Chapter 9
Bioremediation of Heavy Metals

Anamika Das and Jabez William Osborne

Abstract Human activities and industrial processes have led to worldwide heavy metal pollution. Several strategies have been developped for metal remediation. The conventional strategies are expensive, usually low in efficiency and may alter the soil nature. Here we review bioremediation using plants, microbes, e.g. bacteria, fungi, and actinobacteria, earthworms, and algae for metal removal. Bioaugmentation of microbes using plants, earthworms and algae is used to enhance the bioremediation efficiency. We discuss the importance of metagenomics, metabolomics and proteomics approach to assess the response of the living organisms under stress and how they can contribute to the improvement of the already existing strategies.

Keywords Heavy metals • Bioremediation • Biosystems • Bioaugmentation • Metagenomics • Metabolomics • Proteomics

Contents

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9.1 Introduction

Environmental pollution occurs when the natural environment cannot destroy an element without creating harm or damage to itself (Wijnhoven et al. 2007). The elements involved are not produced by nature, and the destroying process can vary from a few days to thousands of years. Current research has documented elemental pollutants as "emerging contaminants" (Yu et al. 2014). Intense industrialization, modern agricultural practices, increased anthropogenic activities, and unauthorized disposal methods have increased the concentrations of elemental pollutants in the environment, creating adverse effects to all the living organisms (Wijnhoven et al. 2007). Heavy metals are one of the major pollutants which has been the chief concern in past decade. They can enter the environment in a single high-level exposure or the cumulative effect of repeated high or low-level exposures but when introduced into an environment, it can stay there in toxic form for a long period of time.

A number of physical, chemical and biological techniques can be used to remediate metal contaminated soils. Physico-chemical methods are, however, not appreciated as they generate a large amount of sludge and result in more contamination (Ahluwalia and Goyal 2007). Thus, bioremediation provides the best answer. Many reports have established the bioremoval of heavy metals by the use of either plants, earthworms or microbes (Wang et al. 2015; Rodriguez-Campos et al. 2014; Dharni et al. 2014; Ma et al. 2015). But recent reports have studied the uptake studies by using more than one living organism and have come out with more efficient and improved results (Emenike et al. 2016; Wood et al. 2016; Lemtiri et al. 2016). Thus, they have opened the gate of exploring the more diverse flora and fauna for achieving the best result in bioremediation. Scientists have also developed and studied the three main 'omics' approach for understanding the response of the organism under the stressed condition, i.e., metagenomics, metabolomics and proteomics (Gillan et al. 2015; Tomanek 2014). The integrated 'omics' analysis can be a powerful technique to identify the vast microbial communities which are unculturable but still possess the ability of bioremediation and the various metabolites released under stress along with their function. This approach has brought a revolution in the field of bioremediation. Figure 9.1 summarizes the bioremediation technologies described in this review.

This review emphases on the utilization of different tactics of bioremediation using plants, bacteria (rhizobacteria, actinobacteria), earthworms, algae, fungi and highlights the advantages of the integrated approach of using multi-biosystem for

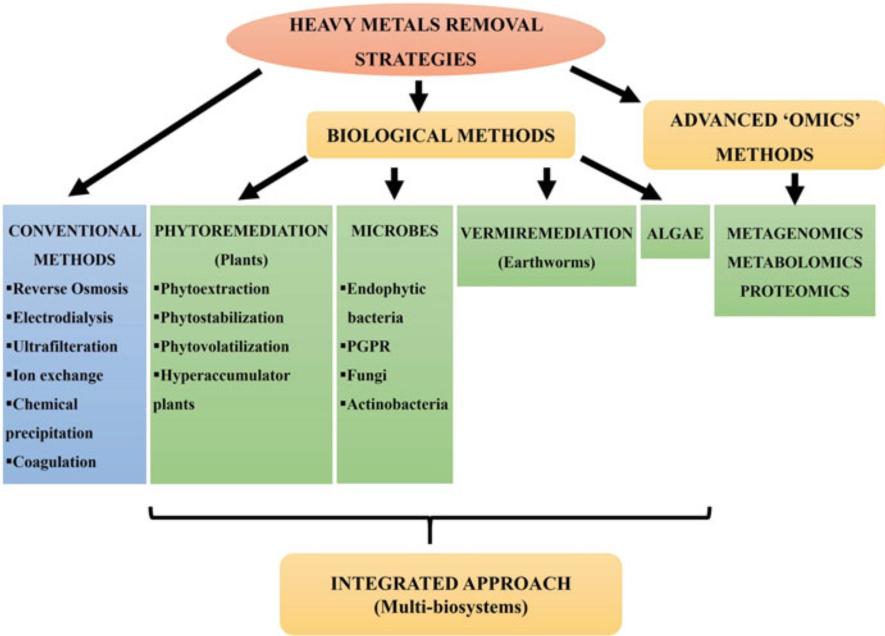

Fig. 9.1 An outline of the remedial strategies applied for bioremoval of heavy metals. The conventional methods are ineffective or expensive when the concentration of heavy metals is very low and produces a large amount of derivatives of contaminants. Alternately, biological methods with the usage of living biosystems has proven efficient in heavy metals bioremediation. The 'omics' approach also enhanced the understanding of the living biosystems under stressed condition (*PGPR* Plant growth promoting rhizobacteria)

the bioremediation of Heavy metals. To support the statement, many evidence has been provided representing different case studies along with their mechanism and limitations. In this context, the scope of 'omics' tool to enhance the overall bioremediation process has also been discussed.

9.2 Heavy Metals

Heavy metals represent a class of metallic element present abundantly in the earth's crust (Yu et al. 2014). They are defined as the metals possessing density greater than 5 gm/cm^3 (Das et al. 2014). Different from other organic pollutants, heavy metals are harder to be chemically or biologically degraded. Irrespective of the origin of the metals in the soil, excessive levels of many metals can result in the deprivation of soil quality, crop yield and agricultural products and can be significantly hazardous to human, animal and ecosystem health (Das et al. 2014). The metals or metalloids including arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), selenium (Se), silver (Ag), and zinc (Zn) can be of severe threat to human and animal health due to its intensified long-term persistence in the environment (Gisbert et al. 2003). Toxic heavy metals are also known as cumulative poison because of it persistence in nature and the ability to get transferred and accumulated in various tropic levels causing DNA damage and carcinogenic effects by their mutagenic ability (Knasmüller et al. 1998). Therefore an alarm has been triggered for the researchers to conserve the environment from toxic heavy metals. The Agency for Toxic Substances and Disease Registry (ATSDR) in Atlanta, Georgia, (a part of the U.S. Department of Health and Human Services) compiled a Priority List called the "Top 20 Hazardous Substances." The heavy metals arsenic, lead, mercury, and cadmium appear on this list (ATSDR 2011).

9.3 Conventional Strategies for Detoxification of Heavy Metals

In order to make the environment healthier, contaminated water bodies and land need to be remedied to make them free from heavy metals and trace elements. There are several conventional techniques to remove these heavy metals, including chemical precipitation, oxidation or reduction, filtration, ion-exchange, reverse osmosis, membrane technology, evaporation and electrochemical treatment. But most of these techniques become ineffective when the concentrations of heavy metals are less than 100 mg/L (Ahluwalia and Goyal 2007). Additionally, physico-chemical methods are ineffective or expensive. Some of the techniques are mentioned in Table 9.1 with their drawbacks.

Biological methods for removal of heavy metals has become an attractive alternative to physico-chemical methods. Bioremediation has proved to be an innovative and promising technology available for removal of heavy metals and recovery of the heavy metals in polluted water and lands.

