascorbate oxidase (Apx) system. Actually, transfer of signal for translation of Apx transcript is inhibited by high levels of GSH (Karpinski et al., 1997). Under conditions of oxidative stress when Apx activity is required, GSH levels would be depleted to reduce dehydroascorbate (DHA) to ascorbate (AA) by means of DHA reductase. There are reports on correlation of acute metal stress and decreased reduced glutathione pool as found in treated cells in *Gonyaulax polyedra* exposed to the toxic metal Pb^{2+} (Okamoto et al., 1999). Nagalakshmi and Prasad (2001) observed progressive depletion of GSH content with increasing concentration of Cu.

The harmful H_2O_2 is removed catalytically by catalase (CAT) and ascorbate peroxidase (APX). Catalytic breakdown of H_2O_2 to H_2O and O_2 is induced by CAT and this occurs in peroxisomes (Halliwell and Gulleridge, 1999), but in cyanobacteria catalase activity is located in cytosol (Regelsberger et al., 1999). Catalysis of H_2O_2 is done differently by APX, which removes it by using it to oxidize ascorbate, producing mono-dehydro-ascorbate (MDHA) and H_2O. There are at least three distinct isoenzymes of APX: thylakoid bound, stromal and cytosolic APX which are restricted to higher plants, algae and some cyanobacteria (Mittler and Zilinskas, 1993). These enzymes counteracting H_2O_2 exposure, is part of the integrated net of strategies that make the redox status of algal cells (Barros et al., 2003). An increase in peroxidase activity is regarded as a reliable indicator of stress from toxicity of heavy metals/metalloids, which may cause disruption of the plasma membrane by lipid peroxidation and the ROS production (Macfarlane and Burchett, 2001).

Dismutation of O_2^- by SOD produces H_2O_2, a weak oxidizing agent that can cross the cell membrane easier than $O_2.^-$ and possesses a steady state high concentration (Chance et al., 1979). A 7-fold increase in catalase activity (90.7 nmol min^{-1} mg^{-1} proteins) was observed by Loretto et al. (2005) in *Scytosiphon lomentaria* inhabiting copper enriched coastal environments indicating involvement of catalase in buffering oxidative stress *in vivo*. The SOD generally catalyses superoxide anion radicals produced in different compartments of plant cells to H_2O_2. On the other hand, transition heavy metals (e.g. Cu, Fe) catalyse the formation of •OH radicals from O_2^- (superoxide) in the nonenzymatic Fenton reaction. The protective function of CAT is limited due to its localization mainly in peroxisomes. Ascorbate (ASC) is known as a major primary antioxidant, reacting directly with •OH, superoxide (O_2^-) and singlet $•O_2$ (Buettner and Jurkiewicz, 1996). Ascorbate peroxidase (APX) and glutathione reductase (GR) are vital constituents of the ascorbate-glutathione pathway which are required to scavenge H_2O_2 and to maintain the redox state of the cell (Asada, 1992). Under oxidative stress increased GR activity could be required to supply reduced glutathione (GSH) to the ascorbate-glutathione cycle.

Flavonoids are found in higher plants and brown algae and are directly linked with scavenging •OH, ONOOH (peroxynitrous acid) and HOCl (hypochlorous

acid) in order to inhibit lipid peroxidation. Since flavonoids bind to metal ions, the scavenging efficiency of flavonoids is directly proportional to the number of hydroxyl groups (Rimbach et al., 2003).

Under unfavourable environmental conditions, among all amino acids, proline is accumulated rapidly and more frequently. It acts as a powerful secondary antioxidant reducing the oxidized form of α-tocopherol (Buettner and Jurkiewicz, 1996).

Lipid peroxidation of membranes is an indicator of oxidative damage, which is caused by free radicals and hydroperoxides (Smirnoff, 1993). It involves oxidative degradation of polyunsaturated fatty acyl residues of membranes (Girotti, 1990). A reduced level of saturated fatty acids and high levels of unsaturated fatty acids of membranes in several plant species are brought about by metal ions through lipid peroxidation (Halliwell and Gulleridge, 1999). These results suggest that decreased activities of antioxidant enzymes could result in an increased level of lipid peroxidation, thus contributing to damage of cell membranes leading to cell death (Blum and Ebercon, 1981; Marcum, 1998; Abernethy et al., 1989).

