

Study of Microremediation, Phytoremediation & Vermiremediation Biotechnologies for Contaminated Lands & Soil by Environmentally Adapted Microbes, Plants & Earthworms

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Transgenic (Genetically Engineered) Plants Combating Soil Pollution; Phytoextraction; Phytovolatilization; Phytostabilization; Phytostimulation; Rhizofiltration; Phytotransformation; Phytodegradation; Excluder Plants; Hyper-accumulator Plants Species; Symbiotic Engineering; Biotransformation; Microbial Detoxification of Metals; Microbial Destruction of Toxic Organics & Hazardous Wastes; Plant – Assisted Microbial Destruction of Toxic Compounds; Earthworms Bio-accumulate & Bio-degrade & Bio-transform Toxic Chemicals; Earthworms Immobilize Soil Contaminants;

ABSTRACT

Bioremediation is a soft bioengineering technique to clean up contaminated lands & soils using microbes, plants and earthworms. It is also a technique to stabilize the eroded lands and prevent soil erosion. Microbes are adapted to thrive in 'adverse conditions' of high acidity / alkalinity / toxicity and high temperature. Under favorable conditions of growth microbes can biodegrade / biotransform the complex hazardous organic chemicals into simpler and harmless ones. Environmentalists are viewing microbes such as yeast, bacteria, algae, diatoms and actinomycetes as an 'eco-friendly nano-factories' for metal remediation. Some bacteria can also ingest the most toxic 'cyanide' from water. After the use of 'super bug' in cleaning up oil spills, there have been several successful stories of microbial technique in clean-up of contaminated lands and soils. The Microbiological Resource Centers (MIRCENS) in Egypt is examining the use of microbes in degrading persistent pesticides pollutants.

Plants involved in bioremediation are adapted to thrive in very harsh environmental conditions of soil and water; absorb, tolerate, transfer, assimilate, degrade and stabilize highly toxic materials (heavy metals, radionuclides and organics such as solvents, crude oil, pesticides, explosives, chlorinated compounds and polyaromatic hydrocarbons) from the polluted soil and water. The organic pollutants may ideally be degraded to simpler compounds like carbon dioxide (CO₂) and water (H₂O). More than 400 plant species ranging from herbs to trees have now been identified which are hyperaccumulators of toxic metals They can accumulate 100 - 500 times higher levels of metal concentrations in their above-ground parts. The brake fern (*Pteris vittata*) can reduce the concentration of 200 ppm of arsenic to nearly 100-fold (10 ppm) within 24 hours.

Earthworms in general (especially *E. fetida*) are highly resistant to many chemical contaminants including heavy metals and organic pollutants in soil. They have been found to remove heavy metals, pesticides and lipophilic organic micropollutants like the polycyclic aromatic hydrocarbons

(PAH) from the soil and have been used for land recovery, reclamation and rehabilitation of sub-optimal soils such as poor mineral soils, polder soils, open cast mining sites, closed landfill sites and cutover peats. Worms have been shown to bio-accumulate even the most toxic 'dioxin' in their tissues and concentrate it on average 14.5 fold. They can bio-accumulate heavy metals cadmium (Cd), mercury (Hg), lead (Pb) copper (Cu), manganese (Mn) and zinc (Zn), particularly extremely high amounts of Zn and Cd. Cadmium levels up to 100 mg per kg dry weight have been found in tissues.

1. Introduction

Large tract of arable land is being chemically contaminated due to mining activities, heavy use of agro-chemicals in farmlands, landfill disposal of toxic wastes and other developmental activities like oil and gas drilling. No farmland of world especially in the developing nations are free of toxic pesticides, mainly aldrin, chlordane, dieldrin, endrin, heptachlor, mirex and toxaphene. According to National Environment Protection Council there are over 80,000 contaminated sites in Australia- 30,000 in NSW, another 30,000 in Queensland, 10,000 in Victoria, 4000 in South Australia, another 4000 in Western Australia, 1000 in Northern Territory and 500 each in Tasmania and the Australian Capital Territory (ACT). There are 40,000 contaminated sites in US; 55,000 in just six European countries and 7,800 in New Zealand. There are about 3 million contaminated sites in the Asia-Pacific. These also include the abandoned mine sites along with the closed landfills. The contaminated sites mostly contain heavy metals cadmium (Cd), lead (Pb), mercury (Hg), zinc (Zn) etc. and chlorinated compounds like the PCBs and DDT. Cleaning them up mechanically by excavating the huge mass of contaminated soils and disposing them in secured landfills will require billions of dollars. There is also great risk of their leaching underground (aggravated by heavy rains) and contaminating the groundwater. Contaminated soils and waters pose major environmental, agricultural and human health problems worldwide.

Traditionally, remediation of chemically contaminated soils involves 'off-site' management by excavating and subsequent disposal by burial in secured landfills. This method of remediation is very costly affair and merely shifts the contamination problem elsewhere. Additionally, this involves great risk of environmental hazard while the contaminated soils are being transported and 'migration of contaminants' from landfills into adjacent lands and water bodies by leaching. Soil washing for removing inorganic contaminants from soil is another alternative to landfill burial, but this technique produce a 'residue' with very high metal contents which requires further treatment or burial.

Since the late 1980s, after the chemical and mechanical treatments of lands and water bodies and thermal treatment (incineration) of hazardous wastes proved economically and environmentally unsustainable, focus shifted towards the biological methods which are cost-effective as well as environmentally sustainable and also socially acceptable. Bioremediation is a soft bioengineering technique to clean up contaminated lands / sites using microbes (bacteria or fungus), plants (terrestrial and aquatic) and earthworms. It is also a technique to stabilize the eroded lands and prevent soil erosion. Bioremediation works carried out by the microorganisms are called 'micro-remediation' while those performed by plants are called 'phyto-remediation'. Earthworms have also been found to perform some environmental cleaning jobs and is termed as 'vermi-remediation'.

I. Microbial Remediation of Polluted Lands & Soils by Adapted Microbes

Microbes are adapted to thrive in 'adverse conditions' of high acidity / alkalinity / toxicity and high temperature. They can develop 'biological resistance' against any toxic substance in the environment due to special 'jumping genes'. Hence while a number of them may be killed due to high toxicity, some resistant microbes survive and are cultured for further use. Under favorable conditions of growth e.g. pH, temperature and moisture and adequate supply of nutrients like vitamins, magnesium, manganese, copper, sulfur, potassium, phosphorus and nitrogen, microbes can biodegrade / biotransform the complex hazardous organic chemicals into simpler and harmless ones. After the use of 'super bug' in cleaning up oil spills, there has been several successful stories of microbial technique in clean-up of contaminated lands and soils. (USGS,

1997). The Microbiological Resource Centers (MIRCENS) at Cairo, Egypt is examining the use of microbes in degrading persistent pesticides pollutants. (UNEP Reports, 1996-2006).

Whilst microbial remediation (bioremediation) is a well established technology for the removal of organic soil contaminants, the use of microorganisms to transform inorganic contaminants like heavy metals is still being investigated. Environmentalists are viewing microbes as an 'eco-friendly nano-factories' for metal remediation through biotechnological applications employing microbes, such as yeast, bacteria, algae, diatoms and actinomycetes.

There are bacteria which can also ingest the most toxic 'cyanide' from water.

Microbial Bioremediation of Heavy Metals

Metals play important role in the life processes of microbes. Some metals such as chromium (Cr), calcium (Ca), magnesium (Mg), manganese (Mn), copper (Cu), sodium (Na), nickel (Ni) and zinc (Zn) are essential as micronutrients for various metabolic functions and for redox functions. Other metals have no biological role e.g. cadmium (Cd), lead (Pb), mercury (Hg), aluminum (Al), gold (Au) and silver (Ag). They are non-essential and potentially toxic to soil microbes. Some of them e.g. Cd^{2+} , Ag^{2+} , Hg^{2+} tend to bind the SH groups of enzymes and inhibit their activity (Turpeinen, 2002).

Soil contamination by heavy metals may repress or even kill parts of the microbial community in soil. Interaction of metals with cellular proteins / enzymes are more commonly implicated in causing toxicity than interaction with membranes. Binding affects the structure and function of proteins and enzymes.

Metal Tolerance & Resistance by Microbes

It is generally assumed that the exposure to metals leads to the establishment of a tolerant / resistant microbial population. Microbial 'resistance' is defined as the ability of a micro-organism to survive toxic effects of metal exposure by means of a detoxification mechanism produced in direct response to the 'heavy metal species' concerned. Microbial 'tolerance' is defined as the ability of a micro-organism to survive metal toxicity by means of intrinsic properties and or environmental modification of toxicity.

Soil micro-organisms have been shown to bio-accumulate metals in tissues in concentrations up to 50 times higher than the surrounding soil.

Micro-organisms employ a variety of mechanisms to resist and cope with toxic metals. The principal mechanism of resistance of inorganic metals by microbes are metal oxidation, metal reduction, methylation, demethylation, enzymatic reduction, metal-organic complexation, metal ligand degradation, intracellular and extracellular metal sequestration, metal efflux pumps, exclusion by permeability barrier and production of metal chelators such as metallothioneins and biosurfactants.

Process and Mechanism of Microbial Remediation of Contaminated Soil & Water

Microbial remediation of toxic metals occurs in two ways –

1). Direct reduction by the activity of the bacterial enzyme 'metal reductase'. It is applied for groundwater decontamination, using bioreactors (pump & treat) and also for soils after excavation (pulping or heaping and inoculation with appropriate microbial consortium). These techniques are *ex-situ* methods, and very expensive and has low metal extraction efficiencies.

2). Indirect reduction by biologically produced hydrogen sulfide (H_2S) by sulfate reducing bacteria to reduce and precipitate the metals. This is an *in-situ* method, and an environmentally sound & inexpensive alternative to pump & treat (for contaminated groundwater) or excavate & treat (for contaminated soils). Microbial growth is induced in sub-surface zones by injecting substrates. The migrating metals are intercepted and immobilized by precipitation with biologically produced H_2S .

There are at least three major microbial processes that influence the bioremediation of metals and these are –

1). Biosorption & Bioaccumulation:

Biosorption is sequestration of the positively charged heavy metal ions (cations) to the negatively charged microbial cell membranes and polysaccharides secreted in most of the bacteria on the outer surfaces through slime and capsule formation. From the surface the metals are transported into the cell cytoplasm through the cell membrane with the aid of transporter proteins and get bioaccumulated.

2). Biologically Catalysed Immobilization

Inside the microbial cells, metal ions gets fixed to Iron (Fe)-Oxides and into organic colloids and becomes immobilised. This is achieved by enzymatic reduction by microbes (described below).

3). Biologically Catalysed Solubilization

Metal reducing bacteria enzymatically reduce and also under appropriate conditions, solubilize oxide minerals. Such dissolution reaction have been shown to release cadmium (Cd), nickel (Ni) and zinc (Zn) into solution during reduction of goethite (a form of Fe-oxide) by anaerobic bacterium *Closteridium* spp.

Microorganisms do not actually biodegrade inorganic metals, but changes (bio-transform) their oxidation state. This can lead to an increase in solubility (and subsequent removal by leaching), or precipitation and reduction in bioavailability. Metallic residues (heavy metals) may be converted into 'metal-organic combinations' that have less bioavailability (to pass into human food chain) than the 'metal-mineral combinations' of the heavy metals. Microbes transform the oxidation states of several toxic metals and increase their bioavailability in the rhizosphere (root zone) thus facilitating their absorption and removal by hyper-accumulating plants by phytoremediation.

Many divalent metal cations like Mn^{2+} , Fe^{2+} and Zn^{2+} are very similar in structure. Also, the structure of oxyanions, such as chromate, resembles that of sulphate. Evolution has endowed microorganisms with effective mechanisms to distinguish between similar metal ions and between toxic and non-toxic metals. Microbes have solved this problem by developing two types of uptake mechanisms and systems for metal ions -

- 1). Selective, substrate-specific uptake system that are slow and require considerable cellular energy (ATP) and is only produced by the cell in times of need;
- 2). Substrate-non-specific rapid system, that transport metals using a chemiosmotic gradient across the cytoplasmic membrane of the bacteria rather than using ATP. (Nies, 1999).