9.4 Bioremediation of Heavy Metals

According to Environmental Protection Agency (EPA), bioremediation is a technique that uses naturally occurring organisms to break down hazardous substances into less toxic or nontoxic substances (Agouborde and Navia 2009). Various living biosystems can be utilized for the bioremoval of heavy metals. The biomass-based systems are more satisfactory compared to the conventional treatment methods as it is cost effective with high efficiency of detoxification of dilute effluents and reducing the quantity of sludge disposal in the environment. There are many reports about biodegradation and bioremediation strategies being utilized by bacteria or

Table 9.1 Conventional techniques for heavy metals removal and their drawbacks

Technique	Application to heavy metal	Drawback	References
Reverse osmosis- A semi permeable membrane is used to separate the heavy metal at a pressure greater than the osmotic pressure	Cu^{2+}, Ni^{2+}, Zn^{2+}	High power consumption due to the pumping pressures, and the restoration of the membranes	Fu and Wang (2011)
Electrodialysis-Ion selective semi permeable membrane are used to separate heavy metals by applying electrical potential between two electrodes	Cr(III), Cu, Fe	The separation percentage decreased with an increasing flow rate	Sadrzadeh et al. (2009)
Ultrafiltration- A porous membrane is used to remove heavy metals by applying pressure.	Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}	If the surfactant and heavy metals are not disposed of, it lead to secondary pollution by generating sludge	Landaburu-Aguirre et al. (2009)
Ion exchange- From the dilute solution containing heavy metal, the metal ion gets exchanged to the exchange resin by the ions held by electrostatic force	Ce^{2+}, Fe^{2+} and Pb^{2+}	It can be used only with low concentrated metal solution and is highly sensitive with the pH of the aqueous phase.	Gunatilake (2015)
Chemical precipitation- Chemicals react with heavy metal ions to form insoluble precipitates	Cu2+, Cd^{2+} and Pb^{2+}	Generates large volumes of low density sludge, which can cause disposal problems	Kongsricharoern and Polprasert (1995)
Coagulation- Removal of heavy metals by charge neutralization of particles	Ni^{2+}	Unable to treat the heavy metal wastewater completely	Chang and Wang (2007)

plant species (Wang et al. 2015; Ma et al. 2016; Glick 2010) but so far very few investigations have been carried out using other living biosystems such as earthworms, algae, fungi and their integrated approach.

9.4.1 *Phytoremediation of Heavy Metals*

The word "phytoremediation" is derived from Greek word phyto (mean plant) and Latin word Remedium (to remove an evil). Phytoremediation utilizes a variety of plant processes and the physical characteristics of plants to aid in remediation of contaminated sites. It is an *in situ* remediation technology driven by solar energy. Remediation of metals using plants seems an effective approach in the present scenario since plants are the primary recipients of heavy metals (Ali et al. 2013;

Wang et al. 2015). Phytoremediation technique includes processes such as phytoextraction, phytostabilization, phytovolatilization (Alkorta et al. 2004).

9.4.1.1 Phytoextraction

It is the process of uptake of contaminants from soil or water by plant roots and their accumulation in biomass, *i.e.*, shoots (Seth 2012). Generally shoot metal concentration and shoot biomass mainly determine a suitable plant species for phytoextraction of metals. Depending upon these parameters, two different phytoextraction approaches have been used, i.e., use of hyperaccumulator plants with relatively low biomass production and use of plants with relatively higher above ground biomass production but lesser metal accumulation such as *Brassica juncea* (Robinson et al. 1998; Ali et al. 2013). A recent report by Ma et al. (2016) suggested that the highly developed root system of Napier grass makes it an ideal candidate for phytoextraction process by absorbing, transporting and storing both contaminants and nutrients into the plant tissue.

9.4.1.2 Phytostabilization

Phytostabilisation is a method where the plants are used to immobilise metals in the rhizosphere and reduce the above ground wind and water erosion (Gil-Loaiza et al. 2016). There are two main factors which are considered when determining the aptness of plants with a large biomass for phytostabilisation: root accumulation and rhizosphere immobilisation (Sun et al. 2016). The plants selected must be able to develop abundant root systems, and translocate metals from roots to shoots at as low concentrations as possible (Mendez and Maier 2008). Giant reed (*Arundo donax*) and silvergrass (*Miscanthus sinensis*) genotypes are bioenergy crops well suited for the phytostabilisation of metal(-loid)-contamination of dry land (Barbosa et al. 2015). But phytostabilization is not a permanent solution as heavy metals remains in the soil as it is; only with restricted movement and needs to monitor regularly.

9.4.1.3 Phytovolatilization

This approach involves conversion of heavy metals into volatile forms by plants and subsequently released into the atmosphere. This process has been used for removal of some volatile heavy metals like Hg and Se from polluted soils (Karami and Shamsuddin 2010). However, this is limited by the fact that it does not remove the metals completely but rather transfers them from one medium (soil or water) to another (atmosphere) from which they can re-enter soil and water.

9.4.1.4 Hyperaccumulator Plants

Recently, removal of heavy metals through hyperaccumulators to degrade the contaminants, has received wide attention due to its efficacy and cost efficiency (Ahemad 2014). Hyperaccumulators have been found to exhibit higher heavy metal tolerance and accumulating abilities compared to other plants (Prasad and Freitas 2003). Many reports are provided for hyperaccumulators being utilized such as *Arabidopsis halleri* and *Solanum nigrum* L. for uptake of Cd (Dahmani-Muller et al. 2000; Wei et al. 2005), *Zea mays* for uptake of Pb, Cd and Zn (Meers et al. 2010), *Brassica juncea, Astragalus bisulcatus* for uptake of Se (Bitther et al. 2012). However, the disadvantages that limit the use of hyperaccumulators include difficulty in finding heavy metal hyperaccumulators, slow growth and lower biomass yield. This makes the process quite time-consuming and therefore not feasible for rapidly contaminated sites or sewage treatments (Xiao et al. 2010).

9.4.1.5 Mechanism of Heavy Metals Phytoremediation

The uptake of heavy metals by plants depends mainly on the bioavailability of the heavy metals in the soil as well as the plant nutrients. The heavy metals either gets accumulated in the root tissues or get translocated to the aerial regions of the plants through xylem vessels by symplastic and/or apoplastic pathways (Sarwar et al. 2016). The tolerance against heavy metals is a prerequisite for phytoremediation process to minimize the adverse effects on the plants. The tolerance potential of the plant depends on mechanisms like cell wall metal binding, active transport of metal ion into the vacuoles, chelation of metal ions with proteins and peptides and complex formation (Memon and Schroder 2009).

9.4.1.6 Challenges in Phytoremediation

Phytoremediation, no doubt, is an attractive process for heavy metals uptake but the researchers have confronted several limitations when only plants were used for the bioremediation (Karami and Shamsuddin 2010; Naees et al. 2011; Ramamurthy and Memarian 2012) which has been summarized in Fig. 9.2.