Enhanced ROS level generally induce antioxidant synthesis in algal cultures, depending on the duration and severity of the stress applied (Okamoto et al., 1996). Generally the type and duration of exposure to metal/metalloid ions, either acute or chronic, alter the level of antioxidants and create different oxidative status (Okamoto and Colepicolo, 2001). Therefore, higher level of cellular antioxidants could allow cells to combat chronic stress, whereas, sometimes a sudden generation of high levels of ROS over a short period can surpass the total antioxidant capacity resulting toxicity. Antioxidant capacities of GSH, NADPH and ascorbate are likely to occur first in acute stress, which lowers the GSH pool and depletes NADPH levels.

In contrast to information pertaining to antioxidative defense in microorganisms to the effects of metals on bacteria, fungi, dinoflagellates and diatoms, very little is known about antioxidant defense system in other algae by arsenic stress. Pandey et al. (2012) studied the upregulations and downregulations in antioxidant system of *Anabaena* sp. PCC 7120 exposed to arsenic and found that an up-regulation of CAT, peroxiredoxin (Prx), thioredoxin (Trx) and oxidoreductase, and also an appreciable induction in phytochelatin content, GST activity and transcripts of phytochelatin synthase, arsenate reductase and arsenite efflux genes—*asr*1102, *alr*1097 which echoed their role in As sequestration and shielding of the organism from As toxicity. They established that, up-regulation in metabolic and antioxidative defense proteins, phytochelatin and GST together with the *ars* genes play a central role in detoxification and survival of *Anabaena* under As stress. *In vitro* study was done by Zutshi et al. (2014) to determine the toxic effect of sodium arsenate (0–100 mM) on an aquatic cyanobacteria *Hapalosiphon fontinalis*-339. At this level they found that MDA production was enhanced that probably resulted in decreased growth of the test organism.

Accumulation of enzymatic and non-enzymatic substance e.g. SOD, CAT, APX activities and proline, total glutathione showed an efficient antioxidative potential mechanism in *Hapalosiphon fontinalis*-339. Srivastava et al. (2009) provided comprehensive information on arsenic induced oxidative stress and changes in antioxidative defense system of *Anabaena doliolum*. They concluded that the cyanobacterium may survive better in As(V) than As(III) contaminated fields

because of its low toxicity and pronounced induction of antioxidative defense system. The present authors already reported arsenic induced changes in stress enzymes and other stress related compounds in *Phormidium laminosum* (Bhattacharya and Pal, 2011). Therefore, metals and metalloids lead to the activation of a defense mechanism inside the cells in the form of antioxidants, which lead to the reduction of toxicity. Thus it is important to study the biochemical modulations of the antioxidant defense system of cyanobacteria in Arsenic stress and to fully understand its potential as a suitable material for bioremediation process.

19.4 Genetic Tolerance to Arsenic

Rosen and his group (1994, 1997, 2002) did an extensive study on biochemistry of As detoxification and its genetic control in *E. coli*. In prokaryotic system, several authors opined the fact that Pit and Pst are the two PO_4^- transporters and both of them catalyse AsO_4^- uptake, where the Pit system appears to be predominant (Willsky and Malamy, 1980). In the eukaryote system, like *Saccharomyces cerevisiae* different PO_4^- transporters participate in AsO_4^- accumulation (Yompakdee et al., 1996). Sanders et al. (1997) recorded the glycerol facilitator of *E. coli* which transports both As III and Sb III—the trivalent metalloid transported as Glp F being a member of the aquaporin superfamily. Aqua-glyceroporins transport neutral organic solutes like glycerol and urea. The Fps 1p, the homologue of Glp F, has recently been discovered as the route of uptake for arsenite in *S. cerevisiae* (Wysocki et al., 2001). Liu et al. (2002) have also shown that mammalian aqua-glyceroporin catalyses uptake of trivalent metalloids. The genes responsible for As transport and detoxification have also been characterized by several authors (Liu et al., 2002).