Even highly evolved, substrate-specific uptake mechanisms may not prevent entry of toxic metals in cells. Once inside, metal cations can interact with various cellular components including cell membranes, proteins and nucleic acids. Incompletely filled d-orbitals allow metals to form complex compounds with organic ligands, such as the proteins, nucleic acids & cell wall materials of microorganisms. The ability of microbial cell surfaces to form complex with metals lies in their net negative charge at normal growth pH. The outer membrane of Gram negative bacteria effectively complexes metals including magnesium (Mg), nickel (Ni), strontium (Sr), manganese (Mn), lead (Pb), iron (Fe), sodium (Na) and calcium (Ca). In Gram –eve bacteria the, the net –eve charge results from the phosphates and carboxyl groups of lipopolysaccharide molecules, while the –eve charge in Gram positive bacteria results largely from teichoic acid. A more negative cell surface charge may more effectively attract and bind toxic metal cations. Toxic metals readily binds to sulfhydryl group of proteins. (Nies, 1999; Sandrin & Hoffman, 2006).

The resemblance of some toxic heavy metals to essential metabolite (minerals) allows them to readily enter into the microbial cells. Thus, chromate (Cr) is often mistakenly taken up in place of sulphate (S), arsenate (As) is mistaken for phosphate (P), cadmium (Cd) is used as an enzyme co-

factor instead of zinc (Zn) or calcium (Ca), nickel (Ni) and cobalt (Co) is mistaken for iron (Fe), and zinc (Zn) is very commonly mistaken for magnesium (Mg). (Nies, 1999; Sandrin & Hoffman, 2006).

Microbial Detoxification of Metals

One or more of the resistance mechanisms allows microbes to function in metal contaminated environments and detoxify them. Micro-organisms can detoxify metals by 'valence transformation', by 'extracellular chemical precipitation' or by 'volatilization'.

Generally, microbial transformations & detoxifications of metals occur either by redox conversions (reduction) of inorganic forms or conversions from inorganic to organic forms and *vice versa*. Most toxic heavy metals are less soluble and less toxic when in 'reduced state' than in 'oxidised state'. Reduction of metals can occur through dissimilatory metal reduction, where microbes utilize metals as terminal electron acceptor for anaerobic respiration.

To date arsenic (As), chromium (Cr), mercury (Hg), uranium (U) and selenium (Se) have responded well to detoxification by microbial reduction. *Oscillatoria* spp. (a blue-green algae), *Chlorella vulgaris* & *Chlamydomonas* spp. (green algae), *Arthrobacter*, *Agrobacter*, *Enterobacter* & *Pseudomonas aeruginosa* are some metal reducing microbes. (Ramasamy et al., 2006).

1). Microbial Reduction of Chromium (VI) to Cr (III):

Chromium is widely used in many industrial & developmental activities, such as in leather & tannery industries, electroplating, steel and automobile manufacturing, production of paint pigments and dyes, refractory and in wood preservation. Its world production is in the order of 10,000,000 tons per year. It is a hazardous contaminant and is a serious threat to human health as it readily spreads beyond the site of initial contamination through aquatic ecosystems and groundwater. (Viti & Giovannetti, 2006).

Chromium in environment is able to exist in several oxidation states, ranging from Cr (II) to Cr (VI), but in soils the most stable & common forms are trivalent Cr (III) and hexavalent Cr (VI) species. The trivalent & hexavalent forms can inter-convert. Cr (III) is essential for animal and human nutrition. Utmost consideration is given to Cr (VI) because it is water-soluble, highly toxic and mutagenic to most organisms and carcinogenic for humans. It is also involved in causing birth defects and the decrease of reproductive health.

A wide range of microbes have been found to have chromium (Cr) tolerance, resistance and reducing ability. The blue-green algae *Nostoc* have been reported to exist in a soil chronically polluted by chromium (about 5000 mg/kg of soil) from leather tannery. Other microbes tolerating / resisting Cr (IV) levels are *Arthrobacter crystallopoites* (500 mg/L), *Pseudomonas* spp. CRB 5 (520 mg/L), *Bacillus maroccanus* ChrA21 (1040 mg/L), *Corynebacterium hoagii* Chr B20 (1144 mg/L), *Bacillus cereus* ES04 (1500 mg/L). (Viti & Giovannetti, 2006). Anaerobic sulfate reducing and methanogenic bacteria possess inherent abilities to sorb more than 90 % of chromium to its cell biomass. Microbe reduce the highly soluble chromate ions to Cr (III), which under appropriate conditions precipitates as Cr(OH)₃. Organic matter (carbon sources) of the soil plays an important role in the reduction of Cr (VI) to Cr (III) by creating reducing conditions, such as increasing activities of soil microbes and by acting as an electron donor, & also by indirectly lowering the oxygen level of the soil (due to increased microbial respiration). Carbon sources, such as organic acids, manure, molasses, have been proposed to improve Cr (VI) reduction, that otherwise is very slow.

2). Microbial Reduction of Uranium (VI) to U (IV):

Microbe reduce uranyl carbonate, which is exceedingly soluble in carbonate-bearing groundwater, to highly insoluble U (IV) which precipitates from solution as the uranium oxide mineral uraninite. Recently scientists have succeeded in microbial binding of U (IV), which is then converted by the living cell to U (IV) and precipitated intracellularly. The *Geobacter* spp. have been found to work well to remove uranium (U) from groundwater. (Anderson et al., 2003).

3). Microbial Reduction of Selenium (VI) to elemental Se (0):

It causes precipitation of the selenium metal and reduced bioavailability. In addition, SeO_4 can be microbially methylated to volatile dimethyl selenide which escape from soil.

Role of Iron & Sulfate Reducing Bacteria in Reducing Toxic Metals

The iron (Fe) reducing bacterium e.g. *Geobacter* spp. and the sulfur reducing bacterium e.g. *Desulfuromonas* spp. are also capable of reducing the toxic metals. They have been found to colonise habitats with elevated metal concentrations. *G. metallireducens*, a strict anaerobe is able to reduce manganese (Mn), such as toxic Mn (IV) to Mn (II), and reduce uranium (U), such as toxic U (VI) to U (IV). The *G. sulfurreducens* & *G. metallireducens* are able to reduce chromium (Cr) such as highly toxic Cr (VI) to harmless Cr (III). The sulfur reducing bacterium is strictly anaerobic and gram-negative. It acquires its energy from sulfur (S) respiration and completely oxidises acetic acid (organic acid) with sulfur (S) to carbon dioxide (CO_2). Reduction of sulfur (S) produces hydrogen sulfide (H_2S) which reacts with heavy metal ions to form less toxic insoluble metal-sulfides. Furthermore, these bacteria are also able to enzymatically reduce and precipitate these heavy metals. (Bruschi and Goulhen, 2006). This approach of indirect metal reduction by biologically produced H_2S by sulfur reducing bacteria was developed in the 1980s and has been used commercially up to industrial scale. They lead to selective metal precipitation, such as copper (Cu) and zinc (Zn) sulfates and acidity removal.

Some Success Stories

- 1). Pilot plants developed by Shell Research Ltd. and Budelco BV, France, using an undefined consortium of SRB (sulfate reducing bacteria), have been used successfully to remove zinc (Zn) and sulfate. The metals were precipitated as sulfides. Acetic acid, produced by the SRB was removed by the 'methanogenic bacteria' present in the consortium. This technology has been scaled up and now capable of treating 7000 cubic meter (cum) of contaminated soil per day. (Bruschi and Goulhen, 2006)
- 2). In-situ bioremediation of uranium contaminated sites have been conducted successfully with *Desulfosporosinus* spp. and *Closteridium* spp. (Bruschi & Goulhen, 2006).
- 3). Treatment of other metals using anaerobic bioreactor with SRB community culture has been reported for 'phosphogypsum', a hazardous waste from fertilizer industries and for 'lead wastes' from car batteries. Lead waste is reduced to less hazardous PbS (Galena).

Microbial Methylation of Metals : Another Mechanism of Detoxification

Microbial methylation also plays an important role in metal detoxification, because methylated compounds are often volatile. Mercury, Hg (II) can be biomethylated by a number of bacteria e.g. *Pseudomonas* spp., *Bacillus* spp., *Closteridium* spp. and *Escherichia* spp. to gaseous methyl mercury. Biomethylation of arsenic (As) to gaseous arsines, selenium (Se) to volatile dimethyl selenide, and lead (Pb) to dimethyl lead has been observed in various contaminated soils. (Ramasamy et, al., 2006).

Acidophilic iron and sulfur oxidizing bacteria are able to leach high concentrations of arsenic (As), cadmium (Cd), copper (Cu), cobalt (Co) and zinc (Zn) from contaminated soils. Also heavy metals can be precipitated as 'insoluble sulfides' indirectly by the metabolic activity of sulfate reducing bacteria. (Ramasamy et, al., 2006).

4. Microbial Destruction of Toxic Organics

Several microbes have been identified in nature which can break down the hazardous organic substances in the environment (soil & water) including the xenobiotic compounds such as pesticides, polycyclic aromatic hydrocarbons (PAHs) and the chlorinated substances like polychlorinated biphenyls (PCBs) in due course of time. Majority of the organochlorines appears to be biotransformed, forming conjugates with the soil humic matter. Scientists at IIT, Madras have discovered a bacterial mixture which breaks down the deadly pesticide 'endosulphan' into simple inorganic chemicals under both aerobic and anaerobic conditions.

1). Bacterial Degradation of Toxic Organics

Naturally occurring aerobic bacteria have been found to decompose both natural and the synthetic hazardous organic materials to harmless CO₂ and water. However, anaerobic microbes are important for degrading the halogens (reductive dehalogenation) and nitrosamine, reduction of epoxides to olefins, reduction of nitro groups and ring fission of aromatic structures.

Table - 1
Naturally Occurring Bacteria Capable of Destroying Some Hazardous Wastes and Chemicals by Biodegradation

Organisms	Chemicals Degraded
<i>Flavobacterium</i> spp. <i>Cunniughamela elegans</i> & <i>Candida tropicalis</i>	Organophosphates PCBs (Polychlorinated Biphenyls) & PAHs (Polycyclic Aromatic Hydrocarbons);
<i>Alcaligenes</i> spp. & <i>Pseudomonas</i> spp.	PCBs, halogenated hydrocarbons and alkylbenzene sulphonates, PCBs, organophosphates, benzene, anthracene, phenolic compounds, 2,4 D, DDT and 2,4,5-trichlorophenoxyacetic acid etc.
<i>Actinomycetes</i> <i>Nocardia tartaricans</i> <i>Closteridium</i> <i>Arthrobacter</i> & <i>Bacillus</i> <i>Trichoderma</i> & <i>Pseudomonas</i>	Raw rubber Chemical Detergents (Ethylbenzene) Lindane Endrin Malathion

 Sources: Various Publications of UNEP, WWF and WHO (1992-2002)

2). Fungal Degradation of Toxic Organics

All fungus, but more specifically the wood-decaying members of Basidiomycetes called the white – rot fungus (WRF) are most efficient microbe that de-polymerize and even mineralize the ‘lignin’ contents which is hard to be degraded. The fungus produce a powerful ‘extra-cellular ligninolytic enzyme system’ which is a strong oxidant. Three main types of oxido-reductase activities can be found in this enzymatic system – the ‘polyphenoloxidases’, ‘peroxidases’ and auxiliary ‘H₂O₂ generating oxidases’. More specifically, the key lignolytic enzymes synthesized by WRF are ‘laccase’(polyphenoloxidase). Both, whole fungal cultures of WRF as well as the lignin-degrading enzymes were found to be useful to the bioremediation of a wide number of toxic pollutants including the recalcitrant ‘xenobiotics’ and more importantly the ‘textile dyes’ which is highly resistant to washing, chemical solvents, action of sunlight and microbial attack. Rate of degradation is affected by atmospheric oxygen concentration, nutrient nitrogen and the source of carbon.

