Bioremediation of mercury: not properly exploited in contaminated soils !

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Abstract

Contamination of land and water caused by heavy metal mercury (Hg) poses a serious threat to biota worldwide. The seriousness of toxicity of this neurotoxin is characterized by its ability to augment in food chains and bind to thiol groups in living tissue. Therefore, different remediation approaches have been implemented to rehabilitate Hg contaminated sites. Bioremediation is considered as cheaper and greener technology than the conventional physico-chemical means. Large scale use of Hg volatilizing bacteria are used to clean up Hg contaminated waters, but there is no such approach to remediate Hg contaminated soils. This review focuses on recent uses of Hg resistant bacteria in bioremediation of mercury contaminated sites, limitation and advantages of this approach and identifies the gaps in existing research.

Key words: Soil, water, monitoing, *mer* operon.

Introduction

Mercury (Hg) is a global threat to human and environmental health because of its toxicity, mobility and long residence time in the atmosphere. This metallic element has been ranked 3rd in the "priority list of hazardous subtances" by the Agency for Toxic Substances and Disease Registry (ATSDR 2015). Recent reports show that the majority of global Hg is released by natural processes such as oceanic emission and biomass burning (combustion of organic substances) whereas the other significant portion is released due to human activities predominantly by mining, metal manufacturing and fossil fuel burning (Nelson et al. 2012; Pirrone et al. 2010; Serrano et al. 2013).

In both terrestrial and aquatic systems, Hg exists in elemental, inorganic, and organic forms. Inorganic Hg has two valences, +1 and +2, mostly found as salts (Wang et al. 2004). Hg with valence +2 is more widely spread in the environment. In anaerobic sediments and water logged soils, methylation is the most toxic tansformation which resluts in formation of organic - monomethyl or dimehtyl Hg (MeHg), which are neurotoxins. Due to inherent toxicity of both inorganic and organic forms of Hg, the US Environmental Protection Agency (EPA) recommends a limit of 2.0 μg L^{-1} in water (EPA 2016). In soils, these recommended precautionary Hg limits vary in different industrial countries from 6.6 to 3600 mg kg^{-1}, depending on the land use (Mahbub et al. 2016c). Generally, the average background concentration of Hg in soil ranges from 0.03 to 0.1 mg kg^{-1} with an average value of 0.06 mg kg^{-1} (Wang et al. 2012). Recent reports shows that Hg can exert deleterious effects on soil health at concentrations even lower than current recommended safe limits, sometimes at background concentrations (de Vries et al. 2007; Mahbub et al. 2016b; Mahbub et al. 2016f; Tipping et al. 2010), which warrants more effective remediation technologies.

Being considered as the cheapest and most environmentally friendly technology, the application of bioremediation for cleaning up Hg from polluted areas has been applied widely in treating Hg loaded waste waters. There are many examples of successful pilot scale applications of Hg volatilizing bacteria for the removal of Hg from contaminated industrial waters (Velásquez-Riaño and Benavides-Otaya 2015; Wagner-Döbler 2003; Wagner-Döbler 2013). The aim of the present work was to review current knowledge (a total of 793 articles recovered using a Scopus search between the years 2000 – 2016) on the Hg remediation with an emphasis on the bioremediation technologies in soil and their potential use in the detoxification of Hg contamination. We have identified that although Hg resistant (HgR) bacteria are ubiquitous in terrestrial environments, there is almomst no knowledge about the application of HgR microorganisms as bio-control agents for remediating Hg contaminated soils.

The mercury cycle in the environment

Most of the mercury released to the atmosphere is gaseous elemental Hg^0 which can travel a long distance from its origin for 6 – 12 months before becoming deposited into aquatic or terrestrial environments. During atmospheric travel, the elemental Hg^0 is oxidized to highly soluble toxic divalent Hg^{2+} by atmospheric oxidants such as bromine, ozone, HClO, HSO^{3-}, OH in fog and cloud droplets (Munthe 1992; Munthe and McElroy 1992). The oxidized Hg (Hg^{2+}) subsequently accumulates in aquatic and terrestrial bodies. A small portion of atmospheric Hg^{2+} is reduced in the atmosphere by the reductant SO^{3-} or by photo-reduction to $Hg(OH)_2$ (Ariya et al. 2015; Munthe et al. 1991). Some of the deposited oxidized Hg^{2+} is reduced to Hg^0 and goes back to the

atmosphere. The major portion cycles through soils and waters, becoming transformed to more toxic organic forms and subsequently intoxicates organisms and concentrates up the food chain (Amos et al. 2013). The overall process of emission of Hg and its transformation in the environment is depicted in Figure 1.

The accumulation of oxidized Hg^{2+} from the atmosphere to soil (60%) and waters (30%) occurs mainly by wet deposition (Mason et al. 1994). In oceanic waters, Hg^{2+} undergoes a series of chemical and biological reactions which leads to volatilization of a major portion of Hg to the atmosphere; whereas a small amount is taken into the sediments. In terrestrial bodies, a smaller portion of Hg returns to the atmosphere in a reduced form and the major portion becomes permanently accumulated in soils. Mercury resistant microbial communities with a *mer* operon can produce a mercuric reductase enzyme which reduces soil Hg^{2+} to volatile less soluble Hg^0 that returns to the atmosphere. In soil, a major portion of Hg is bound to soil organic matter (SOM), sulphide anions, soil minerals and clay particles (Mahbub et al. 2016b; Skyllberg 2012; Tazisong et al. 2012). In low pH soils Hg^{2+} is mainly complexed to the SOM, whereas in neutral to alkaline soils mineral components also offer complexation. Fulvic acid and humic acids play important roles in the complexation of Hg in soil (Dunham-Cheatham et al. 2015). The complexation of Hg^{2+} mainly acts through the C=O, COO^-, and O-H groups of organic matter (Ma et al. 2015). Therefore only a negligible amount of Hg (0.00001 to 1.5% of total Hg) is available in soil solution (Mahbub et al. 2016b) to be transported into resistant microbial cells and subsequent volatilization as Hg^0. Hence most of the soil Hg accumulates and increases Hg load in terrestrial bodies which can subsequently transfer to the food chain. Apart from reduction and subsequent volatilization, methylation and formation of HgS are also evident in soil environments. Monomethyl Hg formation is favoured in low pH soil and dimethyl Hg formation is facilitated in neutral to alkaline soil (Stein et al. 1996). Formation of HgS is common in sulfidic soils which is less mobile and a less reactive form of Hg as a result of adsorption to iron sulphide and pyrite (Stein et al. 1996). Hg can also strongly complex with reduced sulphur groups in SOM in highly aerobic environments (Skyllberg et al. 2006). In addition to chemical reactions, HgS can also be produced aerobically under controlled pH as a result of microbial activity (Kelly et al. 2006; Kelly et al. 2007; Lefebvre et al. 2007).