It is known that the ancient environment was not oxidizing and As(III) was the most dominant form, therefore, early organisms have evolved with a detoxification mechanism of As(III), mainly the extrusion system. According to Dey and Rosen (1995), bacteria show two basic mechanisms of arsenite extrusion—one is with carrier protein, where energy is supplied by the membrane potential of the cell and the other by an AsO_3^- translocating ATPase. For arsenate reduction process, three independently evolved families of arsenate reductase enzymes have been recognized whose sequences also have been identified as a product of ars operon (Mukhopadhyay et al., 2002). Cytosolic arsenite is also detoxified by removal process (Rosen, 1999). Cole et al. (1994) reported that members of multidrug resistance associated protein (MRP) is responsible for AsO_3^- resistance in eukaryotic As extrusion systems. Not much is however known about extrusion mechanisms in algae. Some work has been done by the present group which is illustrated in Fig. 19.3.

As mentioned earlier, three different families of arsenate reductase are reported in different organisms. The product of the *arsC* gene from the *E. coli* plasmid R773 was reported to be the first family of arsenate reductases. Several Gram-negative bacteria harbour this enzyme, which uses glutaredoxin as a source of reducing equivalents. The other two types of pI258 from *Staphylococcus aureus* and *Bacillus subtilis* differ significantly from *E. coli*. In these cases, instead of glutaredoxin, the

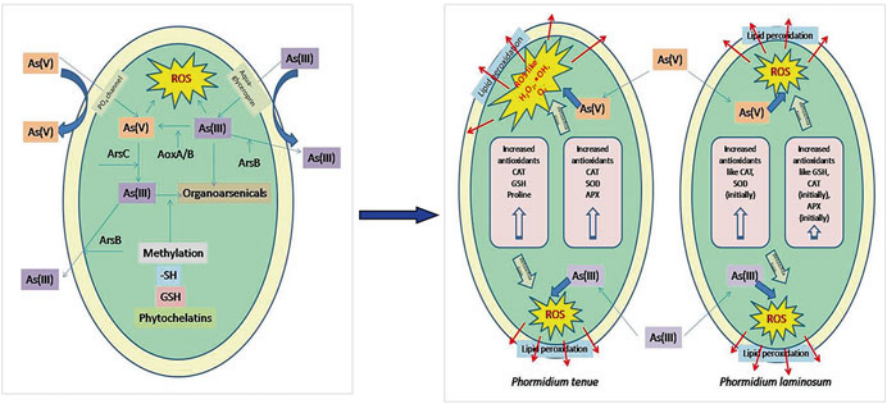

Fig. 19.3 Arsenic toxicity and defence mechanism in cyanobacteria Phormidium spp. (as studied by our group).

arsenate reductase is related to low-molecular-weight protein tyrosine phosphatases and uses thioredoxin as the source of reducing equivalents. On the other hand another family of arsenate reductase from *Saccharomyces cerevisiae*, represented by the Acr2p enzyme, is also similar to a protein phosphatases which contains CDC25a. Generally these *arsC* are responsible for reduction to the more toxic form arsenite, than exported by the carrier protein ArsB. The *E. coli* plasmid R773 and *Staphylococcus aureus* pI258 bearing *arsB* gene show similar activity encoding an integral membrane protein that expel arsenite. When ArsB interacts with ArsA, an arsenite-stimulated ATPase, proteins can also function as an arsenite pumps. The ARR3 protein from *S. cerevisiae* (formerly ACR3) and the ArsB gene of the *B. subtilis* of *ars* operon are considered as second family of arsenite carriers. In addition, another protein ArsH from gene *arsH* has been found to be essential for resistance to arsenite and arsenate both in *Yersinia enterocolitica* and *Acidothiobacillus ferroxidans* (Lopez-Maury et al., 2003). In some plasmid-determined systems of Gram-negative bacteria, the arsenic efflux pump consists of a two-component ATPase complex. The *arsA* gene product is a soluble ATPase subunit (Rosen et al., 1999), which physically associates with an integral membrane protein, the product of the *arsB* gene (Tisa and Rosen, 1990; Gladysheva et al., 1994). In most chromosomal arsenic resistance systems of Gram negative bacteria and the plasmids and chromosomes of Gram-positive bacteria, though adjacent *arsB* and *arsC* genes are found, but there is no *arsA* gene (Silver et al., 2001).