Fungal Destruction of Chlorinated Aromatic Compounds

A very promising fungus which has the arsenal to degrade and destroy an assortment of persistent ‘chlorinated aromatic compounds’ have been identified. It is the white-rot fungus (*Phanerochaete chrysosporidium*). In Turkey, scientists have found that *P. chrysosporidium* can biodegrade LDPE plastic bags containing 12 % starch. Scientists in Japan have used biochemical machining and enzymes found in filamentous fungi to biodegrade or cut down the molecular chains present in biodegradable plastics.

Table - 2
Naturally Occurring Fungus Capable of Destroying Some Hazardous Wastes and Chemicals by Biodegradation

Organisms	Chemicals Degraded
<i>Phanerochaete chrysosporium</i> , <i>P. sordida</i> & <i>Trametes hirsute</i>	Halocarbons such as lindane, pentachlorophenol, DDT, DDE, PCBs, 4,5,6-trichlorophenol, 2,4,6-trichlorophenol, dichlorophenol, and chlordane
<i>Zylerion xylestrix</i>	Pesticides / Herbicides (Aldrin, dieldrin, parathion and malathion)
<i>Mucor</i>	Dieldrin
Yeast (<i>Saccharomyces</i>)	DDT

Sources: Various Publications of UNEP, WWF and WHO (1992-2002)

Use of Yeast for Removal of Textile Dyes from Polluted Waters

Industrial textile dyes have been designed and synthesized to be highly resistant to washing and action of chemical solvents and sunlight. There are currently more than 10,000 different textile dyes commercially available in the world markets. Azo, indigoid and anthraquinone are the major chromophores used in the textile industries. They are complexed with heavy metals copper (Cu), cobalt (Co) and chromium (Cr) and are of considerable public health concern. Conventional treatments like 'adsorbent by activated carbons' is very expensive and do not remove heavy metals.

Living yeast biomass specifically the strains of *Candida tropicalis* isolated from sewage has been shown to bio-absorb various textile dyes – the reactive black and red, and the remazol blue. Maximum bio-accumulation capacity range from 79 mg/g (per gram of yeast biomass) for reactive red to 112 mg/g for remazol blue. It has significantly lower operational costs and the heavy metals are also absorbed and removed. (Donmez, 2002).

Microbial Destruction of Polycyclic Aromatic Hydrocarbons (PAHs)

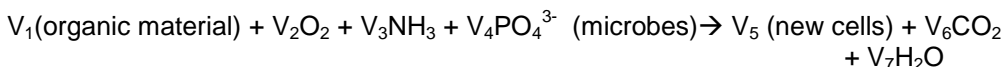
Among the most abundant environmental pollutants, the aromatic compounds are of major concern because of their persistence and toxicity. PAHs are ubiquitous in nature found throughout the environment – in air, water and soil. PAHs are group of over 100 chemicals of which anthracene, benzo (a) pyrene, naphthalene, pyrene are common. They are formed by the incomplete combustion of fossil fuels (coal & oil) and wood. They are emitted from petroleum refineries, coal and oil power plants, coking plants, asphalt production plants, aluminum plants, industrial machinery manufacture and paper mills. They readily evaporate in air. People may be exposed to PAHs in the soil where coal, wood or petrol has been burnt. Food grown on such soils may contain PAH. However, several PAHs breaks down in sunlight in air over a period of days to weeks. Soil microbes can degrade them in weeks to months.

US EPA has identified 16 PAHs as 'priority pollutants' made of two or more fused benzene rings with potential for high 'bio-magnification', and therefore, toxic to living organisms. PAHs are able to 'bio-accumulate' in plants and animals including human beings. Benzo (a) pyrene, benzo (a) anthracene and chrysene are carcinogenic to humans, mutagenic in animal and bacterial cells, and also teratogenic, causing genetic defect in humans. Some PAHs can also induce 'immunodepressive effects'. These chemicals are thermodynamically stable, have very low aqueous solubility, are highly hydrophobic, and tend to be strongly associated with particles surface in the environment. Most of them are resistant to volatilization and photolysis and are therefore, very 'persistent' under natural conditions with 'half-lives' in soils ranging from 26 days for 'phenanthrene' (a volatile and degradable compound) to 6,250 days for 'benzo (a) anthracene' (a recalcitrant compound). (Sims & Overcash, 1983).

Bacteria capable of destroying PAHs are species of *Pseudomonas*, *Alcaligenes*, *Rhodococcus*, *Sphingomonas* and *Mycobacterium*. They utilize PAHs as their sole carbon and energy source. A diverse group of fungi, both ligninolytic and non-ligninolytic, are able to degrade PAHs. But they transform them co-metabolically to detoxified metabolites and do not use them as source of carbon and energy like bacteria. (Sutherland, 1992). Both prokaryotic and eukaryotic algae have been found to degrade PAHs in aquatic environments. The blue-green algae (Cyanobacteria) oxidize PAHs under photoautotrophic conditions to form hydroxylated intermediates.

Mechanism of Microbial Action in Degradation of Toxic Compounds

This is achieved by biodegradation and biotransformation of complex toxic chemicals into harmless simpler biochemical products by heterotrophic microbes which cannot produce their own food and in order to survive extract 'growth nutrients' from the host material (which may be an organic or inorganic waste) by decomposing it. Microbes can also transform toxic substances into energy source. Microbes grow geometrically and in the process of decomposition produce a huge biomass of 'new cells'. Hence the biodegradation process become faster with time.



Oxygen (O₂), ammonia (NH₃), and phosphate (PO₄³⁻) are the nutrients needed by the microbes to oxidize the organic matter in the waste.

Biodegradation by microbes is system-specific. Unless a proper microbial consortium is both developed and maintained, a compound may not degrade in that system. Microorganisms adapt to degrade 'new synthetic compounds' either by utilizing catabolic enzymes they already possess or by acquiring new metabolic pathways. In any event the biological systems can lower the cost of downstream processes by reducing organic load. While a microbial community that is resistant to moderate levels of heavy metals can be developed, the accumulation of metal precipitates on the bacterial biomass may severely inhibit their activity. Hence, a pretreatment system to remove metal contaminants may be needed. If the toxin is non-biodegradable, microbial strains that are resistant to the toxins must be enriched.

Microorganisms break down the complex hydrocarbons in the hazardous waste by using the three general mechanisms- aerobic and anaerobic respiration and fermentation. Aerobic process requires adequate supply of oxygen and the biodegradation process is rapid and more complete and there is no problematic end products like methane and hydrogen sulfide.

(a). Anaerobic Degradation:

It is a sequential, biologically destructive process in which the complex 'hydrocarbons' of hazardous wastes are converted into simpler molecules of 'carbon dioxide' and 'methane' by anaerobic microbes. The anaerobes use oxygen that is combined chemically with other elements, such as nitrates, carbonates, or sulphates. If nitrates are source of oxygen, nitrogen is formed and if sulfates, hydrogen sulfide is formed under anaerobic process. They also require complete absence of 'free oxygen' molecules. They require a narrow pH range than the aerobic systems. Anaerobic microbes are important for degrading the halogens (reductive dehalogenation) and

nitrosamine, reduction of epoxides to olefins, reduction of nitro groups and ring fission of aromatic structures. Several microbes have been identified which can break down the chlorinated substances like PCBs. Majority of the organochlorines appears to be biotransformed, forming conjugates with the soil humic matter.

The microbiology of anaerobic digestion consists of two distinct groups of anaerobic microorganisms- the '*acetogens*' and the '*methanogens*' present at several trophic levels. At the higher levels, organisms attack waste molecules through hydrolysis and fermentation, reducing them to simpler hydrocarbons methane (CH₄) and carbon dioxide (CO₂) in the last two steps of the destructive process. It is crucial that a delicate balance of population must be maintained between the two distinct groups of essential anaerobes- the *acetogens* and the *methanogens* in the final stages of reaction. The *acetogens* convert most of the hydrocarbons of waste to acetate, CO₂, and H₂ and some organic acids. The *methanogens* convert organic acids and CO₂ to CH₄ in the presence of the available H₂.

Hydrocarbons of Waste Organics (*Acetogens*) → Acetate + Organic Acids + CO₂ + H₂

Organic Acids + CO₂ + H₂ (*Methanogens*) → CH₄ + Byproducts + Energy

Anaerobic microbes are important for degrading the halogens (reductive dehalogenation) and nitrosamine, reduction of epoxides to olefins, reduction of nitro groups and ring fission of aromatic structures. Several microbes have been identified which can break down the chlorinated substances like PCBs. Majority of the organochlorines appears to be biotransformed, forming conjugates with the soil humic matter.

Advantages of Anaerobic Treatment

Anaerobic treatment has several attributes that are considered favorable in biological treatment-

1. Less energy is required as mechanical aeration is avoided. On the contrary a potential energy source methane (CH₄) is produced.
2. Significantly less land is required with smaller reactor volumes;
3. Extremely high destruction rates of wastes molecules can be achieved;
4. Solids (biological sludge) generation from the growth of biological cells (biomass production) is about 20 times less than the aerobic process, and therefore cost of disposal of solids are reduced;
5. Certain toxic organic wastes can only be destroyed by anaerobic digestion;
6. Many industrial wastewater lacks sufficient growth nutrients to support aerobic bacteria. Fewer nutrients are required to support the anaerobes;
7. There is complete elimination of 'off-gas' air pollution.

Disadvantages

1. Longer start-up time. While it is in days for aerobic process, it may be in months for anaerobic process;
2. May be more susceptible to upsets due to toxic substances;
3. It has potential for production of odors and corrosive gases;
4. It is not possible to remove biological nitrogen (N) and phosphorus (P);
5. May require alkalinity addition.

Anaerobic Digestion

Anaerobic digestion is a process of oxidizing chemically contaminated materials in closed vessels in the absence of air.

Table - 3
Some Hazardous Organic Chemicals Degradable By Anaerobic Digestion

1. Acetylsalicylic acid	13. Dimethoxy benzoic acid	25. Nitrobenzene
2. Acetaldehyde	14. Dimethylphthalate	26. Pentanol
3. Acetic anhydride	15. Ethyl acetate	27. Phenol
4. Acetone	16. Ferulic acid	28. Phthalic acid
5. Benzoic acid	17. Formaldehyde	29. Polyethylene glycol
6. Benzyl alcohol	18. Glycerol	30. Propanal
7. Butanol	19. Hexanoic acid	31. Propanol
8. Butylene glycerol	20. Methanol	32. Pyrogallol
9. Butyric acid	21. Methyl acetate	33. Sorbic acid
10. Catechol	22. Methyl acrylate	34. Valeric acid
11. Crotonic acid	23. Methyl ethyl ketone	35. Vanillic acid
12. Diethylphthalate	24. Methyl formate	36. Vinyl acetate

Sources: Various Publications of UNEP, WWF and WHO (1992-2002)

Table - 4
Some Organic Compounds Transformed through Anaerobic Process

Toxic Chemicals	Biological Transformation to Harmless Products
2,4-Dinitrophenol	→ Methane
2,4-Dimethyl phenol	→ Methane
Carbon tetrachloride	→ Degrade slowly
Aldrin	→ Dieldrin
DDT	→ Dechlorinated to DDE
Toxaphene	→ Reduced and dechlorinated
Halogenated Benzoates	→ Degraded after dechlorination to CO ₂ and CH ₄
Benzene	→ Degraded to CO ₂ and CH ₄
Isopropylbenzene	→ Degraded to CO ₂ and CH ₄
Ethylbenzene	→ Degraded to CO ₂ and CH ₄
Toluene	→ Degraded to CO ₂ and CH ₄

Sources: Various Publications of UNEP, WWF and WHO (1992-2002)

(b). Aerobic Degradation

Aerobic process requires adequate supply of molecular oxygen to decompose the organic matters and retrieve energy from it, which is needed by the bacteria to grow and multiply. The aerobic biodegradation process is rapid and more complete and there is no problematic end products like methane (CH₄) and hydrogen sulfide. Naturally occurring aerobic bacteria can decompose both natural and the synthetic hazardous organic materials to harmless CO₂ and water.