The most important transformation of Hg in anoxic aquatic sediments is methylation which requires transfer of methyl ion (CH_3^-) by anaerobic sulphate reducing bacteria (SRB). The biotic methylation of Hg is the predominant mechanism for transformation but there is some evidence for abiotic transformation (Barkay and Wagner-Döbler 2005; Celo et al. 2006; Fitzgerald and Lamborg 2007; Fleming et al. 2006; Gårdfeldt et al. 2003). The physicochemical characteristics of water bodies (pH, ligands, sulphates, nutrients) and impact from anthropogenic activities play major roles in the formation of methyl Hg (CH_3Hg^+) and maintaining the relative proportion of Hg^{2+} and CH_3Hg^+ in aquatic environments. Methyl Hg (MeHg) is the only form of Hg which is augmented in the food chain (Celo et al. 2006) as a thiolate complex (Harris et al. 2003), where it represents 95% of total Hg in the top predators of a food chain (Celo et al. 2006). Demethylation of MeHg is another important transformation process. Reductive demethylation converts MeHg to Hg^0 where it is volatilized to the atmosphere; the reduction reaction is governed by Hg resistant anaerobic bacteria which produces organomercury lyase (OL) enzyme in Hg rich conditions. On the other hand oxidative demethylation produces Hg^{2+} in low Hg containing anaerobic environments, which then serves as a substrate for methylation. Photo-oxidation plays a major role in oxidative demethylation in low MeHg contaminated waters, whereas when

MeHg contamination is high in sediments, microbial methylation plays the dominant role. The photolytic demethylation rate (due to formation of singlet oxygen generated by sunlight falling on dissolved organic matter) is faster when the MeHg species are bound to dissolved organics such as sulphur containing ligands in fresh waters, rather than inorganic bound MeHg found in marine waters (Zhang and Hsu-Kim 2010).

Traditional approaches for mercury remediation from soil and water

The primary concern of industries and regulatory agencies is to remediate Hg polluted soils and waters and reduce any potential risks of toxicity. Unlike organic pollutants, Hg cannot be mineralized. Therefore transformation of the toxic ionic and organic forms to less toxic or less reactive species such as elemental Hg or Hg sulphides (which are not accumulated into food chain) is becoming an essential approach for remediating Hg contaminated sites. Recent approaches for Hg remediation are summarised in Table 1. The traditional physico-chemical processes of Hg remediation can produce large volumes of Hg-loaded biomass, the disposal of which is not always environmentally friendly and may be expensive (Wagner-Döbler 2013). These are briefly described in the following sections.

Treatment technologies for water

Precipitation is the most common technology for remediating Hg contaminated ground water and waste water. The principle of precipitation is to mix a chemical precipitant (commonly sodium sulphide in the case of Hg) into the water, coagulating the soluble form of inorganic Hg to insoluble HgS under controlled neutral to alkaline pH condition (Findlay and McLean 1979; Hansen and Stevens 1993; O'rear et al. 2015). The precipitated HgS is later separated by filtration or clarification. Another precipitation approach uses lignin derivatives to form lignin-Hg complexes which are removed by gravity settling in a clarifier. The disadvantages of precipitation approaches are that the precipitated sludge may be hazardous, requiring further solidification/stabilization treatment prior to disposal. Excessive use of sulphides can form soluble HgS_2 species which can leach into ground water from disposed sludge (USEPA 1997).

Hg is also removed from water by adsorption onto granular activated carbon or sulphur-impregnated activated carbon and functionalized multiwall carbon nanotubes which are packed on a column through which contaminated water is passed (Asasian et al. 2012; Hadavifar et al. 2014; Musmarra et al. 2013). Prior to adsorption, pre-treatment technologies such as flocculation, precipitation, settling, and filtration may be required. Fouling and plugging caused by suspended solids, dissolved organic compounds and biological growth are limitations of this approach.

Microfiltration and ultrafiltration have been used on small scales to physically separate Hg after it has been precipitated from wastewaters (Urgun-Demirtas et al. 2012). This approach has disadvantages similar to adsorption when suspended solids, organic compounds, colloids, and other contaminants can cause membrane fouling.

Apart from these physical separation methods, bioremediation of Hg has been successfully implemented to remove Hg^{2+} from contaminated waters. Bioremediation is mainly based on two approaches – microbial volatilization and bio-sorption (Wagner-Döbler 2003). Microbial volatilization utilizes activities of a number of

genes in the bacterial "*mer*" operon which transports Hg^{2+} and organic MeHg into the bacterial cytoplasm where it is then reduced to elemental Hg^0 by mercuric reductase enzyme (MerA) and subsequently volatilised from the cell. (Mahbub et al. 2016a; Santos-Gandelman et al. 2014). Although *merA* is the dominant pathway for volatilization, *mer* independent volatilization is also evident in some studies (Wiatrowski et al. 2006a).