Eukaryotes such as *Saccharomyces cerevisiae*, the arsenic resistance gene cluster is similar to that of bacteria (Bobrowicz et al., 1997). Here, three adjoining genes remain in cluster, ARR1, ARR2 and ARR3. The first gene, ARR1, appears to produce a yeast transcriptional regulator and its disruption leads to hypersensitivity to arsenite and arsenate. Mukhopadhyay et al. (2000) found functional yeast ARR2 arsenate reductase gene in *E. coli.* Galperin et al. (1998) recorded Arr3p (the protein product of ARR3) as a member of a family of arsenite carrier efflux bacterial and archaeal members and is unrelated to the larger family of ArsB proteins found in

many bacterial *ars* operons (including in *E. coli* and *Staphylococcus aureus*). According to them they evolved as a result of convergent evolution. In addition to the three ARR gene products, another yeast protein, Ycf1p, which is an ABC ATPase, also contributes to resistance to As(III) and Sb(III), being located in the vacuolar membrane and by pumping glutathione adducts, $As(GS)_3$ and presumably $Sb(GS)_3$ from the cytoplasm into the vacuole.

But very little work has been reported in cyanobacteria and algae where the function and regulation of these *ars* genes are still unknown (Cervantes et al., 2006). In *Synechocystis* PCC 6803 an arsenic and antimony resistance operon reported showing *arsC*-encoding a putative arsenate reductase, *arsB*-encoding a putative arsenite-antimonite carrier and *arsH*-encoding a protein of unknown function (Lopez-Maury et al., 2003). It was reported that *arsC* mutants were sensitive only to arsenate, while *arsB* mutant strains were sensitive to arsenite, arsenate, and antimonite. They also observed that purified recombinant ArsR protein bound to the *arsBHC* promoter-operator region and dissociated in the presence of Sb(III) or As(III) but not in the presence of As(V), suggesting that trivalent metalloids are the true inducers of the system.

Proteomics study in combination with morphological, physiological and biochemical variables have been employed by Pandey et al. (2012) in arsenic treated cyanobacterium *Anabaena* sp. PCC7120 to unravel its survival strategies. In this study it was revealed that 13 were novel (hypothetical) ones out of total 45 differentially expressed proteins. They also proposed hypothetical model which explains the interaction of metabolic proteins associated with the survival of *Anabaena* sp. PCC7120 under As stress.

19.5 Model Developed for Bioremoval of Metal/Metalloids

Various conventional physical, chemical and biological methods have so far been practiced to remove pollutants from industrial wastewater. Sometimes these methods are costly due to large chemical requirements and excessive sludge production and are with operational difficulties. There is, therefore, always a requirement for a low cost simple to operate system for treating industrial wastewaters. Use of activated algae-reactor is in well practice over the past few years (McGriff and McKinney, 1972; McShan et al., 1974; Lee et al., 1980).

A few models have already been proposed for metal removal process and use of 'algae-pond' being a popular and widespread technology for last few decades (McGriff and McKinney, 1972; McShan et al., 1974; Lee et al., 1980). A continuous flow system consisting of three rectangular algae reactors, connected in series was designed by Aziz and Ng (1993) for removing organic and synthetic pollutants along with metals from industrial wastewater. Experimental data obtained suggested that activated algae-reactor was successfully able to remove organic pollutants, colour, nutrients and toxic metals from wastewater in a cost effective manner. Bender et al. (1994) also used glass column packed with cyanobacterial for removing metals like zinc and manganese from contaminated water. Within 3 h retention time 96 % Zn and 86 % Mn could be removed by the column. Boi-filter, AlgaSORB-

Fig. 19.4 Column type algae based Biofilter for metal removal from contaminated water showing a glass column with live algal mat enmeshed with glass wool, collection flask and pump.

scy (*Scytonema*-dimethyl-formamide slurry) over a polymer-modified silica gel, was suitable for 100 % As(III) removal (Prasad et al., 2006). The present group already reported 95.8 % removal of the Pb from 5 mg L^{-1} Pb solution using *L. majuscula* as bioreagent (Fig. 19.4; Chakraborty et al., 2011).

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