9.4.2 Microbial Remediation of Heavy Metals

Microorganisms as metal accumulators possess an inherent novel remediation property for toxic metals in the soil with increased crop productivity. Many researchers have studied the close interactions among plants-microorganisms heavy metals in rhizosphere soils to enhance phytoremediation process (Glick

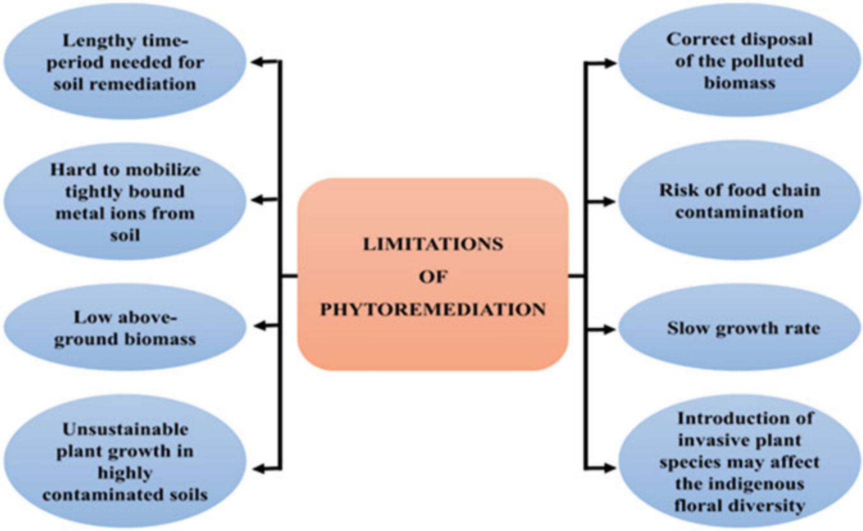

Fig. 9.2 An overview of the common problems faced by the plants in bioremediation. These limitations inhibit the application of the traditional phytoremediation techniques on large scale applications. The limitations can be overcome by synergistic integration of the plants with other living organism for bioremediation by advanced bioremediation research

2010; Dharni et al. 2014; Ma et al. 2015). Inoculation of plants with selected and acclimatized microbes (bioaugmentation) has attained prominence for phytoremediation of metal polluted soils (Lebeau et al. 2008; Glick 2010; Ma et al. 2011). Some microorganisms live in association with plant roots while others are free living. A recent report by Abd-Elnaby et al. (2016) identified three marine Psychrobacter strains which were able to resist and accumulate several metals (Pb^{2+}, Cu^{2+} and Cd^{2+}) with variable degrees, depending on bacterial strains and metal ion species. There are few bacterial species such as Alphaproteobacteria and *P. aeruginosa* which are isolated from sponge genera such as *Sarcotragus* sp. *Suberites clavatus* and *Crella cyathophora* and have been recognized as a source for secondary metabolites having the potential for heavy metal bioremediation (Saurav et al. 2016a, b).

9.4.2.1 Endophytic Bacteria

Endophytes mostly lives under the epidermal cells of plant tissues and colonize (Schulz and Boyle 2006). The extensive co-evolution of plants and endophytes has developed an intimate ecosystem which helps the plants to survive in stressed conditions and helps in enhanced bioremoval of Heavy metals (Ryan et al. 2008).

Bioaugmentation with such endophytic bacteria can diminish the metal phytotoxicity and alter the phytoavailability of heavy metals in contaminated soils, making them ideal for microbial assisted phytoremediation studies (Weyens et al. 2009; Ma et al. 2011). The hyperaccumulator plants constitute a complex and specialized endophytic bacterial flora such as *Pseudomonas koreensis*, *Bacillus* sp., *Rahnella* sp. with high levels of resistance to heavy metals such as Pb, Mn and Cd (Babu et al. 2015; Luo et al. 2012; Yuan et al. 2014).

9.4.2.2 Plant Growth Promoting Rhizobacteria

Plant growth promoting rhizobacteria (PGPR) are a group of microbial community which can improve the growth of the host plant in heavy metal contaminated soils by mitigating toxic effects of heavy metals on the plants (Seth 2012). These may be free- living bacteria, in symbiotic associations, or endophytic bacteria (Glick 2012). Some important genera of PGP bacteria include *Bacillus, Pseudomonas, Enterobacter, Erwinia, Klebsiella, Flavobacterium* and *Gluconacetobacter* (Dardanelli et al. 2010; Nadeem et al. 2010). PGPR improve plant growth and effect heavy metals mobility by atmospheric nitrogen fixation, production of phytohormones and siderophores and solubilisation of insoluble phosphate (Ullah et al. 2015). A wide range of PGPR has been identified which aid in uptake of Heavy metals (Glick 2010). A report by Jing et al. (2014) showed enhanced accumulation of Cd, Pb, Zn in *Brassica napus* when inoculated with PGPR strains such as *Enterobacter* sp. and *Klebsiella* sp..

9.4.2.3 Fungi

Fungi have been chiefly ignored as constituents of the host microbiota and their role in bioremediation (Moyes and Naglik 2012). Fungi have emerged as potential biocatalysts to access heavy metals and transform them into less toxic compounds. They possess metal sequestration and chelation systems to increase their tolerance to heavy metals. Moreover, their high biomass makes them suitable for bioremediation of Heavy metals (Aly et al. 2011). Some fungi such as, *Allescheriella* sp., *Stachybotrys* sp., *Phlebia* sp. *Pleurotus pulmonarius*, have metal binding potential (D'Annibale et al. 2007). Fungi of the genera *Penicillium*, *Aspergillus* and *Rhizopus* have been studied extensively as potential microbial agents for the removal of heavy metals from aqueous solutions (Volesky and Holan 1995; Huang and Huang 1996). Pb (II) contaminated soils can be biodegraded by fungal species like *Aspergillus parasitica* and *Cephalosporium aphidicola* with biosorption process (Tunali et al. 2006; Akar et al. 2007). Recent reports identifies 20 fangal taxa in which *Alternaria,* and *Peyronellaea* are the dominant genera and shows excellent uptake of Pb^{2+} and Zn^{2+} (Li et al. 2012).

9.4.2.4 Actinobacteria

Actinobacteria are a group of bacteria which play an important role in recycling substances, since they are able to metabolize complex organic matter (Kieser et al. 2000). They prove to be an important ecological agent by possessing the ability to remove Heavy metals (Albarracín et al. 2005; Polti et al. 2009). Several reports signifies *Corynebacterium* strain tolerant to heavy metals such as Cd(II), Co(II), Cr (VI), Hg(II), and Ni(II) (Oyetibo et al. 2010). Other reports by Mangold et al. (2012) demonstrates the strain *Acidimicrobium ferrooxidans* tolerant to higher concentrations of Zn(II) and adapting to the adverse environment. Although the bioremediation skills of the genera such as *Streptomyces, Rhodococcus,* and *Amycolatopsis* were extensively studied but the lack of information to enhance the bioremediation process of actinobacteria through pathway engineering techniques did not supported their further use (Alvarez et al. 2017).

9.4.2.5 Mechanism of Bioremediation by Microbes

We know microorganisms are omnipresent and reside in heavy metal contaminated soil. The bioremediation strategy for Heavy metals depends on the active metabolizing capabilities of microorganisms. The microbes mineralize the organic contaminants to end-products such as carbon dioxide and water which are used as substrates for cell growth. The production of degradative enzymes by the microbes for the target pollutants is one way to resist against Heavy metals. Microbes are capable of dissolving metals and reducing or oxidizing transition metals. A short summary of microbial mechanism for Heavy metals tolerance with some examples are provided in Table 9.2.

9.4.2.6 Challenges in Microbial Bioremediation

The lack of information on the cellular responses of microbes towards utilization and interaction with trace heavy metal pollutants restricts their successful execution (Boopathy 2000). Large-scale application of microbes is limited because of their requirements for extra nutrients which in turn increases the biological oxygen demand in the waste (Dixit et al. 2015). Few challenges in bioremediation by microbes has been summarized in Fig. 9.3.