Live or dead microbial biomass from bacteria, fungi or algae has been used for bio-sorption of volatilized Hg^0 to restrict it from re-exposure to the atmosphere (Ahluwalia and Goyal 2007; François et al. 2012). This is achieved by designing a packed bed bioreactor where Hg resistant bacterial biofilm is grown on porous carrier material to trap Hg^0 produced from microbial reduction reactions (Wagner-Döbler 2003). Under certain conditions some resistant bacteria can secrete exo-polymers that adsorb Hg^{2+} (François et al. 2012). Precipitation of Hg^{2+} as insoluble HgS (cinnabar) is a potential bioremediation technology under aerobic conditions but not under anaerobic conditions, because in anoxic environment the precipitated HgS is taken up by SRB and methylated (Lefebvre et al. 2007). Since the microbial reduction of Hg^{2+} to Hg^0 is an energy driven metabolic process, continuous nutrient feeding and maintaining optimal conditions for microbial growth is necessary. Moreover, high concentration of contaminants may inhibit microbial activity and the bioreactor effluent normally requires further precipitation treatment. Despite some limitations, this technology has been considered as a cheaper and greener technology compared to previously described technology to clean up Hg contaminated waste waters (Wagner-Döbler 2013).

Treatment technologies for soil

For remediating Hg contaminated soils, common strategies currently in use include –

- extraction of Hg from soil to lower the bioavailable portion within the soil,
- immobilization of reactive forms of Hg by encapsulation to reduce its mobility in soil,
- thermal treatment to volatilize as elemental Hg and
- vitrification (immobilization of Hg containing waste into a glass matrix)

Physical separation of Hg from soil by soil washing is a widely used approach which sometimes combines chemical extraction (with acid or alkali and chelating agents) when Hg is strongly bound to soil organics and when the soil clay content is 30 to 50% and the Hg content is more than 260 mg/kg (Dermont et al. 2008b; Wang et al. 2012). This technology is easily applied and has been established in several industries, but the cost of chemicals increases processing costs. Moreover, strongly complexed Hg is difficult to remove in this process requiring longer processing times and multiple processing steps, and soil cations may interfere with the extraction process. Unfortunately, soil washing generates a large volume of Hg containing waste water which is difficult to recycle (Abumaizar and Smith 1999).

Hg can be stabilized and encapsulated in a rigid and durable matrix (Cho et al. 2014a; López et al. 2015). Stabilization/solidification is the most utilised *in situ* approach for remediation of Hg contaminated soils where the Hg load is less than 260 mg/kg (Wang et al. 2012). This process can decrease the bioavailable portion of soil Hg and slow the release of Hg to surface and ground waters. Phosphates, lime, fly ashes, alumino-silicates, powder re-activated carbon, ceramics and sulphur polymer are widely used stabilizing agents (Cho et al. 2014b; López et al. 2015; Zhang et al. 2015). This strategy is common in the USA and there is no risk of secondary

waste; but leachability, increased volume of the treated material, interference by soil organic matter and long-term monitoring are the limiting factors (Guo et al. 2011).

Another important *in situ* technology is immobilization of Hg by sulphur containing ligands, reducing agents and absorbing agents that decreases mobility, toxicity and solubility of reactive forms of Hg in soil (Bower et al. 2008; Kot et al. 2007). Adding reduced sulphur to Hg containing soil is a method to precipitate HgS - which is relatively insoluble and less volatile than other forms of Hg. Soil contaminated with 2300 mg/kg Hg has been treated by this method (Piao and Bishop 2006). Field scale use of this approach has been successfully applied (Zhuang et al. 2004). The advantages of this approach are that the remediated soil can be re-vegetated and the approach is applicable to large sites. But the amount of HgS loaded in the soil (which may serve as substrate for methylation) and long term monitoring are disadvantages of this approach.

High temperature with reduced pressure has been employed as thermal treatment to volatilize Hg from soil and to condense the Hg vapour to liquid form (Busto et al. 2011; Ma et al. 2014a) but this process is not suitable for organic or clay rich soil, the capital cost for maintaining this approach is very high, and the treated soil is not suitable for agricultural re-use as the high temperature alters soil quality (Dermont et al. 2008a). Moreover, hazardous gas produced from the process requires further treatment (Mulligan et al. 2001). Vitrification has also been used to immobilize soil bound Hg mainly in organic rich soils *in situ* and *ex situ*. This approach is not cost effective for soils with excessive organic content, high moisture, high metal content and halogens (USEPA 2007). Moreover, these two approaches are still at the experimental stage for field use.

Other than these physico-chemical approaches, biological methods such as phytoremediation has recently been introduced to remediate Hg from contaminated soils. Phytoremediation works in three ways – phyto-stabilization, phyto-extraction and phyto-volatilization (Tangahu et al. 2011). For example, it has been demonstrated that willow species stabilize Hg by adsorption and accumulation in the root system which inhibits the level of bioavailable Hg in the rhizosphere (Wang et al. 2005). Certain plant species such as *Polypogon monospeliensis, Brassica juncea, Pteris vittata* can accumulate Hg from contaminated soils. These plants accumulate Hg in their roots and shoots which are subsequently harvested, removed to an isolated area and then incinerated (Su et al. 2008; Su et al. 2007). However, recent studies show that the efficiency of Hg sequestration in plant is low because it is restricted to only leached and bioavailable Hg (Pant et al. 2010). Therefore, some strategies like compost amendment have been introduced recently to increase soluble Hg portion in soil which would be subjected to phyto-extraction (Smolinska 2015).

Additional approaches have included the modification of plants such as *Oryza sativa* with Hg reductase gene (*merA*) from bacteria. These genetically engineered plants were observed to reduce ionic Hg to less toxic elemental Hg which was subsequently volatilized (Heaton et al. 2003) leading to a secondary pollution problem. The future application of phytoremediation of Hg is limited by the scarcity of suitable hyper-accumulator resistant plant species, and the disposal of contaminated plant biomass (Xu et al. 2015).

Although microbial volatilization has been successfully applied to remediate Hg contaminated waters, there is no evidence of small or large scale utilization of this approach to clean up contaminated soils except our recent study (Mahbub et al. 2016f), where successful removal of approximately 60% of soil bound Hg from a contaminated site was achieved with bio-augmentation and nutrient amendment. The study also demonstrated better growth of lettuce and cucumber in the bio-augmented soils. However, the application of bio-augmentation is limited in soil due to some or all of the following issues:

- poor bioavailability of Hg in soil,
- presence of mixed contaminants which may interfere with the metabolic activity of Hg resistant microorganisms,
- inadequate supply of nutrients and
- poor biochemical potential for effective bioremediation (USEPA 2007).