Organics + Molecular Oxygen (Aerobes) → CO₂ + H₂O + Other Products + Energy

Many hazardous organics are readily degraded aerobically, but because the depth of oxygen penetration in soils is limited, the rate and extent of biological detoxification is also limited. Mechanical aeration may sometimes be needed in biological treatment of landfill leachates and heavily contaminated wastewaters. The dissolved molecular oxygen concentration must be maintained at least at 2 pounds of oxygen per pound of solids destroyed. If the oxygen content is too low, portions of the solids may become anaerobic and adversely affect biological destruction.

Microbial Destruction of Hazardous Wastes

Microremediation technology is now used to detoxify the hazardous chemical wastes before dumping in the land-fills and closing it finally. It has also been proposed for application *in-situ* by injecting naturally occurring microbes, cultured bacteria, nutrients and oxygen into the soil column or groundwater to form zones of microbial activity. The chlorinated and non-chlorinated pollutants

(hazardous wastes) are broken down into carbon dioxide and water and a bacterial biomass is generated. Microremediation is typically able to reduce up to 90 % of some contaminants in field systems. The technology has been successfully used in Australia for treatment of PCBs at a site in NSW and for cleaning up 'chlorinated phenols' at a herbicide manufacturing plant near Melbourne. Animal manure were spread over the contaminated sites and in about an year full degradation of the contaminants occurred by mixed species of bacteria.

Table - 5
Degradable Organic Hazardous Wastes and the Associated Microorganisms

Hazardous Organic Compounds	Microorganisms Involved in Degradation
Phenylmercuric acetate	<i>Pseudomonas, Arthrobacter, Citrobacter, Vibrio</i>
Raw rubber, hevea latex	<i>Actinomyces</i>
Detergents	<i>Nocardia, Pseudomonas</i>
PCBs	Not identified
Malathion	<i>Trichoderma, Pseudomonas</i>
Endrin	<i>Arthrobacter, Bacillus</i>
Lindane	<i>Closteridium</i>
DDT	<i>Escherichia, Hydrogenomonas, Saccharomyces</i>
Dieldrin	<i>Mucor</i>

Sources: Various Publications of UNEP, WWF and WHO (1992-2002)

Plant – Assisted Microbial Degradation

Many organic compounds are degraded by microorganisms located in the rhizospheres (in the root zone) of plants. Microbial activity in the rhizosphere of plants is several-fold higher than in the bulk soil. The population of microflora present in the rhizosphere is much higher than present in the vegetation-less soil. (Suthersan, 1997). Certain plant roots release substances that are nutrients for microorganisms, bacteria and fungi, that provide favorable habitats for soil microbes to act (Cunningham and Ow, 1996).

A typical microbial population present in the rhizosphere, per gram of air-dried soil comprises –

- 1). Bacteria: 5×10^6
- 2). Actinomyces: 9×10^5
- 3). Fungi: 2×10^3

This results in increased biological activity of the microbes in the area immediately surrounding the root zone (rhizosphere). By encouraging a microbiologically active rhizosphere, the plants facilitate accelerated digestion (biodegradation) of wide variety of organic contaminants in the upper soil layers and / or wastewater / polluted water (Anderson *et al.* 1993). Interestingly, gram-negative bacteria appear to have some important metabolic capabilities for degrading xenobiotic chemicals not found in gram-positive bacteria.

A large number of bacteria and fungi are capable of degrading the hazardous chemical 'chlorobenzene' and mineralizing it. The end products are 2 & 4 – chlorophenol. Researches indicate that certain herbs stimulates microbial degradation of 'chlorinated organics' primarily via rhizospheric reductive dechlorination biodegradation processes. Chlorobenzene can undergo microbial dechlorination and the benzene ring can be converted to catechol, followed by ring fission or oxidation of the side chain.

Role of Environmental Biotechnology & Genetic Engineering In Improving Microbial Degradation by Producing Genetically Engineered Bacteria

With the advancement in our knowledge in environmental biotechnology some bacterium have been genetically engineered (tailored) to biodegrade those hazardous wastes and chemicals which otherwise cannot be degraded by the naturally occurring bacteria. In Germany, genetically engineered bacteria have been used to clean up polluted soils and oil spills in water. These bacteria eat pollutants, gobble them up and chemically alter them in their bodies. When pollutants

are finished the bacteria die leaving clean soil and a biomass of dead bacteria containing harmless minerals.

Table - 6

Genetically Engineered Bacteria Capable of Destroying Some Hazardous Chemicals

Bacterium	Chemicals Destroyed
1. <i>P. putida</i>	Camphor degradation
2. <i>P. oleovarans</i>	Alkane degradation
3. <i>P. cepacea</i>	2,4,5 – Trichlorophenoxyacetic acid degradation
4. <i>P. mendocina</i>	Trichloroethylene degradation
5. <i>P. diminuta</i>	Parathion (pesticide) degradation

Source: *Biotechnology and Biodegradation* ; Kamely et,al. (1990)

With genetic engineering it will be possible to enhance the reduction activities of hexavalent chromium {Cr (VI)} of the indigenous bacterial strains that express such activities at high levels under poor nutrient and stressful environmental conditions.

Development of Microbial Biosensors by Genetic Engineering

Genetic engineering has led to development of microbial 'biosensors'. These are 'genetically engineered bacteria'. They offer the potential to measure quickly, cheaply and accurately the degree of contamination of environmentally contaminated sites. More specifically, biosensors have been developed to detect heavy metals in contaminated sites. These microbial biosensors have used two distinct methods to detect heavy metal ions-

- 1). Protein (antibodies & enzymes) or
- 2). Whole cells (genetically modified or not)

Various biosensors have been designed to evaluate the heavy metal concentrations of metals like cadmium (Cd), mercury (Hg), nickel (Ni), copper (Cu), arsenic (As) and iron (Fe). (D' Souza, 2001; Verma & Singh, 2005; Bruschi & Goulhen, 2006).

Factors Affecting Microbial Remediation

Microbial remediation depends upon the presence of appropriate micro-organisms in the correct amounts and in combinations and in appropriate environmental conditions. Bio-stimulation & bio-augmentation are two essential factors influencing bioremediation by microbes.

Bio-stimulation

It is the addition of nutrients (usually source of carbon, nitrogen & phosphorus), oxygen or other electron donors or acceptors. These amendments serve to increase the number or activity or both, of naturally occurring micro-organisms available for bioremediation. Amendments can be added in either liquid or gaseous forms, *via* injection. Liquids can be injected into shallow or deep aquifers to stimulate the growth of micro-organisms involved in bioremediation. Anderson et al. (2003) removed uranium (U) from groundwater of an uranium-contaminated aquifer by biostimulating the *in-situ* activity of *Geobacter* spp..

Bio-augmentation:

It is the addition of microorganisms that can bio-transform (usually toxic metals) or biodegrade (toxic organic compounds) a particular contaminant.

Success Story of Microbial Remediation for Environmental Cleanup in UK

The town of Salem in New Hampshire, UK, faced a big environmental problem from the decommissioned wastewater treatment plant in 1987 (constructed in 1964) which showed symptoms of soil and groundwater contamination by 'chlorinated solvents' e.g. the chlorinated aliphatic hydrocarbon (CAH) at unacceptable levels. The potential impact of the CAHs could have

been on the 30 bedrock water supply wells located within a 3 km radius of site. The major challenge for the cleanup was the large area (2-3 ha) of contaminated land and groundwater.

Bioremediation program was started in the year 2000 and included injections of 5 bio-stimulant (mixture of yeast & lactose) at the site. A total of more than 200,000 lb (90,720 kg) of bio-stimulant was injected during the program to enhance the anaerobic destruction of chlorinated solvents in groundwater. Bioremediation successfully destroyed the chlorinated solvents mass at an expenditure of just US \$ 300,000.

It saved the municipalities an estimated US \$ 2 million in cleanup cost by 'conventional treatment' approach (pump and treat) that would have required up to 20 extraction wells, a treatment building with air-stripper, treatment of the air stripper discharge gas, and at least 10 years of system operation and monitoring. (Schaffner, 2004).

The Limitations of the Microbial Technology

A limitation of the use of microbial technology for metal remediation is that although the metals are bound to microbes, they can be released back into the soil soon after decomposition of the microbes upon their death and decay and still be present in soil. The *in-situ* microbial remediation strategies may need to combine with phyto-remediation strategies by suitable hyper-accumulator plants that can effectively uptake the metals (made bioavailable by the microbes) from soil and bioaccumulate them in their roots & shoots, thus preventing their recycling in soil. Genetic engineering can resolve the problem by engineering 'bacteroids' (with metal binding peptides) in root nodules of plants. This has been termed as 'symbiotic engineering'.

II. Phytoremediation of Polluted Lands & Soils by Adapted Plants

Phytoremediation (Greek: *phyton* = plant; Latin: *remediare* = remedy) is emerging 'green bioengineering technology' for environmental cleanup that uses plants to remove pollutants from the soil or to render them harmless. It takes advantage of the natural abilities of plants to take up, bioaccumulate, store or degrade organic or inorganic substances. They are cost-effective, aesthetically pleasing, passive, solar-energy driven and pollution abating nature's biotechnology meeting the same objectives of fossil-fuel driven and polluting conventional technology. Plants involved in phytoremediation are adapted to thrive in very harsh environmental conditions of soil and water; absorb, tolerate, transfer, assimilate, degrade and stabilize highly toxic materials (heavy metals, radionuclides and organics such as solvents, crude oil, pesticides, explosives, chlorinated compounds and polyaromatic hydrocarbons) from the polluted soil and water. The organic pollutants may ideally be degraded to simpler compounds like carbon dioxide (CO₂) and water (H₂O), thus reducing the environmental toxicity significantly. Possibly due to their static (non-mobile) nature, plants had to evolve their survival modes even in odd environments including lands contaminated with xenobiotic substances.

Large scale phytoremediation of the contaminated sites has been achieved for heavy metals , organic xenobiotics, and radionuclides. Phytoremediation has been carried out commercially or has been demonstrated as pilot scale studies on nearly 200 contaminated sites (involving all categories of organic and inorganic chemical and radiological contaminants) in the U.S. The plant biomass eventually becomes valuable biological source for the community or for the plant based industries. The roots, shoots and leaves may be collected (harvested) and incinerated to decompose the contaminants.

Contaminants Suitable for Removal by Phytoremediation

The following recalcitrant organic & inorganic pollutants & radioactive contaminants are best suited for phytoremediation technology-

1. Benzene
2. Toluene

3. Ethylbenzene
4. Xylene
5. Chlorinated Solvents (TCE & PCE)
6. Chlorinated Pesticides
7. Organophosphates
8. Insecticides
9. Polyaromatic Hydrocarbons (PAHs)
10. Polychlorinated Biphenyls (PCBs)
11. Nitrotoluene ammunition wastes
12. Petroleum Hydrocarbons
13. Nutrients like nitrate, phosphate and ammonium
14. Toxic heavy metals (Cadmium, Lead, Nickel, Chromium, Mercury, Arsenic etc.)
15. Hazardous Air Pollutants e.g. Oxides of Sulfur & Nitrogen
16. Radionuclides (Cesium, Strontium, Uranium etc.)

Commercial Organizations Involved in Phytoremediation & Business Potential

In the last few years, several commercial companies practicing phytoremediation biotechnology for environmental cleanup have come up in US and Europe. Important among them are Phytotech (USA), Phytoworks (USA), Earthcare (USA), Ecolotree (USA), BioPlanta (Germany), Piccoplant (Germany), Plant techno (Italy), Slater (UK) and Aquaphyte Remediation (Canada). A number of large industrial companies, mainly the oil and chemical industry, are also either employing phytoremediation technology or supporting it through appropriate funding.