9.4.3 Bioremediation of Heavy Metals Using Earthworms

As one of the most important species in soil fauna, earthworms play a major role in the functioning of the soil ecosystem (van Gestel et al. 2009). They have been

Table 9.2 Summary of microbial bioremediation mechanisms

Microorganisms	Mechanism of bioremediation	References
Endophytic bacteria	Bioremoval of Heavy metals in metal amended medium; Increased biomass, chlorophyll content, nodule number and metal accumulation	Babu et al. (2013)
Endophytic bacteria	Increased root elongation of plant; Reduced metal phyto-toxicity and increase metal accumulation	Shin et al. (2012)
Endophytic bacteria	Improved heavy metal availability in soil, shoot dry bio-mass and uptake of Heavy metals	Chen et al. (2014)
PGPR	Produce metal chelating agents termed siderophores, which are able to bind metals and thus enhance their bioavailability in the rhizosphere through a complexation reaction	Rajkumar et al. (2013)
PGPR	Decrease the level of ethylene in plants, which increases plant growth. This attributed to ACC deaminase, which hydrolyzes ACC, the biosynthetic precursor for ethylene in plants, into ammonia and α ketobutyrate	Ullah et al. (2015)
PGPR	Phosphate solubilization and nitrogen fixation which affect heavy metals mobility and availability to the plant	Gadd (2010)
Fungi	Extracellular metal sequestration and precipitation, metal binding to the fungal cell walls, intracellular sequestration and complexation, compartmentation, and volatilization	Fomina et al. (2005)
Fungi	Fungi can compete with roots and other microorganisms for water and metal uptake, protect the roots from direct interaction with the metals and impeded metal transport through increased soil hydrophobicity	Wenzel (2009)
Fungi	Fungal endophytes possess chelation systems to increase the tolerance of host plants to heavy metals	Aly et al. (2011)
Actinobacteria	Upregulation of genes to antioxidant proteins like super-oxide dismutase, alkyl hydroperoxide reductase and mycothiol reductase,	Costa et al. (2012)
Actinobacteria	Use of immobilized microbial cells provides high degra-dation efficiency and good operational stability	Ahamad and Kunhi (2011)
Actinobacteria	Production of 'Surface active compounds' which form complexes with pollutants attached to soil matrix and promote their desorption	Shafiei et al. (2014)

Heavy metals Heavy metals, *PGPR* Plant Growth Promoting Rhizobacteria, *ACC* 1-Aminocyclopropane-1-Carboxylate

described as the soil ecosystem engineers with physical, chemical and biological effects on plants and the environment (Lavelle et al. 2006). The potential use of worms in so-called vermiremediation process was recently reviewed (Rodriguez-Campos et al. 2014). Indeed, earthworms can be exploited in the process of remediation of contaminated soils due to their ability to enhance the removal of some heavy metal trace pollutants. Earthworms can survive in heavy-metal contaminated soils, can accumulate efficiently high tissue metal concentrations such as Pb, Cd, and Zn using a variety of sequestration mechanisms (Sinha et al. 2008; Andre et al. 2009). They may expose to heavy metals through their intestine and

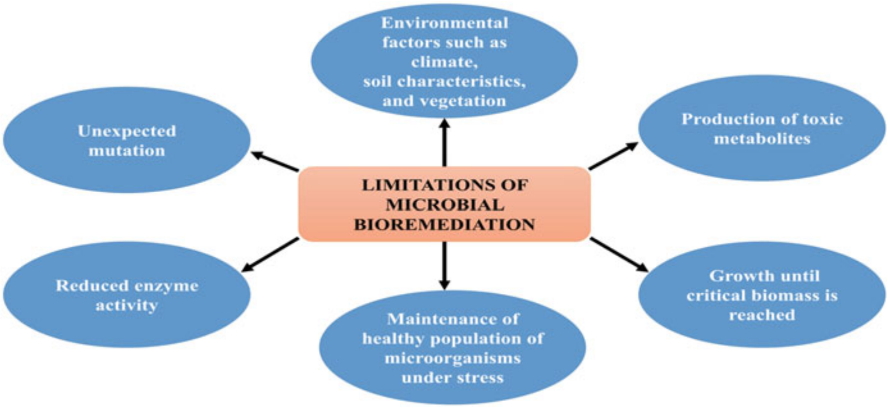

Fig. 9.3 Limitations of the microbial remediation. It is difficult to maintain the healthy condition of microbes in contaminated soil throughout as it is exposed to various environmental factors which inhibit the bacterial growth. Unexpected mutation in microbes can lead to loss of their enzymatic activity which will affect their heavy metal degrading property

skin via alimentary and dermal uptake routes (Homa et al. 2010). There are reports which indicates that earthworms reduced the concentrations of Cr, Cu, Pb and Zn in the vermicomposted sludge below the limits set by the USEPA in 60 days (Contreras-Ramos et al. 2006). Earthworms collected from the roadsides and mining sites show higher amounts of heavy metals than those from the other sites and hence can be a 'bioindicator' of heavy metal contamination in soil. The choice of the right species of earthworm and proper selection of earthworm for vermicomposting is the prime step as it affects the rate of waste stabilization. For eg. a recent report by Sizmur et al. (2011) showed that *Lumbricus terrestris* decreased water soluble Cu and As but increased the solubility of Pb and Zn in soil but at the same time, Natal-da Luz et al. (2009) did not observed an influence of *Dendrobaena veneta* on the solubility of Cr, Cu, Ni, and Zn in soil. A brief report on successful bioremediation cases of Heavy metals by earthworms is provided in Table 9.3.

Earthworm, no doubt, is beneficial candidate for bioremediation as they easily available, easy to handle and to measure the toxic parameters such as growth, reproduction and biochemical responses but taking into account the indicator role of earthworms in contaminated environments is a topic of limited practicality.

9.4.3.1 Mechanism of Vermiremediation

Earthworms ingests a large amount of different substrates and thus, concentrates Heavy metals in their body through their skin and intestine (Mohee and Soobhany 2014). Thus, vermicomposting can be used to breakdown the toxic metals into its non-toxic forms. Dia et al. (2004) suggested that bioaccumulation of metals in

Table 9.3 A report on bioremoval of heavy metals by earthworms

Earthworm species	Heavy metals uptake	References
Eisenia andrei	Body accumulation factor exceeded 1 only for Cd (17.4 4). BAFs calculated for all analyzed metals can be ranked as follows: Cd > Cu > Zn > Ni > Cr > Pb	Rorat et al. (2017)
Eisenia fetida	A slight reduction of total Pb in a binary biological system was observed with an adverse impact of Pb on the morphological parameters of the earthworms	Liu et al. (2017)
Eisenia fetida and *Metaphire guillelmi*	*M. guillelmi* accumulated more Cd than *E. fetida* but at higher doses of Cd, inverse results were obtained. This behavioural response indicates higher bioaccumulation at low-dose exposure and to the lower detoxification ability of *M. guillelmi*	Chen et al. (2017)
Eisenia fetida	Co uptake was higher than Hg which proves that Hg is more toxic to earthworms as it effects coccon production, coelomocytes, body weight and length also	Jatwani et al. (2016)
Eudrilus eugeniae	An increased concentration of Cd, Co and Ni were obtained in the tissue of the earthworms after the vermicomposting processes which showed that vermicomposting can efficiently remove heavy metals	Soobhany et al. (2015)
Metaphire posthuma and Eisenia fetida	The removal efficiency of *M. posthuma* was positive for Zn but it was negative in *E. fetida*	Sahariah et al. (2015)
Eisenia fetida	Indicated a reduction in As mobility and bioavailability in all matured composts and vermicomposts.	Maňáková et al. (2014)
Lumbricus rubellus	The heavy metals Cr, Cd and Pb contained in vermicompost of sewage sludge were lower than initial concentrations, with 90–98.7% removal	Azizi et al. (2013)
Eisenia fetida	Cu and Zn appear to be less toxic to earthworms than Cd and Pb referring to Cytochrome P450 monooxygenase activity.	Cao et al. (2012)
Eisenia fetida	Bioaccumulation of Cu and Zn within 10 weeks of experiment	Malley et al. (2006)

BAF Bioaccumulation factor

earthworms is their ability to eliminate the excess of metals. Sizmur and Hodson (2009) suggested four prime mechanisms of metal bioremoval by earthworms (Fig. 9.4).