Importance of bacterial *mer* operon in bioremediation of mercury

Functions of *mer* operon

Hg resistant bacterial species contain a cytoplasmic enzyme "mercuric reductase", encoded by the *mer* operon which reduces soluble Hg^{2+} to insoluble elemental Hg^0 (Adeniji 2004) which subsequently diffuses from the cell (Wagner-Döbler 2003). Volatilization of Hg from the bacterial cell is a well-known resistance mechanism attributed to the genetic determinant, the *mer* operon (Felske et al. 2003; Nies 1999; Summers and Lewis 1973) The *mer* operon has been found in a wide range of Gram-negative and Gram-positive bacteria (Dash and Das 2012) where it can be located on plasmids (Brown 1985; Griffin et al. 1987; Rådström et al. 1994), chromosomes (Inoue et al. 1991; Mahbub et al. 2016e), transposons (Kholodii et al. 1993) or integrons (Liebert et al. 1999). A number of bacteria have been reported to have the *mer* operon system, including *Shigella flexneri, Pseudomonas aeruginosa, P. putida, P. stutzeri, P. fluorescence, Klebsiella pneumonia, Morganella morgani, Xanthomonas, Achromobacter, Acinatobacter calcoaceticus, Serratia marcesense, Mycobacterium marinum, Staphylococcus aureus, Bacillus* sp., *Enterobacter*, *Sphingobium* sp., *Sphingopyxis* sp. *Luteimonas* sp., *Psychrobacter* sp. (Cabral et al. 2012; Chien et al. 2012; Giri et al. 2014; Mahbub et al. 2016a; Mahbub et al. 2016d; Pepi et al. 2013; Singh et al. 2011; Sinha and Khare 2012; Sinha et al. 2012). The presence of the *mer* operon has also been detected in thermophilic bacteria and archaea such as *Brevibacillus* sp., *Anoxybacillus* sp. and *Geobacillus kaustophilus* isolated from Hg rich geothermal springs and deep ocean (Barkay et al. 2010; Sar et al. 2013).

The *mer* operon is probably of ancient evolutionary origin and it is highly conserved in bacteria (Wang et al. 2004). There are two types of *mer* determinants; narrow-spectrum and broad-spectrum. The narrow-spectrum *mer* determinant confers tolerance to inorganic Hg only whereas the broad spectrum *mer* determinant is for resistance to both organic and inorganic forms of Hg (Bogdanova et al. 1998; Misra et al. 1984; Silver and Phung 1996). This is a positively regulated operon that consists of

- operator and promotor region, encodes specific regulatory protein MerR,

- uptake proteins at the downstream of operator-promotor region (translated by structural genes) namely MerT, MerP and MerC, MerF, MerG, MerE,
- reduction enzyme MerA and
- lyase enzyme MerB in broad spectrum resistant organisms (Table 2).

The transcription of the "*mer*" operon is suppressed if no Hg is present because the repressor MerR binds to the promoter region and inhibits transcription. When Hg is available in inducible concentrations, it binds to the MerR repressor and releases it from the promoter and transcription begins. At the protein sequence level, MerR's closest homolog is ZntR, the regulator of Zn^{2+} homeostasis in many bacteria (Summers 1986). Another regulatory gene in the *mer* operon, *merD*, encodes a protein that in small concentrations is antagonistic to MerR by competing for the promotor-operator region through weak binding (Nucifora et al. 1989).

Although Hg can get into bacterial cells at the pico-molar level without any transport proteins, there is a dedicated specific Hg transport machinery which utilises the *merT*, *merP*, *merC*, *merF* and *merE* genes (Nascimento and Chartone-Souza 2003). Hg binds to the periplasmic space with MerP and is then transported across the membrane using proteins encoded by the *merT* or *merF* genes. Both *merT* and *merP* are required for full expression of Hg resistance, but loss of *merP* is less deleterious than loss of *merT*. In contrast, mutating *merC* has no effect on Hg^{2+} resistance. In the cytosol, Hg^{2+} is transferred from MerT to MerA.

Within the *mer* operon the *merA* gene is of greatest significance being translated into a mercuric reductase, which catalyses the NADPH dependent reduction of thiol-avid Hg^{2+} to Hg^0 and expels toxic Hg out of the cell. The broad spectrum *mer* operon containing *merB* encodes the organomercurial lyase enzyme which breaks the carbon-Hg covalent bond present in organic Hg transported into the cell by MerE or MerG activity to produce Hg^{2+} (Curran and Franza Jr 1991; Summer and Silver 1978; Wang et al. 2004) which is then reduced to Hg^0 by the enzyme mercuric reductase (*merA*) with NADPH, –SH compounds and FAD (Schottel 1978). The enzymatic reaction takes place within minutes. The reduced Hg diffuses from the cell and can readily be volatilized. This volatilized Hg either can be retained in a packed bed bioreactor consisting of inert porous carrier material such as siran, pumice, synthetic fibres, activated carbon, wood chips, cellulose fibres (Nascimento and Chartone-Souza 2003) or is trapped in the remediating microorganisms intra or extracellularly either by bioaccumulation or biosorption (Sinha et al. 2012).

Applications of mercury resistant bacteria in bioremediation

As a result of understanding the mechanisms of the *mer* operon, a number of strategies have evolved exploiting Hg resistant microorganisms and cloned *mer* genes with various degrees of success. The most frequently applied approach is to pass Hg contaminated water through a bioreactor containing resistant bacteria which volatilize Hg^{2+} that is subsequently trapped in activated carbon or some other suitable material (USEPA 2007; Velásquez-Riaño and Benavides-Otaya 2015). A pilot plant bioreactor for treating wastewaters from a chlor-alkali plant was designed with a packed bed biofilm consisting of both Hg resistant and Hg-volatilizing bacterial biofilm (Wagner-Döbler 2003). The packed bed was composed of an inert porous carrier material and the biofilm included seven different species of Hg resistant *Pseudomonas*. The bacteria present in biofilms reduced Hg^{2+} to volatile Hg^0 which were subsequently trapped in the carrier material. During the whole testing period of eight

months this bioreactor remediated 98% of Hg in the effluent (28.8 kg out of 29.3 kg Hg). The volatile Hg collected in the bioreactor was recovered by distillation (Wagner-Döbler 2003).