Table - 7
Phytoremediation Markets in the U.S. in 2005

Phytoremediation Works Carried Out	Value in Million US Dollars
1. Removal of Heavy Metals from Contaminated Soil	70 – 100
2. Removal of Heavy Metals from Contaminated Groundwater	1 – 3
3. Removal of Heavy Metals from Wastewater	1 – 2
4. Removal of Radionuclides	40 – 80
5. Removal of Organics from Contaminated Groundwater	35 – 70
6. Others	65 – 115
Total	214 – 370 Million Dollars

Source: Eapen et al. (2006)

Sources & State of Heavy Metals in Soil and their Bioavailability for Phytoremediation

Developmental activities and their byproducts are the sources of metal contamination of our soils & water bodies. Main sources are -

- 1). Metalliferous Mining & Smelting : Arsenic (As), cadmium (Cd), lead (Pb) & mercury (Hg);
- 2). Industries (Metal & Electroplating, Saw Mills etc.) : Chromium (Cr), cobalt (Co), copper (Cu), Zinc (Zn), nickel (Ni), As, Cd & Hg;
- 3). Atmospheric Deposition from Industries & Automobiles: Uranium (U), As, Cd, Cr, Cu, Pb & Hg;
- 4). Agricultural activities: Selenium (Se), As, Cd, Cu, Cr, Pb, Zn & U
- 5). Waste disposal (MSW landfills & wastewater treatment plants): As, Cd, Cr, Cu, Pb, Hg, & Zn
- 6). Wastewater from electroplating, paint and cement industries discharge heavy metals like cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg) nickel (Ni), zinc (Zn) and arsenic (As).

Heavy metals are defined as metals having density more than 5 g/cm³. The group of heavy metals are about 65. Some heavy metals, such as cobalt (Co), chromium (Cr), copper (Cu), nickel (Ni) & zinc (Zn) are essential and serve as micronutrients for plants like calcium (Ca), potassium (K), magnesium (Mg), manganese (Mn), iron (Fe) and sodium (Na). They are used for redox-processes, as components of various enzymes and for regulation of osmotic pressure in cells. Other metals have no biological role e.g. cadmium (Cd), lead (Pb), mercury (Hg), aluminum (Al), gold (Au) and silver (Ag). Some of them e.g. Cd²⁺, Ag²⁺, Hg²⁺ tend to bind the SH groups of enzymes and inhibit their activity (Turpeinen, 2002).

Heavy metals are one very significant category of the industrial pollutants which are unique being selectively toxic, persistent and non-biodegradable. At high concentrations, both essential and non-essential metals can damage cell membrane, alter enzyme specificity, disrupt cellular function, and even damage the structure of DNA. They have been linked to birth defects, cancer, skin lesions, retardation leading to disabilities, kidney & liver damage and several other health problems. Remediation of metals presents a different set of problems when compared to organics. Organic compounds can be degraded while metals normally need to be physically removed by excavation or immobilized by plant roots.

Metal Concentrations in Soil & Their Bioavailability for Uptake by Plants

Metal concentration in soil can range from < 1 mg / kg to as high as 100,000 mg / kg (Eapen et al. 2006). Metals are often tightly bound to soil particles. Cations of heavy metals are often bound to soil particles because of soil cations exchange capacity. Binding mechanisms of heavy metals is complex and vary with composition of soil, soil acidity and redox conditions. The binding affinity of cations reduces cation movement in vascular plants, particularly in the negatively charged xylem cells. The slow desorption of heavy metals in soil has been a major impediment to the successful phytoremediation program of the metal contaminated sites.

Metal bioavailability is often low in soil systems. Metals are more bioavailable at acidic pH values. Generally, only a fraction of soil metal is readily available (bio-available) for the plant uptake. In the soil, the organic matter and the clay mineral content are important factors that can reduce metal bioavailability. Clays, with high cation exchange capacities, such as montmorillonite, appear to reduce metal bioavailability & toxicity. In a study used to investigate the effect of cadmium (Cd) on microbial biodegradation of toxic organic compound 'phenanthrene', a total of 394 mg cadmium (Cd) per kg of soil was added, but only 3 mg cadmium (Cd) / L were actually bioavailable. Similarly only 1 % of the total zinc (Zn) used in a study was present in the aqueous phase. In another study, 20 mg / L of soluble metal initially amended was below the detection limit i.e. 0.03-0.04 mg/L. At 100 mg / L of total metal amended, only 1mg cadmium (Cd) / L, and less than 0.12 mg copper (Cu) / L and chromium (Cr) /L were found in the aqueous phase. (Sandrin & Hoffman, 2006). Plant roots also increase metal bioavailability by extruding protons to acidify the soil and mobilize the metals. (Zhao et al., 2001). By decreasing the pH below 5.5, metal bioavailability for plant roots can be enhanced. But that may also inhibit plant growth.

The Soluble & Mobile Metals

The bulk of the metal in soil is commonly found as 'insoluble' compounds unavailable for transport into the plant roots from the aqueous phase. Metals, which are taken up by plants are those which exist as soluble components in the soil solution or are easily desorbed or solubilized by root exudates. Solubility of metals is dependent on soil characteristics and is strongly influenced by pH of the soil and the degree of complexation with soluble ligands.

However, some metals, such as zinc (Zn) and cadmium (Cd) are considered as 'easily mobile' heavy metals as they occur primarily as 'soluble or exchangeable' form and readily bioavailable. Copper (Cu), chromium (Cr) and molybdenum (Mo) are mainly bound in silicates and thus are 'slightly mobile' and available. Others such as lead (Pb), occur as 'insoluble precipitate' (phosphates, carbonate & hydroxy-oxides) in soil which is significantly much less mobile and largely unavailable for plant uptake.

Table - 8
Range of Heavy Metals in Contaminated Soil

Metals	Contamination Range ($\mu\text{g}/\text{kg}$)	Regulatory Limit (mg/kg)
Lead (Pb)	1000 – 6,900,000	600
Arsenic (As)	100 – 102,000	20
Cadmium (Cd)	100 - 345,000	100
Chromium (Cr)	5.1 – 3,950,000	100
Copper (Cu)	30 – 550,000	600
Mercury (Hg)	0.1 - 1,800,0000	270
Zinc (Zn)	150 – 5,000,000	1,500

Source: Eapen et, al; In SN Singh & RD Tripathi (ed.) ‘ *Environmental Bioremediation Technologies*’; Springer (2006).

Environmentally Adapted Plants Used in Phytoremediation: Gift of Nature

Several plants are being identified and trialed to be used in phytoremediation task. The most versatile plant species that has been identified after rigorous laboratory and field experiments are-

Table - 9

Some Environmentally Adapted Plant Species Identified for Phytoremediation Works

1. Sunflower (*Helianthus anus*);
2. Vetiver grass (*Vetiveria zizanioides*);
3. Indian Mustard (*Brassica juncea*)
4. Poplar tree (*Populus Spp.*);
5. Brake fern (*Pteris vittata*)
6. Barmuda grass (*Cynodon dactylon*);
7. Bahia grass (*Paspalaum notatum*);
8. Cumbungi (*Typha angustifolia*);
9. Redroot pigweed (*Amaranthus retroflexus*);
10. Kochia (*Kochia scoparia*);
11. Foxtail barley (*Hordeum jubatum*);
12. Switch grass (*Panicum variegatum*);
13. Musk thistle (*Carduus nutans*);
14. White raddish (*Raphanus sativus*);
15. Catnip (*Nepeta cataria*);
16. Big bluestem (*Andropogan gerardii*)
17. Alpine pennycress (*Thlaspi Spp.*);
18. Canada wild rye (*Elymus candensis*)
19. Nightshade (*Solanum nigrum*);
20. Wheat grass (*Agropyron cristatum*)
21. Alfa-alfa (*Medicago sativa*);
22. Tall Fescue (*Festuca anundinacea*)
23. Lambsquarters (*Chenopodium berlandieri*);
24. Reed grass (*Phragmites australis*);
25. Tall wheat grass (*Thynopyron elongatum*);

26. Rhodes grass (*Chloris guyana*);
27. Flatpea (*Lathyrus sylvestris*);
28. Carrot (*Daucus carota*)
29. Willows (*Salix viminalis*)
30. Periwinkle (*Cathranthus roseus*);

A number of them are still wild, while others have been domesticated for their food value. They are highly salt and toxicity tolerant, have extensive root binding system and were tried in the rehabilitation works. Number of them readily absorb, volatilise and / or metabolise compounds such as tetrachloroethane, trichloroethylene, metachlor, atrazine, nitrotoluenes, anilines, dioxins and various petroleum hydrocarbons. Ideal species for the job are members of the grass family Graminea and Cyperacea and the members of families Brassicaceae (in particular the genera *Brassica*, *Alyssum* and *Thlaspi*), and Salicaceae (in particular willow and poplar trees). Grasses such as the vetiver, clover and rye grass, Bermuda grass, tall fescue etc. have been particularly effective in the remediation of soils contaminated by heavy metals and crude oil (Kim, 1996). Hybrid poplar, willows, sunflower, alpine pennycress, clover, Indian mustard, redroot pigweed and ferns have been plants of choice for many commercial phytoremediation applications.

The Hyper-accumulator Plants

There are species which can bioaccumulate very high concentrations of metals in their stem and leaves. These are 'hyper-accumulators' species. More than 400 plant species ranging from herbs to trees have now been identified from the families of Euphorbiaceae, Asteraceae, Brassicaceae and Rubiaceae which are hyperaccumulators of metals (Eapen et al., 2006). Most have been found in the contaminated areas of temperate Europe and the USA, New Zealand and Australia. They can accumulate 100 - 500 times higher levels of metal concentrations in their above-ground parts. (Chaney et al. 1997). Plants are called hyperaccumulators when they can accumulate more than 0.1% Pb, Co, Cr or more than 1 % Mn, Ni, or Zn in plant shoots when grown in their natural habitats (Baker & Brooks, 1989). The degree of accumulation of metals like Ni, Zn, and Cu observed in hyperaccumulator species often reaches 1 – 5 % of their dry weight. (Raskin et al., 1997). Hyper-accumulator plants tends to be contaminant specific. No plant species has been found that demonstrate a wide spectrum of hyper-accumulation (Watanabe, 1997).

These hyper-accumulator plants possess 'genes' that regulate the amount of metals taken up from the soil by roots and deposited other locations within the plants. There are number of sites in the plant that could be controlled by different genes contributing to the hyper-accumulation genetic traits. These genes govern processes that can increase the 'solubility of metals' in the soil surrounding the roots as well as the 'transport proteins' that move metals into the root cells.

Table - 10

Some Known Hyperaccumulator Plants for Different Metals		
Metals and Plant Species		Concentration of Metal Accumulated (mg/kg)
A. Nickel (Ni)		
<i>Thlaspi spp.</i>	(Brassicaceae)	2000 – 31,000
<i>Alyssium spp.</i>	(Do)	1280 – 29,400
<i>Berkheya codii</i>	(Asteraceae)	11,600
<i>Pentacalia spp.</i>	(Do)	16,600
<i>Psychotria corinota</i>	(Rubiaceae)	25,540
B. Zinc		
<i>Thlaspi caerulescense</i>	(Brassicaceae)	43,710
<i>Thlaspi rotundifolium</i>	(Do)	18,500
<i>Dichopetalum gelonioides</i>	(Do)	30,000
C. Cadmium		

<i>Thlaspi caerulescense</i>	(Brassicaceae)	2,130
D. Lead		
<i>Minuartia verna</i>	(Caryophyllaceae)	20,000
<i>Agrostis tenuis</i>	(Poaceae)	13,490
<i>Vetiveria zizaniodes</i>	(Cyperaceae)	> 1500
E. Cobalt		
<i>Crotolaria cobalticola</i>	(Fabaceae)	30,100
<i>Haumaniastrum robertii</i>	(Lamiaceae)	10,232
F. Copper		
<i>Ipomea alpina</i>	(Convolvulaceae)	12,300
G. Manganese		
<i>Maystenus sebertiana</i>	(Celastraceae)	22,500
<i>Maystenus bureaviana</i>	(Celastraceae)	19,230
<i>Macadania neurophylla</i>	(Proteaceae)	55,200
H. Selenium		
<i>Astragalus racemosus</i>	(Leguminosae)	1,49,200
<i>Lecithis ollaria</i>	(Lecithidiaceae)	18,200
I. Arsenic		
<i>Pteris vittata</i>	(Fern)	

Source: Eapen et, al; In SN Singh & RD Tripathi (ed.) 'Environmental Bioremediation Technologies'; Springer (2006).