Few reports on mechanism of vermiremediation suggested by various scientists are given below:

(a) The heavy metal accumulation in the tissue of earthworms is the result of their detritivorous lifestyle coupled with their highly permeable body walls and Chloragosomes (phosphate- sulphur rich stuctures) which function as metal sequestering organelles (Morgan et al. 2002).
(b) Some metals are taken up by earthworms and bound by a protein called 'metallothioneins (MT)' which have the capacity to bind metals. Stürzenbaum et al. (2004) found that Cd detoxification in *E. fetida* was due to

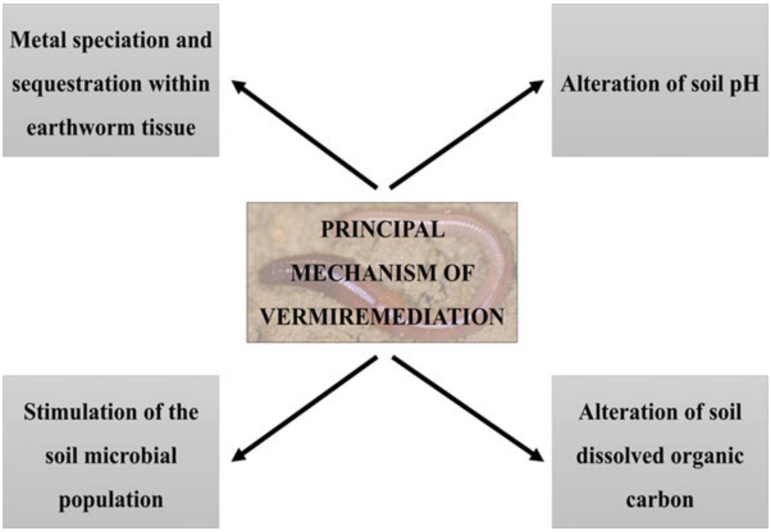

Fig. 9.4 Principal mechanism of vermiremediation. Vermiremediation is very cost-effective, environmentally sustainable way to treat heavy metals polluted soil. It lead to significant improvement in the quality of soil

compartmentalization of the metal by binding it to metallothioneins in the form of Cd-metallothioneins

(c) Wang et al. 2014 studied the relative contribution of the dermal and the gut exposure route to the uptake of heavy metals in earthworms. Using a modeling approach, it was estimated that the dermal exposure route accounted for more than 96% of the total uptake of Cd and Cu in the *E. Andrei*.

(d) Malonylaldehyde (MDA) is an important indicator of lipid peroxidation whose level increases reactive oxygen species stress (produced in response to oxidative stress). Sinhorin et al. (2014) measured decreased malonylaldehyde content in *E. fetida* under Cd stress which may be one of the mechanism to resist against heavy metal.

(e) Earthworms either bio-transform' or 'biodegrade' the contaminants rendering them harmless in their bodies. The process takes place in their gut followed metabolization, complexation and sequesteration in tissues or vacuoles (Gu et al. 2016).

(f) The worm's digestive system is capable of detaching heavy metal ions from the complex aggregates between these ions and humic substances in the waste as it rots. Various enzyme-driven process accumulate the metal ions in the worms' tissues rather than being released back into the environment. The separation of dead worms from compost is a relatively straight forward process allowing the heavy metal to be removed from the organic waste (Jatwani et al. 2016).

9.4.3.2 Challenges in Vermiremediation

Although earthworms are capable of accumulating heavy metal from the soil, it is not considered worldwide as a practical approach of enriching contaminated sludge or soils since there are evidences which indicates that progressive mineralization tends to increase the total metal concentration of metals in the substrates (Sizmur et al. 2011). Moreover, the application of metal-containing vermicomposts, to any contaminated site will inevitably introduce heavy metal into terrestrial food chain by earthworms which are significant prey organisms (Roodbergen et al. 2008). The general concept is that when earthworms are available for their predators with high concentrations of heavy metals in their tissues, the heavy metal should not get transfer to higher trophic levels and lead to biomagnification of heavy metal. There are few reports which exhibits such predator-prey phenomenon along with transfer of metals in terrestrial and aquatic food chains (DeForest et al. 2007). There are no recent cases reported on biomagnification of heavy metal from one trophic level to another via earthworms but that does not mean that the bioaccumulation of heavy metal by earthworms during vermicomposting, or during field exposure has no potentially serious ecotoxicological impacts on consumer species since earthworms can transfer metal fractions both from internal cellular compartments and alimentary canal. Future research is needed to better understand the interaction mechanism between heavy metal exposure and soil macroorganism in polluted soil.

9.4.4 Bioremediation of Heavy Metals by Algae

Accumulation of heavy metal by algae has received attention only in recent years because of its potential for application in environmental protection and recovery of some important metals (Zeraatkar et al. 2016; Malik 2004). The algal biomass may serve as an ecologically safer, cheaper and efficient means to remove heavy metal ions from waste water by biosorption process (Pohl and Schimmack 2006). The metal content of the indigenous algae can be used for biomonitoring metal pollution in a water body since the amount of metal accumulated by algae is related with the concentration of metal in water (De Filippis and Pallaghy 1994). The heavy metal uptake may depend upon the specificity of the algal strain used in the process for interaction. For eg. Monteiro et al. (2010) investigated removal of Cd ions using two strains of *Desmodesmus pleiomorphus* cells and found 25% difference between them for cadmium biosorption. Romera et al. (2007) introduced brown algae as a very good candidate for biosorbents of heavy metal ions. Alginate is one of the main constituents of the cell wall of brown algae and it is well recognized to be involved in metal accumulation (Davis et al. 2003). The phenomenon of remediation by algae can be broadly categorized in two different sets. (i) Bioaccumulation of heavy metal by living cells and biosorption by non-living cells, (ii) Macroalgae and microalgae.

9.4.4.1 Live vs. Non-living Biomass for Heavy Metal Biosorption

Heavy metal ions can be removed from wastewaters by either live cells or dead cells by the usage of inactive biomass. Lamaia et al. (2005) reported the limited sortion capacity of heavy metal ions by live cells as they were harmed by the increased heavy metal ions. The live cells are affected by many environmental factors which influence their sorption capacity. The absorption mechanism of the live cells are more complex as the intracellular heavy metal uptake occur at the growth phase where adsorption occurs whereas in dead algal cells, the heavy metal are absorbed on the surface of the cell and it is an extracellular process (Godlewska-Zyłkiewicz 2001). The non-living algal biomass is an assemblage of polymers such as sugars, cellulose, pectins, etc. which are capable of binding heavy metal ions (Volesky 2007; Arief et al. 2008). Moreover, they do not require a nutrient supply and therefore can be used for multiple sorption desorption cycles (Areco et al. 2012).

9.4.4.2 Macro Algae vs Micro Algae

The green macroalgae (seaweed) exhibit high affinity for many metal ions (Mani and Kumar 2014). The adsorption capacity of the macroalgae is directly related to the alginate content, availability and its specific macromolecular conformation. Lee and Chang (2011) tested the bioremoval capacity of two macroalgae *Spirogyra* and *Cladophora* for Pb(II) and Cu(II) and found that although the functional groups of these two genera of algae were similar but the sorption capacity of *Spirogyra* was superior to *Cladophora*.