In another approach, the wastewater and a Hg resistant bacterial culture were mixed in an aerated bioreactor where Hg reducing bacteria transformed Hg^{2+} to volatile Hg^0 gas which was trapped in an activated carbon filter (Deckwer et al. 2004). An ion exchange membrane bioreactor (IEMB) was developed recently which was coupled with a cation exchange membrane and a bioreactor containing Hg volatilizing bacteria to remove low levels of Hg in drinking water and high levels in industrial water (Oehmen et al. 2014)

Other than using external trapping material, another method for Hg bioremediation requires the accumulation of volatilized Hg in the remediating cells. A Hg resistant strain of *Enterobacter* which completely reduced Hg^{2+} to volatile Hg^0 and subsequently accumulated the volatilized Hg in the cytoplasm has been reported (Sinha and Khare 2012). This kind of approach has been reported by other authors where the resistant bacteria have been immobilized onto alginate beads or biofilms (Anthony 2014; Chien et al. 2012; Dash and Das 2015; Tariq and Latif 2014).

For the removal of Hg from sediments a combination of chemical leaching by hydrochloric acid-ferric chloride solution and subsequent seeding by a Hg resistant strain *Pseudoalteromonas haloplaktis* M1 has been reported; this process resulted in removal of 85% of Hg from Minamata Bay sediments (Nakamura et al. 1999). A similar approach was utilized by Pepi et al. (2011) who developed a laboratory scale pilot plant to treat contaminated sediment. Biofilm of *Pseudomonas* sp. and *Psychrobacter* sp. were formed on pumice particles packed in 100 ml glass column. The immobilized cells completely volatilized Hg from sediments leachate which was trapped by $KMnO_4$ added at outflow. Utilization of immobilized resistant bacterial cells to remediate Hg contaminated sediment leachates is evident in several other laboratory scale pilot studies (Cabral et al. 2013; Jafari et al. 2015; Pepi et al. 2013).

To remediate radioactive Hg contaminated waste a *Deinococcus radiodurans* strain was transformed with *mer* gene from *E. coli* (Brim et al. 2000). *Deinococcus radiodurans* is well known for its radiation resistant characteristics (Daly et al. 1994) and the recombinant strain engineered with *mer* harbouring plasmid became Hg resistant also. This recombinant strain reduced Hg^{2+} to volatile Hg^0 in the presence of 50 Gy/h of gamma radiation (Brim et al. 2000). *Deinococcus geothermalis*, a thermophilic radiation resistant strain was also engineered to harbour *mer* operon to use in Hg remediation in high temperature radioactive Hg contaminated sites (Brim et al. 2003).

Since contaminated sites contain a range of pollutants in addition to Hg, genetic engineering has been used to develop multi-metal resistant bacterial strains with ability to withstand mixtures of environmental pollutants including other heavy metals. For example, a heavy metal resistant *Cupriavidus metallidurans* was transformed with *merB, merG*, *merA* and other *mer* genes that made the strain broad spectrum Hg resistant superbug which could completely volatilize 0.15M Hg from solution contaminated with other metals, such as chromium and copper (Rojas et al. 2011). In another approach, a *Bacillus cereus* strain having Hg bio-sorption properties was transformed with the *mer* operon which made the transgenic strain capable of volatilizing and simultaneous precipitating Hg as HgS and resulting in 100% removal of Hg from solution (Dash and Das 2015)

In addition to the application of MerA, there are applications of MerR in Hg remediation. A temperature responsive biopolymer has been reported for the remediation of Hg from contaminated water without volatilization. The bacterial MerR protein which has high affinity to Hg was extracted from a genetically engineered *E. coli* and fused to elastin like polypeptides for the formation of highly Hg specific biopolymer. This biopolymer reduced Hg concentration to background level (Kostal et al. 2003).

Importance of *mer* operon in monitoring of environmental mercury

To set up a successful bioremediation strategy it is important to have an appropriate monitoring system which measures the bioavailable fraction of a pollutant in the environment. A number of classical analytical methods are available for the detection and quantification of Hg from environmental and biological samples. The most widely used techniques are atomic absorption spectrophotometry (AAS), cold-vapour atomic flame absorption spectroscopy (CVAFS) (BáStockwell and TáCorns 1995), atomic emission spectroscopy (AES) (Jamoussi et al. 1995) and inductively coupled plasma mass spectrometry (ICP-MS) (Hintelmann et al. 2000). These methods are highly sensitive and characterized by low detection limits but the instrumentations are very expensive, require trained operators and laborious sample preparation procedures. Furthermore they cannot be used in field experiments (Bontidean et al. 2004). Some good alternatives to these analytical techniques are electrochemical methods (Turyan and Mandler 1993) such as ion selective electrodes (IES), anodic stripping voltammetry (ASV), potentiometric stripping analysis (PSA), current stripping chronopotentiometry (CSP) and differential pulse voltammetry (DPV). The disadvantage of all these methods is that they cannot detect the bioavailable Hg concentration because the Hg can be in various valences and complexes.