It may be possible to revegetate closed hazardous landfills, industrial sites, and dis-used mine areas with hyper-accumulator species. Recent researches have indicated that the range of soil types suitable to hyper-accumulators can be widened by the incorporation of essential nutrients (e.g. N and P), and also chelating agents like EDTA which increase the plant availability of metals, particularly those in clay-textured soil.

Mechanism of Phytoremediation of Contaminated Soils

The plants act as 'accumulators' and 'excluders'. Accumulators survive despite concentrating contaminants in their aerial tissues. They biodegrade or biotransform the contaminants into harmless forms in their tissues. Adaptations to tolerate toxic compounds in plants appears to be processes like immobilization, exclusion, chelation & compartmentalization. These mechanisms not only control the uptake and accumulation of essential and non-essential heavy metals, but also detoxify them.

Phytoremediation of organic and inorganic contaminants involves either physical removal of compounds (phyto-extraction) or their bioconversion (phyto-degradation or phyto-transformation) into biologically inert forms. The conversion of metals into inert forms can be enhanced by raising the pH (e.g. through liming), or by addition of organic matter (e.g. sewage sludge, compost etc.), inorganic anions (e.g. phosphates) and metallic oxides and hydroxides (e.g. iron oxides). The plants themselves can play a role here by altering soil redox conditions and releasing anions and /or lignins (Salt *et al.* 1995).

Phytoremediation technology works mainly through –

1). Phytoextraction and Phytoaccumulation of Contaminants

Plant roots uptake (extract) metal or radioactive contaminants from the soil, polluted water and the wastewater, translocate and accumulate them in their roots. Plant roots absorb both organics and inorganics. Considerable amount of the contaminants may be translocated above through the xylem and accumulated in the shoots and leaves. The radionuclide uptake by plant roots need

not necessarily result in translocation to shoots. The majority of cesium-137 (Cs^{137}) taken up by plants tends to be localized in roots. However, 80 % of the strontium-90 (Sr^{90}) taken up by the plants, is usually localized in the shoots. (Eapen et al., 2006).

Phytoextraction exploits vascular plant's natural ability to take up variety of chemical elements (macro & micronutrients) through the root system, deliver them to the vascular tissues, and to transport and compartmentalize in different organs.

Major limiting factor for phytoextraction are lower availability of metals in soil and poor translocation from roots to shoots. The bioavailability of a given compound for phytoextraction depends upon the lipophilicity and the soil or water conditions e.g. pH and clay content. Addition of soil amendments increased the metal availability in solutions to more than 10-fold for Cs^{137} and 100-fold for lead (Pb) and uranium (U). (Huang et al. 1998).

This technique has been effectively used by Phytotech Inc. (USA) for removal of lead (Pb) and cadmium (Cd) from contaminated soil. Excessive selenium (Se) in farm soils is also successfully remediated by this technology. (Eapen et al., 2006). Above-ground biomass, loaded with metals or radionuclides is harvested, processed for volume reduction and further element concentrations & safely recycled to reclaim metals of economic importance (industrial uses), or disposed off as 'hazardous waste' in secured landfills or incinerated in case of radionuclides.

Table - 11
Plants Identified With Potential for Phytoextraction of Metals

Plant Species	Metals Extracted
1. Indian mustard (<i>Brassica juncea</i>)	Cd, Cr, Cu, Ni, Zn & Pb
2. Sunflower (<i>Helianthus annuus</i>)	Do
3. Rapeseed	Do
4. Amaranthus (<i>Amaranthus retroflexus</i>)	Do
5. Chenopodium (<i>Chenopodium album</i>)	Do

2). Phytostabilization (Immobilization of Contaminants):

Phytostabilization is stabilizing process for contaminated soils and sediments in place using plants, thus preventing the lateral or vertical migration of toxic metals by leaching. Certain plant species immobilize contaminants in the soil and groundwater through absorption by and adsorption on to roots or precipitation within the root zone (rhizosphere). Plants capable of tolerating high level of metal contaminants and having efficient growth rate with dense root systems (to bind / sorb contaminants) & canopies are preferred. Trees which transpire large amounts of water for hydraulic control (e.g. poplar) and grasses with fibrous roots (e.g. vetiver grass) to bind & hold soil are best suited for phytostabilization. Generally, plants suitable for 'soil conservation' is good for phytostabilization of soil contaminants.

This technique is best applicable in phytostabilizing metal contaminants of waste landfill sites (e.g. Pb, Cd, Zn, As, Cu, Cr, Se, and U) where the best option is to immobilize them *in situ*. Addition of manure, digested sewage sludge, straw etc. to inorganic waste sites may help in binding of metals. Supplementing with limes (CaO) and limestones ($CaCO_3$) may help neutralizing acid soils that help in binding of cationic metals with inorganic wastes.

Phytostabilization is particularly suitable for stabilizing radionuclide-contaminated sites, where one of the best alternative is to hold contaminants in place to prevent 'secondary contamination' and exposure to humans and animals. Plants roots also help to minimize water percolation through soil, thus reducing radionuclide leaching significantly. The technology is very suitable for controlling tailings in arsenic, zinc, cadmium and uranium mining areas.

Success Story

Mine tailings at Superfund site at South Dakota in the US containing up to 1000 mg/kg of arsenic (As) and lower concentrations of cadmium (Cd); smelter in Kansas, U.S, with up to 200,000

mg/kg of zinc (Zn) could be phytostabilized by decreasing vertical migration of leachate to groundwater using hybrid poplar (*Populus* spp.) trees (Hse, 1996).

3). Phytovolatilization (Evapo-transpiration of Detoxified Contaminants):

Phytovolatilization exploits a plant's ability to transpire large amount of water from their leaf pores (stomata). Plants absorb and transpire the impurities from soil through their aerial organs (leaves). This system is a combination of hydraulic control and enhanced evapotranspiration.

Phytovolatilization is a mechanism by which plants convert a contaminant into volatile form and transpire the detoxified vapor through their aerial organs. Rate of transpiration is a key factor for this technology. For a 5 year old poplar tree (*Populus* spp.) it was estimated to be 26 gpd (gallon per day) . A single willow tree (*Salix* spp.) has been found to transpire 5000 gpd and an individual cottonwood tree transpire 50 to 35 gpd.

Some contaminants like selenium (Se), mercury (Hg) and volatile organic compounds (VOCs), can be released through the leaves into the atmosphere. (Cunningham and Ow, 1996). It is also being used for remediation of tritium (H^3) contaminated water. Some transgenic plants e.g. *Arabidopsis thaliana* have been found to convert organic and inorganic mercury salts to the volatile and elemental form. (Watanabe, 1997).

4). Rhizofiltration:

Rhizofiltration is the use of plant roots to sorb, concentrate or precipitate metal contaminants from aqueous medium. Although the technology is more suitable for decontamination of polluted water or removal of organic and inorganic impurities from wastewater by the use of aquatic plants, terrestrial species can also be used by growing hydroponically or on floating platforms in treatment ponds. An ideal plant for rhizofiltration should quickly produce significant amount of root biomass with large surface area when grown hydroponically.

5). Phytostimulation (Microbe Stimulated Phytoremediation)

Soil microbes have been found suitable to enhance the bioavailability of metals for phytoremediation and increase the phytoremediation potential of plants by complimenting the process in many ways. Soil microbes may degrade organic pollutants into simpler organic compounds and supply them as nutrient to plants for enhanced phytoremediation of the polluted site.

Microbial activity in the rhizosphere of plants is several-fold higher than in the bulk soil. The population of microflora present in the rhizosphere is much higher than present in the vegetation-less soil. It is due to the presence of nutrients from the root exudates of plants. (Suthersan, 1997).

A typical microbial population present in the rhizosphere, per gram of air-dried soil comprises – bacteria 5×10^6 , actinomycetes 9×10^5 , and fungi 2×10^3 (Schnoor et al., 1995).

The rhizosphere in plants is divided into two general areas, the inner rhizosphere at the root surface and the outer rhizosphere immediately adjacent to soil. The microbial population is larger in the inner zone where the root exudates are concentrated. A large variety of exudates are secreted from roots in the form of sugars, amino acids and essential vitamins. Root exudates may also include acetates, esters and benzene derivatives. Enzymes are also present in the rhizosphere and this may act as substrates for the microbial population. Enzymes secreted by plant roots or the microbial community in the rhizosphere comprise esterases different oxidoreductases (phenoloxidases and peroxidases). Plant peroxidases are exuded by some members of Fabacea, Graminae and Solanaceae. White radish (*Raphanus sativus*) and horse radish (*Armoracia rusticana*) secrete 'peroxidase' while the aquatic green algae *Nitella* & *Chara* secrete 'laccase'. (Dias et al., 2006).

Chemolithotrophic bacteria have been shown to enhance metal availability. Several strains of *Bacillus* and *Pseudomonas* have been reported to increase cadmium accumulation by *Brassica juncea*. Naturally occurring *Rhizobacteria* were found to promote selenium (Se) and mercury (Hg)

bio-accumulation in plants growing in wetlands. (Singh et. al., 2006). These microbes grow much better if organic manures are added to the soil.

Uptake of hydrophobic xenobiotics of larger size can be facilitated by primary microbial biodegradations in the rhizosphere. The hydrophobic persistent organic pollutants like 'polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with present log K_{ow} value above 4 have been reported to be taken up by roots and transported to shoots in *Cucurbita pepo*. (Singh et. al., 2006).

Plants which have been identified to promote rhizosphere degradation of petroleum compounds are –

1. Big bluestem (*Andropogon gerardii*),
2. Indian grass (*Sorghastrum nutans*),
3. Switch grass (*Panicum virgatum*),
4. Canada wild rye (*Elymus canadensis*),
5. Western wheat grass (*Agropyron smithic*) and some more.

Aprill & Sims (1990) studied the degradation of 4 PAHs viz. benz(a)anthracene, chrysene, benzo(a)pyrene and dibenz (a,h)anthracene in the root zone of mixture of 8 prairie grasses and found that the disappearance of the PAHs were consistently greater in the vegetated columns of soil than in the unvegetated controls.

Plants which have been identified to enhance microbial degradation of PAH compounds in the rhizosphere are –

1. Alfalfa (*Meticago sativa*),
2. Fescue (*Festuca anundinacea*),
3. Big bluestem (*Andropogon gerardii*), and
4. Sudan grass (*Sorghum vulgare sudanense*).

Wheat grass (*Agropyron cristatum*) has been found to enhance microbial degradation of 'pentachlorophenol' (PCP) and *Phragmites australis* the microbial degradation of phenols in the soil. Mackova et al. (1997), reported effective degradation of PCBs (Polychlorinated Biphenyls) by cells of *Solanum nigrum* that were infected with bacterial strains of *Agrobacterium tumefaciens* and *A. rhizogenes*. Hairy root cultures of *S. nigrum* proved capable of transforming PCBs under controlled conditions. Plant cells proved capable of transforming PCBs even after growth had stopped. The red mulberry (*Morus rubra*) appears to be another good plant for enhancing microbial degradation of PCBs.

Root exudates in the form of compounds structurally analogous to the chemicals utilized by microorganisms, would enhance the activity of the bacteria present in the rhizosphere accelerating the degradation process. Certain phenolic compounds in the root exudates has been found to support growth of PCB-degrading bacteria and serve as structural analogs for PCB degradation. (Zhao et al., 2001).

Sometimes inoculation of plant soil by microbes enhance the phyto-degradation of organic contaminants present in the soil. Degradation of 'pentachlorophenol' (PCP) was accelerated by the prosomillet after the soil in which it was growing was inoculated with *Pseudomonas* strain SR 3.

6). Mycorrhiza Assisted Phytoremediation

It is generally considered that the majority of plants growing under natural conditions have symbiotic relationship with mycorrhizal fungi roots, which results in increase in root surface area and enhanced absorption of nutrients. Mycorrhizal fungi have been reported in plants growing on heavy metal contaminated soils/sites indicating its heavy metal tolerance and a potential role in heavy metal phytoremediation. Mycorrhizal species *Glomus*, *Gigaspora* and *Entrophospora*, have been reported to be associated with most of the plants growing in the heavy metal polluted soils indicating its obvious role in assisting the plants in tolerating and removal of metals by phytoremediation. (Khan et. al., 2000).