Microalgae has gained more demand due to the development of innovative mass-production and more efficient biosorption of heavy metal ions. Minimal growth requirements (solar light and CO_2) make them suitable for bioremediation of heavy metal. Microalgae have developed an extensive spectrum of mechanisms (extracellular and intracellular) to cope with heavy metal toxicity (Kumar et al. 2015). *Spirulina* spp. and *Planothidium lanceolatum* are reported to remediate Ni and Zn (Doshi et al. 2008; Sbihi et al. 2012).

9.4.4.3 Mechanism of Algal Bioremediation

The accumulation of heavy metal ions in algae occurs in two phases (Monteiro et al. 2012). The first is a rapid passive biosorption where the metal ions adsorb onto the cell surface within a short span of time, and the process is metabolism independent. The second phase is a slower active sorption of heavy metal ions into the cytoplasm of algal cells. This phase is metabolism-dependent (Talebi et al. 2013).

The biosorption capacity for heavy metal ions has been attributed to presence of various functional groups on the algal cell surface such as hydroxyl (OH), phosphoryl (PO_3O_2), amino (NH_2), carboxyl (COOH), sulphydryl (SH), etc., which confer negative charge to the cell surface (Kaplan 2013). Since heavy metal ions are in the cationic form in water, they get adsorbed onto the algal cell surface. The functional groups are associated with various cell wall components such as peptidoglycan, teichoic acids, polysaccharides and proteins which provide metal binding sites (Kuyucak and Volesky 1988).

Other mechanisms have also been reported like complexation which is important in metal sorption by algae (Davis et al. 2003). Adhiya et al. (2002) reported that Cd biosorption to *Chlamydomonas reinhardtti* involves complexation with carboxylic groups. Electrostatic attraction and covalent binding, respectively, mediate Ni and Zn adsorption on *Chaetophora elegans* (Andrade et al. 2005). Aluminum sorption onto algal cells involves a different kind of mechanism. Aluminium (Al) ions bind to biomass in the form of polynuclear Al species and thus prevents other heavy metal ions from accessing the binding site (Bottero et al. 1980).

9.4.4.4 Challenges in Algal Bioremediation

Use of algae for biosorption of heavy metal ions from wastewaters has shown promising results but an efficient and commercially viable algal technology still need to be developed. There is a need to develop a thorough understanding of the mechanism of metal sorption. Still there are many freshwater and marine algae which has not been explored for their metal binding capacity. Therefore, screening of algae is a necessary step for selection of the best algal species with high affinity for a particular metal. The algal biomass has to be immobilized before passing wastewater through it. For this purpose, alginate is used which is an expensive chemical and thus not feasible for metal removal from wastewater always.

Although the use of inactivated algal biomass has been preferred, there are some limitations to it as well. Dead cells cannot be used where biological alteration in valency of a metal is sought. Moreover, there is no scope for biosorption improvement through mutant isolation. On the other hand, use of live cells also carries some demerits. The metal recovery might be limited since it is bound intracellularly and the metabolic extracellular products may interact with metals and retain them within the solution. However, to achieve the highest removal efficiency, interaction between algal strains, dead or live cells and pollutants should be optimized.

9.5 Integrated Approach Using Multi-biosystems for Remediation of Heavy Metals

There are many cases of heavy metal bioremediation reported using single biosystem but very few reports on biological approaches using multi-biosystems. When compared bioremediation strategies applied to polluted soils between combined and single process, it can be easily concluded that combined multiple bioremediation approaches removed much more heavy metal from the soil and highly efficient hydrophobic than each single process alone.

Bacterial consortia have gained interest of environmentalists where the ultimate aim of the bacterial mixtures system is to deliver benefits environmental applications of cleaning up the contaminants (Emenike et al. 2016). Compared with single strain, the bacterial mixtures showed higher growth rate and a considerably higher heavy metal bioremediation which might due to higher bacterial cell density at high levels of heavy metals (Kang et al. 2016).

Phytoremediation alone sometimes may not be sufficient to bring out the best result and may cause toxic effects to the plants at higher concentrations of heavy metal. Inoculation of the plant rhizosphere with microorganisms is an established route to improving phytoextraction efficiency. The plants are benefited from synergistic effects with rhizobacteria that improve plant growth and metal accumulation, mitigating the toxic effects on plants and increasing their tolerance to heavy metals (Wood et al. 2016; Sumi et al. 2015). PGPBs-legumes associations represent an alternative procedure for phytostabilisation of heavy metals polluted soils mainly generated by industrial and agricultural practices (Hao et al. 2014).

We know microorganisms are responsible for the biodegradation of heavy metal but the combination of earthworms and microbes have shown better results. Tomar and Suthar (2011) have reported a successful treatment of waswater by microbial-earthworm ecofilters as a promising economical process. The concept behind the approach is that microorganisms perform biochemical degradation of waste material while earthworms regulate microbial biomass and activity by directly or/and indirectly grazing on microorganisms (Liu et al. 2012). Earthworms have a complex digestive system in which the earthworm and microbes in the gut are mutually benefited from each other and lead to the degradation of ingested contaminants (Brown et al. 2000). However, it is difficult to differentiate between the metabolism of earthworms microorganisms which contribute to the bioremediation of heavy metal.

Algae and bacteria have coexisted ever since the early stages of evolution. They synergistically affect each other's physiology and metabolism. Many studies have dealt with algae-bacteria consortium for metal bioremediation (Boivin et al. 2007). Higher concentrations of heavy metal can cause toxic effects in algae but the consortia of algae and bacteria overcomes it and they mutually detoxify and assimilate metals from metal rich environments.

Generally, fungi are more tolerant to metals than bacteria (Kidd et al. 2009). They can proficiently explore the soil microbes which are not accessible for plant roots due to their small diameters. Fungi can compete with roots and other microorganisms for water and metal uptake, protect the roots from direct interaction with the metals and inhibit metal transport through increased soil hydrophobicity (Wenzel 2009). The endophytic fungi could increase resistance of the host plant to multimetal contamination. They can also reduce the level of growth-inhibiting stress ethylene within the plants and also provide the plants with iron from the soil. Thus, they prove to be a suitable candidate for remediation of heavy metal in combination with plants with reduced toxicity of plants under stressed condition.

Remediation of contaminated soils using earthworms and plants appears to be cost-effective and environmentally friendly technology. Wang and Li (2006) observed higher uptake of heavy metal by plants under earthworm inoculation which was probably due to the increase in dry matter production stimulated by earthworms. However, further research is needed to optimize the species combinations for suitable heavy metal uptake. A brief summary of the remediation of the heavy metal by integrated approach is demonstrated in Table 9.4.

9.6 Metagenomics

To bioremediate the heavy metal contaminated site, various biosystems are used. But very often, remediation techniques fail because of the difficulty to control and expand key biodegradative processes from bench to full scale (Fantroussi and Agathos 2005; Paerl and Steppe 2003). To get better results, a better understanding of the ecology of microbial communities inhabiting contaminated sites is needed, as well as of their interactions with the environment (Rittmann et al. 2006). But, the complete study of the microbial communities of the environment is challenging as most of them are recalcitrant to conventional cultivation (Stewart 2012). The proper management of microbial resources needs a comprehensive characterization of their genetic pool to measure the fate of contaminants and enhance bioremediation processes (Gillan et al. 2015). The emergence of metagenomics has the potential to revolutionize the overall bioremediation process as it gives direct access to microbial communities inhabiting polluted environments independently of their culturability (Bouhajja et al. 2016).