Quantification of bioavailable Hg is significant because it is the fraction that causes toxicity to plants and animals and is the substrate for biotic methylation and reduction. Information about the concentration of bioavailable Hg is critical for the management of Hg contamination. To detect and quantify the bioavailable Hg in environmental samples, microbial biosensors have been used. A biosensor combines a biological recognition element (biochemical receptor) and a suitable transduction element that can provide specific quantitative and semi-quantitative analytical information about the bioavailable metal. The recognition element can be an enzyme, whole bacterial cell, DNA or antibody and the transducer may be electrical, optical or thermal (Turdean 2011). For the detection of Hg, whole cell bacterial biosensors have been constructed to contain a reporter plasmid that carries a fusion of *merR* regulatory region and the *luxCDABE* operon from bioluminescent bacteria such as *Aliivibrio fischeri* and *Photbacterium luminescens* The combination of these genes in a suitable bacterial host can quantitatively responds to Hg^{2+} and can be detected through production of bioluminescence (Rasmussen et al. 2000) Corbisier et al. 1994). Since sensing of Hg occurs in the cytoplasm it has been established that biosensors detect the concentration of Hg available for binding the internal MerR protein. In biosensors for organic Hg, the biochemical receptor carries an additional *merB* gene encoding the enzyme organomercurial lyase that cleaves the C-Hg bond in organic Hg. When organic Hg is present in the cytoplasm, the organomercurial lyase enzyme cleaves the bond and produce Hg^{2+} which then binds to *merR* gene and induces the expression of the reporter gene (Figure 2) (Barkay and Wagner-Döbler 2005). The MerR protein is the most common sensing element in both types of biosensors and the reporting elements can be bacterial

luminescence (*lux),* green fluorescence protein (*gfp*), β-galactosidase (*lacZ*) or firefly luciferase (*lucFF)* (Hakkila et al. 2004; Hansen and Sørensen 2000).

A recombinant strain of *E. coli* MC1061 containing *mer-lucFF* gene fusion was used in a sensor which could detect Hg from soil sediment samples within a 2h incubation period followed by 30min settling time (Lappalainen et al. 2000). The same strain containing the sensor was reported to respond to $HgCl_2$ with maximum detection limit 0.2 mg/L (Ivask et al. 2002). Recombinant *E. coli* (Hakkila et al. 2002) containing *merR* and *luxCDABE* from *Photorhabdus luminescens* was immobilized on multimode optical fibres. The bioluminescent response of this biosensor started at 0.001 mg/L and reached a maximum of 0.03mg/L Hg^{2+} (Ivask et al. 2007). A number of green fluorescent protein (*gfp*) based Hg biosensors have been reported (Hakkila et al. 2002; Priyadarshi et al. 2012). Fro example, an *E. coli* DH5α biosensor was made with the *merR* gene derived from pDU1358 and the *gfp* gene from plasmid pDB402 inserted into pLDR9 responded to 100-1700 nM ($21.2x10^{-6}$ g/L to $360x10^{-6}$ g/L) concentration of Hg^{2+}, and was stable at very high concentrations of Hg (Priyadarshi et al. 2012).

Although microbial whole cell biosensors offer a convenient, effective, specific and reliable method for monitoring of bioavailable Hg, there are some limitations such as slow response, low sensitivity and poor selectivity. Biosensors with immobilized cells may have measurement problems because of inappropriate attachment of Hg^{2+} to the cells. Another limitation of immobilized whole cell biosensors is their restriction to only aqueous sample (Rasmussen et al. 1997). In lakes where pico molar levels of Hg frequently occur many biosensors are unable to detect these concentrations. This is essential where fish have bioaugmented Hg and residual levels of Hg need to be determined (Selifonova et al. 1993). Sometimes availability of Hg^{2+} to MerR is reduced due to some negatively charged groups and ligands on the cell (Rasmussen et al. 1997), interference of environmental factors such as dissolved organic carbon, salinity and pH (Barkay et al. 1997) leading to reductions in the sensing range.

Emerging technologies

mer operon independent bioremediation approaches

A number of novel mechanisms for Hg bioremediation by volatilization have been reported where the reduction of Hg^{2+} was not due to *mer* operon regulated mercuric reductase enzyme activity, and the bacteria were sensitive to Hg. Iron (Fe^{2+}) oxidizing Hg sensitive acidophilic thiobacilli *Thiobacillus ferrooxidans* was reported to reduce Hg^{2+} by cytochrome c oxidase activity, when the medium was supplemented with Fe^{2+} (Iwahori et al. 2000). Hg sensitive dissimilatory metal reducing bacteria *Shewanella oneidensis* MR-1, *Geobacter sulfurreducens* PCA and *G. metallireducens* GS-15 demonstrated reduction of Hg^{2+} to volatile Hg^0 without mercuric reductase in the presence of ferrous iron. Noteworthy is the activity of these organisms occurs only in very low concentrations of Hg. Since *mer* gene expression requires nM concentrations of Hg, these Hg sensitive bacteria are useful for Hg remediation in anoxic conditions where inorganic Hg^{2+} concentrations is not as high as in oxic environments (Wiatrowski et al. 2006b).

In another study, the gas which was produced by an aerobic culture of *Klebsiella pneumoniae* grown in a broth culture without any heavy metals, when passed through a solution of mixed contaminants including Hg, resulted

in a yellow white precipitate containing 97% of the initial Hg. The gas evolved contained organo-sulphur compounds which immobilized Hg in solution (Essa et al. 2006).

Metallothionein (mt) is a well-known cysteine rich, low molecular weight metal binding protein that can sequester heavy metals in a biologically non-reactive form (Le et al. 2016). The metal sequestration property of metallithionein was utilized in Hg remediation by transforming a Hg sensitive *E coli* with *mt* gene which subsequently became resistant to Hg and could intracellularly accumulate approximately 100 μM Hg from solution (Ruiz et al. 2011).

Application of nanotechnology

Recently some endeavours have been taken to exploit nanoparticles for Hg sequestration from contaminated streams. A novel adsorbent, Thiol Self-Assembled Monolayers on Mesoporous Silica (Thiol-SAMMS) was developed which consisted of a nano-porous ceramic substrate with a high surface area made functional by a monolayer of thiol groups. The thiol functional groups bind with Hg and immobilize it (Mattigod et al. 2007). Colloidal gold nanoparticles, stabilized iron sulphide nanoparticles and Gymnemic Acid-Chitosan nanoparticles have also been utilized as Hg scavengers from water and sediments (Minu et al. 2015; Ojea-Jiménez et al. 2012; Xiong et al. 2009). A Hg resistant *Enterobacter* strain has been reported which exhibited a novel property of Hg immobilization by synthesis of nanoparticles Hg. The strain could intracellularly synthesise uniform sized 2–5 nm, spherical and monodispersed Hg nanoparticles in low Hg containing solution which prevented the reduced Hg from being volatilized (Sinha and Khare 2011).