Cellular Mechanism of Uptake & Detoxification of Heavy Metals by Plants : Role of Metal Chelators & Transporter Proteins

Chelation of heavy metals in the cytosol by high affinity ligands followed by subsequent compartmentalization of the ligand-metal complex is thought to be a potentially very important mechanism of heavy metal detoxification and tolerance by plants. It is a process in which the metal cations binds to a chemical compound, resulting in an 'uncharged metal complexes' that become easily bioavailable for uptake & transport in plants. Several natural and synthetic chelators are known. Among the natural chelators are organic acids, amino acids, phytochelatins (PC) and metallothionein (MT) and among the synthetic ones are ethylene diamine tetra acetic acid (ETDA) & ethylene glycol tetra acetic acid (EGTA). The use of natural & synthetic chelates has been shown to dramatically stimulate lead (Pb) accumulation in plants. They prevent Pb precipitation and keep the metal as soluble chelate - Pb complex bioavailable for uptake by plant roots & its transport to shoots. (Sriprang, 2006). Addition of synthetic chelates like EDTA has been found to be very effective in facilitating the plant uptake of Cd, Cu, Zn & Ni. (Raskin et al. 1997). Malic & Citric acids are two organic acids in plant roots that have been shown to make uncharged complexes with heavy metals.

Uptake and Translocation of Metals from Soil to Roots and to the Shoots : Role of Transporter Proteins

Accumulator plants must have the ability to uptake metals from the soil to roots and translocate them to the shoots at high rates. Because of their charge, metal ions cannot move freely across the cellular membrane, which are lipophilic structures. Membrane proteins mediate ion transport into cells and they are known as 'transporter proteins' (TP). They contain binding domains, which binds to specific ions from extracellular space through the hydrophobic environment of the membrane into cell. (Sriprang & Murooka, 2006). Transporter proteins are usually specific and one TP usually binds with an individual heavy metal only. Thus, Zinc (Zn) transporter protein (ZnTP1) can only uptake and bind with Zn but not with Cd.

Storage of Metals in Plant Cells : The Vacuolar Compartmentalization for Tolerance

The vacuole in plant cells appears to be the main storage site for metals. Compartmentalization of metals in the vacuole appears to be the part of the tolerance mechanism of some metal hyper-accumulator plants. The nickel (Ni) hyper-accumulator *T. goesingensis* enhances its Ni tolerance by compartmentalizing most of the intracellular leaf nickel components into the vacuole. (Sriprang & Murooka, 2006).

Role of Phytochelatins (PCs)

PCs are metal-binding peptides in plants made of three amino acids (Glu, Cys & Gly) and are know to play an essential role in the heavy metal detoxification by chelating heavy metals in the cytosol & sequestering PC- metal complexes in the plant cell vacuoles via transport across the tonoplast. Production of PC is activated / stimulated by heavy metals and the best activator is cadmium (Cd) followed by Ag, Bi, Pb, Zn, Cu, Hg and Au cation. It has been studied that Cd stimulates synthesis of PCs, which rapidly form a low molecular weight Cd-PC complex. The Cd-PC complex is transported into the vacuole by a Cd/H antiport and an ATP – dependent PC-transporter protein. A gene (*Hmt1*), which codes for PC-transporter protein in yeast was isolated. The *Hmt1* gene encodes for a member of family of ATP-binding cassette (ABC) membrane transport proteins that is located in the vacuolar membrane. The gene (*Hmt1*) product i.e. a protein (Hmt1) is responsible for transporting the Cd-PC complex into the vacuole. (Sriprang & Murooka, 2006).

PCs contain strongly nucleophilic sulfydryl group and thus can react with many toxic specie within the plant cell such as the free radicals, active oxygen species, and cytotoxic electrophilic organic xenobiotics and obviously heavy metals.

Role of Metallothioneins (MTs)

MTs are super cysteine-rich metal-chelating proteins in plants meant to sequester toxic heavy metals such as cadmium (Cd). MTs bind readily with Zn, Hg, Cu, Cd & Ag.

Fate of Chemicals Absorbed in Plant Roots & Shoot Cells

Contaminants once absorbed inside the plant roots are either degraded into simple and harmless compounds or transformed into some inert harmless materials. This they do with the aid of 'enzymes' (described as biological catalysts) or symbiotic microbes inhabiting in their roots.

Phytodegradation

Certain plant species breakdown the contaminants after absorbing them. This they do through enzyme-catalyzed metabolic process within their root or shoot cells. Others breakdown the contaminants in the substrate itself by secreting enzymes and chemical compounds. The enzymes secreted are usually dehydrogenases, dehalogenases, oxygenases peroxidases, phosphatases, laccases and reductases. Plants can also degrade 'aromatic rings' in complex organics with the aid of enzymes. Phenols have been found to be degraded by radish (*Raphanus sativus*) and potato (*Solanum tuberosum*) that contain 'peroxidase' enzymes.

The biodegraded constituents are converted into insoluble and inert materials that are stored in the lignin or released as exudates (Watanabe, 1997). Enzymes cytochrome P450, peroxygenases & peroxidases are involved in plant oxidation of xenobiotics. Other enzymes like Glutathione s-transferase, carbo-oxylesterases, o-glucosyltransferases and o-malonyltransferases are associated with xenobiotic metabolism in plant cells & transportation of intermediates.

Several plants biodegrade contaminants with the aid of microbes which live in symbiotic association on their roots.

Phytotransformation

Several inorganic and organic contaminants once absorbed inside the root, may become biochemically bound to cellular tissues (biotransformed), in forms that are biologically inert or less active (Watanabe, 1997). In many cases, plants have the ability to metabolize organic pollutants by phytotransformation and conjugation reactions followed by compartmentalizing products in their tissues. Poplar trees (*Populus* Spp. have been found to transform 'trichloroethylene' in soil and groundwater.

Table - 12
Plant Enzymes Implicated in Phytodegradation & Phytotransformation of Organic & Inorganic Contaminants

Enzymes	Contaminants Degraded / Transformed into Less Toxic Forms
1). Phosphatase	Organophosphates
2). Aromic Dehalogenase	Chlorinated aromatic compounds (e.g. DDT, PCBs etc.)
3). O-demythlase	Metaoalchor, Alachlor
4). Cytochrome 450, Peroxidases & Peroxygenases	PCBs
5). Glutathione s-transferase, carbo-oxylesterases o-glucosyltransferases, o-malonyltransferases	Xenobiotics
6). β -cyanoanaline synthase	Cyanide

Sources : (Sandermann, 1994; Macek et al., 2000; Prasad, 2006).

Phytodetoxification of Toxic Cyanide (CN) by Phytotransformation

Plants possess the necessary mechanism to detoxify the cyanides from mining wastes resulting from gold & silver mining. Cyanide (ammonium thiocyanate) is the leach reagent of choice for gold (Au) and silver (Ag) extraction. During several metabolic functions, plants are confronted with cyanide as a byproduct of metabolism. This occurs particularly during synthesis of 'ethylene' in mature tissues, where 'hydrogen cyanide' (HCN) is formed as byproduct.

Consequently vascular plants have evolved effective strategies for detoxifying the toxic cyanide with the aid of enzymes. Those identified with cyanide detoxification are *Salix* spp. & *Sorghum* spp. Plants only survive cyanide exposure up to the doses they can eliminate. The cyanide detoxifying enzyme system is 'beta-cyanoalanine synthase' which connects free cyanide and cysteine to cyanoalanine. The final metabolic product is 'asparagine', a non-toxic essential amino acids in plants.(Manning, 1988; Trapp et al., 2003).

Summary of the Metal Uptake & Transport in Plants

1. Metal ions are mobilized by secretion of chelators in soil and by acidification of the rhizosphere (soil in the vicinity of root system);
2. Uptake of hydrated metal ions or metal-chelator complexes mediated by the transporter proteins residing in the plasma membrane & energized by ATP (cellular energy molecules- adenosine triphosphate produced by the cell mitochondria);
3. Metals are chelated again in the cell cytosol by various binding natural chelators and ligands (PCs & MTs);
4. PCs quickly form complex with Cd (because cadmium is the best activator of PCs and PCs have greater affinity for Cd) and the PC-Cd complex is transported into the cell vacuole. Other metals also follow the same process and are transported into the cell vacuole.

Some Wonder Plants for Phytoremediation of Contaminated Lands : The Gift of Nature and Potential for Commercialization

1. The Vetiver Grass (*Vetiveria zizanioides*)

The 'wonder grass' vetiver is native of India, and has been used worldwide for phytoremediation. It grows very rapidly and becomes effective for environmental restoration works in only 4-5 months as compared to 2-3 years taken by trees and shrubs for the same job. It can tolerate very high acidity and alkalinity conditions (pH from 3.0 to 10.5); high soil salinity (EC = 8 dScm), sodicity (ESP = 33 %) and magnesium; very high levels of heavy metals Al, Mn, Mg, As, Cd, Cr, Ni, Cu, Pb, Hg, Se, Zn and the herbicides and pesticides in soils.

Vetiver roots can absorb and accumulate several times of some of the heavy metals present in the soil and water. (Truong and Baker, 1998). Studies further indicated that very little (1 to 5 %) of the arsenic (As), cadmium (Cd), chromium (Cr) and mercury (Hg) and very moderate amount (16 to 33 %) of copper (Cu), lead (Pb), nickel (Ni) and selenium (Se) absorbed were translocated to the shoots above. Hence its green shoots can be safely used as feed or grazed by animals or harvested for mulch. Vetiver can be disposed off safely elsewhere, thus gradually reducing the contamination levels. Vetiver also has high capacity to absorb and remove agro-chemicals like carbofuran, monocrotophos and anachlor from soil thus preventing them from contaminating and accumulating in the crop plants.

Success Story

At the Scott Lumber Company site in Missouri, U.S., 16,000 tonnes of soils contaminated with polyaromatic hydrocarbons (PAHs) were biologically treated with VGT. The PAH concentration was effectively reduced by 70 %.(Pinthong *et al.*, 1998).

2). The Brake Fern (*Pteris vittata*)

It is hardy, versatile and fast-growing perennial plant easy to propagate. It can grow on contaminated soils up 1,500 ppm of arsenic and is extremely efficient in extracting arsenic (As) from both soil and water and translocating it into its above-ground biomass mainly the fronds. It can reduce the concentration of 200 ppm of arsenic in water to nearly 100-fold (10 ppm) within 24 hours and can hold up to 22 grams of arsenic per kg of plant matter.

Brake fern has great potential to remediate arsenic contaminated soils and is very cost-effective. Arsenic concentration in fern fronds growing in soil spiked with 1,500 ppm arsenic increased from 29.4 ppm to 15,861 ppm in two weeks. In the same period, ferns growing on soil containing just 6 ppm arsenic accumulated 755 of arsenic in their fronds, a 126-fold enrichment. Arsenic concentration in roots were less than 303 ppm, while in the fronds reached 7,234 ppm. Up to 93 % of the arsenic was concentrated in the fronds. (Ma et al, 2001).

Chinese brake fern has been shown to grow in soils of 50 – 4030 mg/kg arsenic and even in tailings containing arsenic as high as 23,400 mg/kg of soil. (Chen et al, 2002). More wild fern species are coming to light with phytoremediation properties.

Success Story

In 2001, the Edenspace System Corporation in the US used the Chinese brake fern to treat 1.5 acre site contaminated with arsenic (As) in New Jersey, North Carolina. The fern phytoextracted more than 200 fold of arsenic (As) in above ground part. (Singh, et.al., 2006)

3). Hybrid Poplar (*Populus x Canadensis*)

Hybrid poplar trees has assumed great significance in phytoremediation of several kinds of toxic organic pollutants including TCE (trichloroethylene), a potential carcinogen commonly found in groundwater as well as in contaminated sites.(Kassel et al. 2002). While several organic pollutants are metabolized or degraded to less toxic compounds and bioaccumulated in the plant, others like volatile chlorinated organic compounds e.g. BTEX (benzene, toluene, ethylbenzene & xylene) are released to the atmosphere. Poplar trees has also been used in phytoremediation of soil and groundwater contaminated PAHs (polycyclic aromated hydrocarbons), cyanide and pesticides. (Singh et al. 2006).