There are few main metagenomic approaches:

9.6.1 Library-Based Targeted Metagenomics

The environmental DNA is isolated from the environmental samples and cloned inside suitable host (usually *Escherichia coli*), then the clones of interest are selected based on their expression of biodegradative functions or sequence homology with probes and primers, thus establishing a metagenomic library. As host, *Escherichia coli* has been extensively used in metagenomic studies (Gabor et al. 2004) but use of multiple-host systems and broad-host-range vectors can be used to overcome the limitations of gene expression machinery or toxicity of some gene products in a single host (Cheng et al. 2014; Ekkers et al. 2012).

Table 9.4 Some examples of bioremediation of heavy metal by integrated approach

Biosystems	Reports on bioremediation on heavy metal	References
Bacterial consortia- *Bacillus* sp., *Lysinibacillus* sp. and *Rhodococcus* sp	Optimal removal of Pb, Mn and Cu in leachate-polluted soil of a land fill environment. Enhanced metabolic activity due to bioaugmentation of the microcosm using bacterial inoculums	Emenike et al. (2016)
Bacterial consortia- *Viridibacillus arenosi* B-21, *Sporosarcina soli* B-22, *Enterobacter cloacae* KJ-46, and *E. cloacae* KJ-47	Compared with single strain cultures, the bacterial mixtures demonstrated greater resistance and efficiency for the remediation of heavy metals such as Cd, Pb, Cu	Kang et al. (2016)
Plant and bacteria-*Sedum alfredii* and *Burkholderia cepacia*	Increase in the plant biomass and leading to enhanced Zn and Cd uptake	Li et al. (2007)
Plant and bacteria-*Brassica juncea* and *Bacillus* spp.	Increase in the plant dry weight with an increase in Cd uptake	Jeong et al. (2013)
Plant and bacteria-*Vicia faba*, *Lens culinaris* and *Sulla coronaria* co-inoculated with *Enterobacter cloacae, Pseudomonas sp.* and *Rhizobium sullea*	Inoculations decreased heavy metals (Cu and Pb) availability in the soil indicating a positive effect of co-inoculation of legumes by appropriate heavy metals resistant bacteria for the phytostabilisation of mine tailings	Saadani et al. (2016)
Plant and bacteria-*Lepidium sativum* and *Azotobacter*	Stimulate the plant growth and enhance its tolerance to Cr(VI) and Cd(II), to ultimately provide a reliable phytoremediation system.	Sobariu et al. (2016)
Fungi and plant-*Trichoderma atroviride* and *Brassica juncea*	Significantly alleviates the cellular toxicity of Cdand Ni from contaminated soil	Cao et al. (2008)
Fungi and plant- *Cryptococcus sp.* (yeast), *Rhodotorula sp.* and *B. chinensis*	Fungi helps in plant growth in multi-metal contaminated soils and give resistance to Cd, Pb, Zn, and Cu	Deng et al. (2012) and Wang et al. (2013)
Fungi and plant-*Microsphaeropsis sp.* and *Solanum nigrum*	Shows enhanced Cd biosorption capacity	Xiao et al. (2010)
Fungi consortia-*Mucor sp.* and *Fusarium sp.*	Increased metal concentrations in the canola (Cd, Pb, and Zn), elevated the extractable metal amount, and increased metal translocation from roots to shoots	Deng et al. (2014)
Plant and Earthworm-*Vicia faba, Zea mays*, and *Eisenia fetida*	Earthworms and plants increased the uptake of metals (Pb, Cd and Zn) from contaminated soils. The earthworm-plant-soil interaction influence both the health of the plant and the uptake of heavy metals by plants	Lemtiri et al. (2016)

(continued)

Table 9.4 (continued)

Biosystems	Reports on bioremediation on heavy metal	References
Plant and Earthworm-*Lantana camara* and *Pontoscolex corethrurus*	Interaction between earthworm and plant have a positive effect on Pb-phytoextraction yield and was significantly correlated with the increase in total microbial activity and richness index of the fungal community	Jusselme et al. (2015)

9.6.2 Direct Sequencing of Metagenomes

It does not involve a cloning step and has been more often applied to polluted environments for characterization of the taxonomic and functional composition of microbial communities and their dynamics. The analysis has focused on 16S rRNA genes and marker genes of biodegradation.

9.7 Next Generation Sequencing

Next Generation Sequencing (NGS) was introduced in 2005 (Margulies et al. 2005).There has been a remarkable increase in metagenomic studies based on NGS. It includes immense parallel sequencing of clonally amplified or single DNA molecules spatially separated in a flow cell (van Dijk et al. 2014).

Lastly, the huge amount of data generated by metagenomic studies is analyzed using bioinformatic tools to predict the microbial diversity, enhance the discovery and characterization of unknown bacterial and fungal metabolic pathways involved in the degradation of hazardous pollutants. Even though metagenomics is having some technical and computational challenges, the positive claims of it can be used to efficiently monitor the clean-up process of the environment and mitigate the effects of the pollutants on the eco-system.

9.8 Metabolomics

The main challenge faced by plants growing under heavy metal stressed condition is biomass reduction, nutrient deficiency aided with increased toxicity of heavy metal. Plants are considered to biosynthesize specialized (primary and secondary) metabolites to adapt to the environmental stresses (Auge et al. 2014).

Metabolomics is a newly emerging discipline which can serve to analyze the whole set of small molecular weight chemical compounds (<1000 Da) in organism (Ji et al. 2015; Watanabe et al. 2015). It provides a glimpse of dynamic changes in metabolic pathways in the host plant regulated by microbial population and their response to highly dynamic environmental conditions in their unique ecological niches. This field is coupled with functional genomics to understand biochemical phenotypes across a range of biological systems. Metabolomics measures all metabolites at a specific time point, reflecting a snapshot of all the regulatory events responding to the external environmental conditions (Kumar et al. 2016). The metabolites reflect the true integration of gene regulation and protein expression incorporating the impact of the environment and other organisms. The metabolites fate can be employed as bioindicators to monitor the biological effects of the pollutants on living organism and help in better understanding of the environment (Tomanek 2014).

Recent developments in analytical instrumentation and bioinformatics tools has led to evaluate numerous plant metabolites, metabolic changes and finally elucidate metabolic pathways responsible for heavy metal tolerance to plants (Obata and Fernie 2012). Current studies are mostly restricted to targeted metabolomics, which focuses on amino acid and/or lipid metabolism (Kumari et al. 2015; Melo et al. 2015).

9.8.1 Various Metabolomic Platforms to Identify Metabolites

The main strategies engaged to analyse the metabolome of plants include (i) metabolite profiling; (ii) targeted analysis; and (iii) metabolic fingerprinting (Hill and Roessner 2013). Metabolite profiling is a semi-quantitative which allows for detection of a large set of both known and unknown metabolites. Target analysis is an absolute quantitative approach which detects metabolites involved in a particular pathway by utilizing specialized protocols and detection techniques. Finally, metabolic fingerprinting is the highest throughput procedure and generates fingerprints characterizing a specific metabolic state of a sample by non-specific and rapid analysis of crude metabolite mixtures.

Without adequate knowledge of the metabolites under stressful conditions, a targeted metabolomic approach possess a high risk of missing significant changes in the metabolome. In order to achieve desired results, there is a need to expand beyond the known targets that can only be accomplished with non-targeted, unbiased metabolomics also known as global metabolomics (Kueger et al. 2012). Global metabolomics provides a panoramic view covering both primary (including sugars, amino acids and tricarboxylic acids involved in primary metabolic processes such as respiration and photosynthesis) and secondary metabolites (including alkaloids, phenolics, steroids, lignins, tannins, etc.) in a single run and has advantages of uncovering many novel compounds.