Future directions

Bioremediation is considered a greener and cheaper technology to scavenge Hg from contaminated sites compared to physico-chemical means. Although a number of endeavours have been made to implement bioremediation approaches to clean up Hg contaminated waters, there is not enough evidence of the application of Hg resistant bacteria to remediate Hg contaminated soils. As soil is the reservoir of the major portion of Hg contamination it is mandatory to study the viability of the bioremediation technology. Soil organic matter, clay, minerals and other complex soil ligands determines the fate and mobility of Hg in soil, which is crucial for evaluating the implementation of bioremediation techniques. Future research should focus on the implementation of the Hg resistant microorganisms to remove or immobilise Hg from soil. Since each contaminated site has unique characteristics, a detailed evaluation and proper risk assessment should be carried out before implementing bioremediation. However, in addition to Hg, contaminated sites are often polluted with a range of heavy metals and organic substances. Therefore, there is a need to isolate or genetically modify and characterize multi-metal resistant bacterial strains which have resistance to mixed contaminants in soil. As the leachability and bioavailability of Hg in soil is often negligible, bioremediation can be coupled with other techniques which can extract Hg from soil ligands that will be subjected to microbial volatilization and/or precipitation.

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Table 1: Mercury remediation technologies

Treatment	Matrix	Species	Mechanism	References
			Physico-chemical techniques	
Solidification and stabilization	Solid and waste	Elemental Hg and contaminated soil	Reducing the mobility by physically binding within a stabilized mass; or chemically converting into less soluble form	(López et al. 2015)
Amalgamation	Solid and waste	Elemental Hg	Dissolution in other metals, formation of semi-solid alloy	(Kalb et al. 2001)
Soil washing	Soil and sediment	Elemental Hg	Washing the contaminated soil with a soil solution and treating the water by ion exchange and solvent extraction	(Xu 2013)
Acid extraction/chemical leaching	Soil and sediment	Elemental Hg	Extraction by dissolving in acid followed by flocculation	(Xu et al. 2014)
Thermal treatment	Soil, sediment and waste	Elemental Hg	Volatilization by heating at reduced pressure followed by condensation, then amalgamation	(Ma et al. 2014b)
Vitrification	Soil and sediment	Elemental Hg	Immobilization by incorporating in vitrified end products by high temperature treatment	(USEPA 2002)
Precipitation	Water	Inorganic Hg	Transformation of dissolved Hg in insoluble precipitates (sulphide precipitation)	(Patterson and Stein 1997) (USEPA 2002)
Adsorption	water	Inorganic Hg	Reducing concentration by adsorption at the surface of a sorbent packed in a column	(Cyr et al. 2002; USEPA 1998)
Membrane filtration	water	Inorganic Hg	Precipitation or co-precipitation followed by filtration through a semi-permeable membrane	(USEPA 2000)
			Biological Techniques	
Phytoremediation	Sediments, soil, water	Inorganic Hg	Accumulation of Hg in harvested plant; reduction of ionic Hg to elemental Hg by engineered plant	(Heaton et al. 2003; Marrugo-Negrete et al. 2017; Su et al. 2008)
Microbial remediation	Water	Inorganic and organic Hg	Transformation of highly toxic forms to less toxic elemental form by microbial “Hg reductase” enzyme followed by volatilization	(Wagner-Döbler 2013)
Biosorption	Water, sediment	Inorganic and organic Hg	Adsorbing Hg on biological material such as plant, algae, moss, lichen, crab carapace, bacterial biofilm, fungal biomass etc.	(Wagner-Döbler 2013)
			Nanotechnology	
Use of different nano-adsorbent	Water	Inorganic, organic Hg	Thiol group containing nano-adsorbents, alumina nanoparticles etc are being used to trap Hg	(Li et al. 2008; Mattigod et al. 2007)

Table 2: Functional genes present in *mer* operon of mercury resistant bacteria (adapted from (Andréa et al. 2003; Dash and Das 2012; Osborn et al. 1997))

Genes	Encoded protein	Location	Functions
merA	Mercuric reductase	cytoplasm	Reduction of Hg^{2+} to Hg^0
merB	Organomercurial lyase	cytoplasm	Lysis of C-Hg^+ bond
merC	Mercuric ion transport protein	Inner membrane	Transport of Hg^{2+}
merD	Regulatory Protein	cytoplasm	Negatively regulates the *mer* operon
merE	MethylHg transport protein	Inner protein	Uptake of organomercurials into cytoplasm
merF	Mercuric ion transport protein	Inner membrane	Transport of Hg^{2+}
merG	Phenylmercury resistance protein	periplasm	Resistance to phenylmercury by efflux mechanism
merP	Periplasmic mercuric ion binding protein	periplasm	Transfer of Hg^{2+} to integral membrane protein
merR	Regulatory protein	cytoplasm	Positively regulates the *mer* operon
merT	Mercuric ion transport protein	Inner membrane	Transport of Hg^{2+}

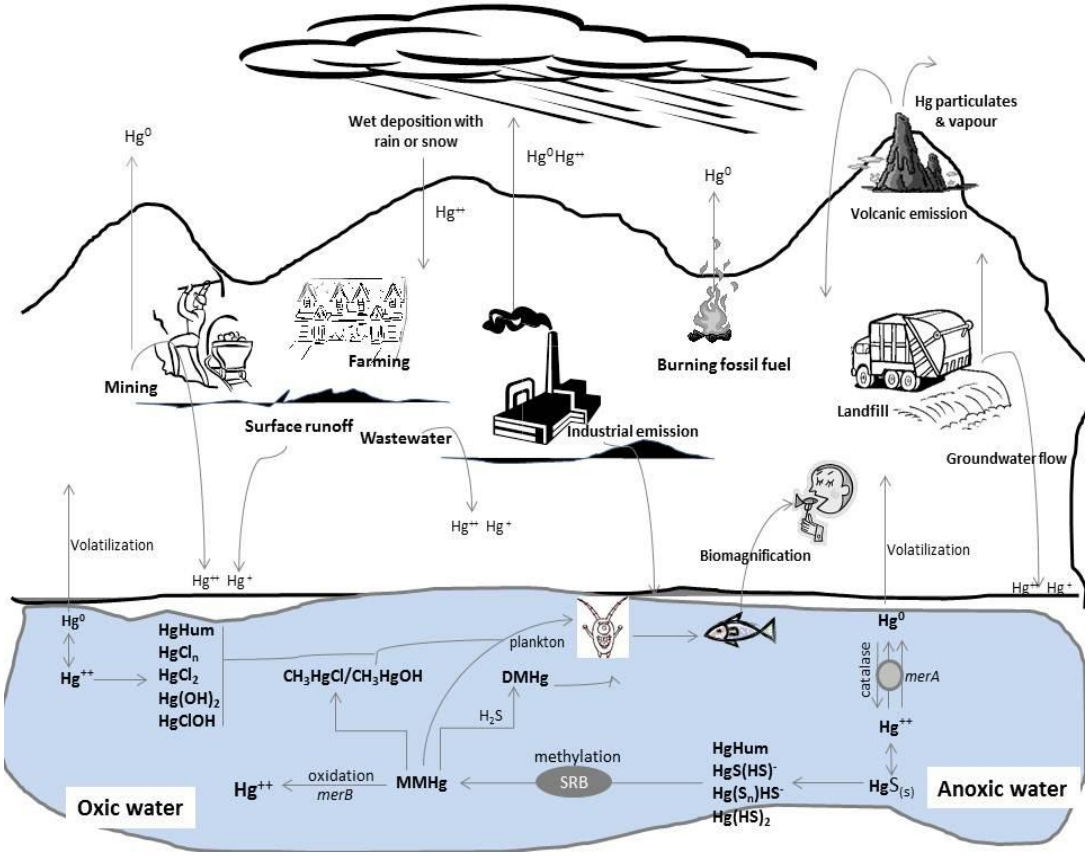

Figure 1: Emission and cycling of Hg in the environment; adapted from (Barkay and Wagner-Döbler 2005)

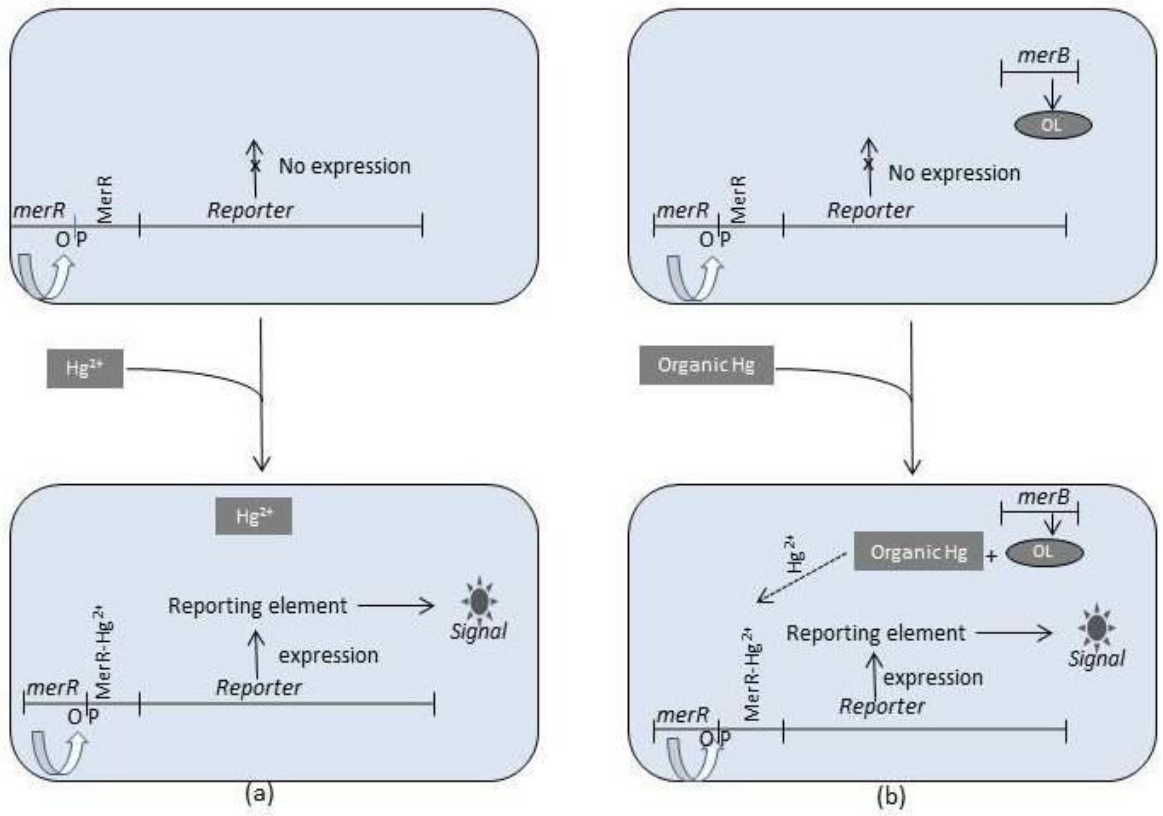

Figure 2: Schematic diagram of biochemical receptor in mercury biosensors (Barkay and Wagner-Döbler 2005): (a) when the biosensor comes into contact with Hg^{2+} it binds with the MerR protein having high affinity to Hg^{2+}, repression is alleviated and the reporter gene is expressed resulting in a detectable signal. (b) for organic Hg detection, biosensors carry an additional gene *merB* encoding the enzyme organomercurial lyase (OL). In the presence of organic Hg, the enzyme cleaves the C-Hg bond to release Hg^{2+} which subsequently interacts with MerR to induce reporter gene expression.