9.8.2 Analytical Platform to Analyze the Metabolites

A range of analytical platforms have been established which includes nuclear magnetic resonance (NMR), Fourier transform ion cyclotron resonance mass spectrometry (FT-ICRMS) and mass spectrometry (MS). MS-based metabolomics combines chromatographic separation with mass spectra and are available in multiple forms such as liquid chromatography (LC –MS), gas chromatography (GC –MS), capillary electrophoresis (CE –MS) and matrix-assisted laser desorption/ionization (MALDI-MS). However, due to high grade of molecular weight and structural diversity between primary and secondary metabolites, a single platform is not sufficient to indentify and quantify the metabolites (Kueger et al. 2012). Therefore, a combination of different techniques will reveal a vast metabolite profile. However, investigations have demonstrated 1H NMR as efficient approach for detection of the metabolites released in responses to metal pollutants whereas, MS-based analytical approaches are preferred to investigate plant responses to environmental cues due to its sensitivity to low abundant molecules and the flexibility for detecting multiple classes of molecules (Hill and Roessner 2013).

9.8.3 Bioinformatics Tools

The vast amount of metabolic data generated need to be archived, managed and integrated for metabolic analysis. So, various bioinformatics tools are designed for processing of raw data, mining, statistical analysis, management and mathematical modelling of metabolomic networks. A range of bioinformatics tools for effective insilico data pre- processing have been designed for this purpose including Analyzer Pro, Automated Mass Spectral Deconvolution and Identification system, and many more (Fukushima and Kusano 2013).

Though metabolomics is a relatively new approach in plant biology, it can be combined with other ‘omics’ disciplines turning out to be a major tool in revealing new knowledge on diverse metabolites produced by plants to heavy metal contaminants, and also on their metabolomic reprogramming for acclimation to extreme perturbations.

9.9 Proteomics

It is very important to understand why a particular metal at a certain concentration can alter from non-toxic to toxic form for other species at a slightly higher concentration (Ge et al. 2009; Vido et al. 2001). In the past years, substantial improvements in protein separation and identification techniques have opened the application of proteomic methods to answer the biological questions along with metagenomics and metabolomics methods (Isaacson et al. 2006). The heavy metal uptake process across a number of unrelated plant species appears to be associated with proteins involved in energy metabolism, the oxidative stress response and abiotic and biotic stress (Visioli and Marmiroli 2013). Examining the toxic effects of heavy metals on protein expression can be useful for gaining insight into the biomolecular mechanisms of toxicity and for identifying potential candidate metal-specific protein markers of exposure and response (Luque-Garcia et al. 2011). Proteomics, an important omic approach facilitates both identification and quantification of differentially expressed proteins. Moreover, the identification of post-transcriptionally regulated array of functionally diverse genes playing a key role in conferring resistance towards stress has also been advanced (Zargar et al. 2017). Proteomic data supplement the huge genomic and transcriptomic data sets in providing a clear picture of the process and thus helps in determination of major genetic determinants of the hyperaccumulation phenomenon (Visioli and Marmiroli 2013). Research analysis has depicted that proteomics in union with bioinformatic tools, can facilitate the discovery of new and better biomarkers of heavy metal toxicity (Zhai et al. 2005).

The current state of knowledge regarding the proteomics of hyperaccumulation is inadequate to understand the role of the large number of proteins involved and the level of cross-talk between different pathways (Visioli and Marmiroli 2013). Few proteomic methodologies appropriate for the identification of key regulators of hyperaccumulation are as follows.

9.9.1 Gel and Non-gel Approaches

For most of the plant proteomics studies, pre-fractionation of the sample prior to mass spectometry (MS) analysis is carried out which can be achieved by gel electrophoresis or by certain gel-free techniques. 2D-Gel electrophoresis (2D-GE) has become the optimum choice for separating complex protein mixtures with respect to achievable resolution and reproducibility (Rose et al. 2004). However, there are certain drawbacks such as limited capacity to fractionate hydrophobic proteins and glycoproteins successfully, detection of small peptide molecules and the risk of quantification (Visioli and Marmiroli 2013). Generally, the reproducibility of LC-based separation is better than that achieved by 2D-GE which is an important advantage for comparative proteomics (Lambert et al. 2005; Pirondini et al. 2006). There are various statistical packages which facilitate semi-quantitative proteomics such as Progenesis (Nonlinear Dynamics), ImageMaster 2D Platinum (Ge Healthcare, Amersham Biosciences) and PDQuest (Bio-Rad).

9.9.2 Mass Spectometry-Based Quantification

The introduction of MS technology has widely enhanced the throughput of proteomic data compared to electrophoretic or chromatographic methods and provides a more reliable characterization of all the protein species along with identification of post-translational modifications such as phosphorylation and acetylation, which are important in cell signalling and various epigenetic phenomena (Bantscheff et al. 2012).

9.10 Conclusion

Heavy metal contamination has taken a serious turn leading to devastating effects on environment and human health. Compared to the complexity and time consumption involved in the conventional methods for remediation of soil, bioremediation techniques has proven to be the best alternative techniques where in addition to bioremoval of heavy metal, it also replenishes the site and maintain the ecological balance of the environment. Plants are the most widely accepted bio-tool for remediation of soil. But the traditional phytoremediation approaches are less economical because the hyperaccumulators are generally slow growing and have less biomass production. Earthworms, being the soil organism, leads to significant improvement in the quality of soil and assist in heavy metal bioremoval in their biomass but higher concentrations of heavy metal produce toxic effects in earthworms. The bioremediation capacity of the algae and fungi have been studied extensively and has been effective remediators in many cases. Use of microbes has arisen as the savior for bioremediation. Recently, the integrated approach of using more than one organism for bioremediation has gained popularity as it helps to overcome the drawback of a single biosystem. Moreover, the symbiosis relation between has resulted in high performances such as more metal accumulation, high biomass production and well adapted to variety of climatic conditions, therefore driving us towards a sustainable environment. A successful bioremediation strategy require a detailed understanding of the functioning of degradative microbial communities which is quite a challenge for microbiologists. Thus, metagenomics, metabolomics and proteomics have come into play and has become the major tool for identification of all the unexplored microbial communities possessing the ability to degrade heavy metal and identification of the diverse metabolites produced by organism to tolerate under stress conditions. Thus, coupling both the 'omics' will give a comprehensive understanding of the microbial communities and their biodegradation pathways.

Moreover, in order to achieve even better results for bioremediation, certain points have to be considered as follows:

(a) The exploitation of the floral diversity should be extended for obtaining the effective hyperaccumulator plant which can maintain effective rate of heavy metal uptake throughout. Prior comprehensive risk assessment studies should be carried out to protect the local plant diversity.
(b) Studies need to be conducted to have a better understanding of the interactions between heavy metal, soil, microbe, earthworm and plant roots to comprehend the fate of metal ions in the soil.
(c) More research is needed to obtain effective and environmentally safe chemicals which can increase the metal solubility in soil and thus, enhanced the bioavailability of metals to the plant roots.
(d) In spite of all the advances, most of the research is still limited to laboratory scale studies. Long-term in-situ field trails are actually required for to prove the efficacy of the strategy in real-contaminated area.
(e) More sophisticated bioinformatics tools should be developed to reconstruct full length metabolic and catabolic pathways. More studies have to carried out opting an integrated approach using the 'omics' tool together for better insights. Thus, it can provide a practical implementation of large-scale application of bioremediation.

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