Normally 1000-2000 hybrid poplar per acre are planted for phytoremediation of contaminated land. Poplar trees grow very fast. They can grow 9 to 15 feet / year. The average life of hybrid poplar is about 30 years, and every 4-6 years, the above ground biomass can be cut and removed and new shoots grow from the cut stem.

Success Stories

1). In Milwaukee, Wisconsin, USA, the Ecolotree Inc. used the hybrid poplar trees to phytoremediate soil and groundwater contamination with petroleum related organics, PAHs and chlorinated organics released by accidental spills in 2000. The poplar trees were buried up to 10 feet below the surface and a sub-surface aeration system was provided to encourage deep rooting into groundwater.

2). In Illinois, USA, in 1999, the Ecolotree Inc. used the hybrid poplar to treat soil contaminated with chemical fertilizer & pesticides. Some 440 trees, of about 12 to 18 feet tall bare root poplar were planted into 6 feet deep trenches.

3). The Occidental Petroleum Corporation, LA & University of Washington, USA, used hybrid polar to treat several sites in the US contaminated with 'trichloroethanol' .

4). Hybrid polar trees were successfully used by other commercial companies like Phytokinetics Inc. in the US to treat groundwater contaminated with chlorinated volatile organics including dichloro-benzidine at several superfund sites.

5). Technical University of Denmark, used poplar trees to phytoremediate soils contaminated by gasoline and diesel compounds at old gas filling station at Axelvej Denmark, and cyanide, PAHs, oil and BTEX (benzene, toluene, ethylbenzene & xylene) contaminated soil at former municipal gas work site in Denmark.

6). The Polish Academy of Sciences, used the poplar trees to remove pesticides stored in bunkers at a resort Niedwiady, Poland.

(Singh, et.al., 2006)

4). Sunflower (*Helianthus annuus*)

Sunflower is economically important as an 'oil yielding' plant and the oil is considered to be very healthy rich in 'polyunsaturated' lipids good for heart. It has gained much importance as

phytoremediator for environmental cleanup of both chemically & radiologically contaminated soil & water bodies (by growing hydroponically). Sunflower plants, when tested in batch experiment significantly reduced the concentrations of heavy metals cadmium (Cd), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni) and lead (Pb) within an hour of treatment. (Eapen et al., 2006).

Success Stories

1). Daimler Chrysler, the car manufacturing company in the US had 4,300 cubic pounds of soil contaminated with lead (Pb) ranging from 75 – 3,450 mg/kg of soil at its Detroit Forge Site in 1998. Sunflower plants were used for phytoremediation. Successive crops of sunflower (with Indian mustard plant) were planted in 24 inches deep ex-situ treatment cell on an impermeable concrete base. In single season of crop growth the lead contents in the soil was brought down to 900 mg/kg of soil and subsequently it was removed completely after successive crop growth. The total cost of phytoremediation treatment by sunflower was US \$ 50.00 per cubic yard, which saved more than US \$ 1.1 million compared to the estimated cost of physio-chemical treatment by soil excavation and disposal in landfills. (Singh, et.al., 2006).

2). Sunflower & the Indian mustard plant (*Brassica juncea*) was used to phytoremediate the lead (Pb) contaminated soil at industrial facility in Connecticut, USA. A combined phytoextraction and phytostabilization treatment for three successive years (1997-2000) costed less than US \$ 40 per cubic yard of treated soil. (Singh, et.al., 2006).

3). The Edenspace System Corporation in the US used the sunflower and the Indian mustard to treat various sites in the USA contaminated with heavy metals. The plants took up heavy metals more than 3.5 % of their dry weight. The company also used this plant to remediate uranium (U) contaminated soils (47 mg/kg of soil) at US Army Sites at Aberdeen, Maryland. The sunflower plants bioaccumulated uranium at the rate of 764 mg/kg – 1669 mg/kg of soil. (Singh, et.al., 2006).

5). Indian Mustard (*Brassica juncea*)

The Indian mustard is an oil yielding plant. Mustard oil is used in the Indian culture for cooking as well as for medicinal purposes. It has large root system with good biomass and can also be grown hydroponically. It has gained much significance as 'phytoremediator' for environmental cleanup of both chemically & radiologically contaminated soil & water bodies (by growing hydroponically). The Indian mustard concentrate toxic heavy metals (Pb, Cu and Ni) from hydroponics solution to a level up to several percent of their dried shoot biomass (Sriprang, 2006). Several superior 'transgenic species' of *B. juncea* have been produced for improved phytoremediation function. (Described below).

Success Stories

1). The Edenspace System Corporation of USA, used the Indian mustard plant to treat the radionuclide strontium ($\text{Sr}^{89/90}$) contaminated soil at Fort Greely in Alaska, USA. The plants bioaccumulated more than 10-15 fold of strontium ($\text{Sr}^{89/90}$) higher than in soil. They also used the Indian mustard with sunflower to treat various sites in the USA contaminated with heavy metals. They accumulated more than 3.5 % of heavy metals of their dry weight. They also used the Indian mustard to remove cesium – 137 (Cs^{137}) from the contaminated pond waters after the Chernobyl Nuclear Power Plant disaster in Ukraine in 1986. (Singh, et.al., 2006).

2). The Brookhaven National Lab, New Jersey, USA, used Indian mustard to remove radionuclides cesium – 137 (Cs^{137}) and strontium – 90 (Sr^{90}) by phytoextraction from contaminated soil. (Singh, et.al., 2006).

3). The Phytotech, Florida, USA used the Indian mustard plant to remediate lead (Pb) and cadmium (Cd) contaminated soil at the Czechowice Oil Refinery, Katowice, in Poland. (Singh, et.al., 2006).

4). As described above the Indian mustard plant was used with sunflower (*Helianthus annuus*) to phytoremediate the lead (Pb) contaminated soil at industrial facility in Connecticut, USA. (Singh, et.al., 2006).

6). Alpine pennycress (*Thlaspi caerulescens*)

It is a small weedy member of the broccoli & cabbage family (Brassicaceae) and can thrive on soils having very high levels of cadmium (Cd) and zinc (Zn). It is a 'hyper-accumulator' plant and can bio-accumulate up to 30,000 ppm (mg/L) Zn and 1,500 ppm Cd in its shoots while exhibiting few or no toxicity symptoms. A normal plant can be poisoned with as little as 1000 ppm of zinc or 20 to 50 ppm of cadmium in its shoots.

7). The Bermuda grass (*Cyanodon dactylon*)

It is very common grass species and can colonize and thrive in soils containing up to several thousands ppm (parts per million) of arsenic (As) (Ashley and Lottermoser, 1999). It also absorb and concentrate arsenic in its above ground parts.

8). Nightshade (*Solanum nigrum*)

Mackova *et al.* (1997), reported effective degradation of PCBs (Polychlorinated Biphenyls) by cells of *Solanum nigrum* that were infected with bacterial strains of *Agrobacterium tumefaciens* and *A. rhizogenes*. Hairy root cultures of *S. nigrum* proved capable of transforming PCBs under controlled conditions. Plant cells proved capable of transforming PCBs even after growth had stopped.

9). Drum-Stick Tree (*Moringa oleifera*)

Some plant extract have metal binding properties and can help remove several contaminants from aqueous solutions. Researches have indicated that the seeds of some trees possess strong anti-coagulant properties like 'alum' and remedify contaminated waters. Others have strong anti-bacterial, anti-protozoal and anti-viral properties and are significantly effective in inactivating bacteria, protozoas and viruses and prevent their re-growth. These trees are drum-stick tree (*Moringa oleifera*) and the other is *Strychnos potatorum*.

10). Strychnos Tree (*Strychnos potatorum*)

A chemical compound from the seeds of *S. potatorum* readily binds with toxic heavy metals like cadmium and mercury and radioactive materials like uranium, thorium and other radioactive isotopes present in the nuclear wastes. *S. potatorum* is highly effective in removing industrial pollutants from the effluents. (Evans, 1991, UNEP Report).

11). The Willow Tree, Alfalfa & Cottonwood

In 1999 - 2001, the native willow tree, alfalfa, cottonwood and several grasses were used to treat 140 acre of land (site) contaminated by wood preservative waste which included PCPs (pentachlorophenol) and PAHs (polyaromated hydrocarbons) at Union Pacific Railroad, Wyoming, USA . (Singh, et.al., 2006)

11. Role of Environmental Biotechnology & Genetic Engineering In Improving Efficiency of Phytoremediation : Some Achievements

Several genes that are involved in metal uptake, translocation, sequestration and bioaccumulation has now been identified. Transfer of these genes into candidate plant will result in developing 'transgenic plants' with enhanced ability for metal uptake and accumulation for removal from environment. Environmental biotechnology provides us the tools to accelerate the phytoremediation process through either 'over-expression' of those genes responsible for the sequestration of heavy metals & radionuclides in plants or through 'gene transfer'. Many appropriate genes of foreign origin have been transferred in plants like *Arabidopsis thaliana*, *Nicotiana tabacum*, *Brassica juncea*, *Brassica oleraceae* var *botrytis*, *Lycopersicon esculentum* etc. to enhance the phytoremediation efficiency of these plants.

Transgenic plants so far have been developed for the hyper-accumulation of toxic heavy metals like Hg, As, Pb, Cd, Co, Ni, Zn, Cu etc. from soil, and from air gaseous pollutants like NO₂, SO₂ and organic pollutants e.g. 2,4,6-trinitrotoluene and organomercurials etc. Higher plants have

evolved many genes (and their products – enzymes), which have potentials to metabolize or degrade different kinds of xenobiotic compounds. (Bizily *et al.*, 2000; Pan *et al.*, 2005).

Many hyper-accumulating plants are rare, with small population occurring in remote places or have restricted distribution. They often have slow growth rate and produce small biomass. Such hyper-accumulator species may provide suitable genes involved in metal uptake, translocation and sequestration for enhancing phytoremediation.

If genes from highly metal tolerant & hyper-accumulator plants are transferred to high biomass yielding & fast growing cultivars, this can do miracle. Some fast-growing hyper-accumulator species with large biomass has also been identified. These are nickel (Ni) hyper-accumulators *Alyssum bertolonii* and *Berkheya coddii*. They produce 9 & 22 tons/ ha of shoot dry matter respectively, in small-scale field experiments. The arsenic (As) hyper-accumulator *Pteris vittata* can also produce large biomass under favorable conditions.

Important Achievements

1). With the aim of creating 'new transgenic phytoremediator plants' that can tolerate and hyper-accumulate high levels of toxic metals, various *MT* (metallothionein) genes were introduced into plants such as in *Nicotiana* spp., *Brassica* spp. and *Arabidopsis thaliana*. Transgenic plants, that express *MT* genes, have shown to be highly tolerant to cadmium (Cd) and other metals in soil. However, the metal uptake was not markedly altered.

Transfer of *human MT-2* gene to tobacco (*Nicotiana tabacum*) resulted in transgenic plant with enhanced Cd tolerance and accumulation. Transfer of *pea MT* gene in *Arabidopsis thaliana* resulted in enhanced copper (Cu) accumulation in the transgenic *A. thaliana*. Transfer of *yeast CUP1* gene in cauliflower (*Brassica capitata*) resulted in 16-fold higher accumulation of cadmium (Cd) in the transgenic cauliflower. (Sriprang & Murooka, 2006; Eapen *et al.* 2006).

2). Certain metals such as mercury (Hg) and selenium (Se) can be phytovolatilised usually through plant-microbe interactions (Cunningham and Owe, 1996). Genes for synthesizing the enzyme 'bacterial mercuric ion reductase' has been engineered into *Arabidopsis thaliana* and the resulting transformant transgenic plant is capable of tolerating and volatilising mercuric ions. The toxic cation is absorbed by the root and reduced to volatile Hg (O) by the introduced mercuric ion reductase. (Rugh *et al.* 1996).

3). Transgenic yellow poplar expressing a modified *MerA* gene and *MerB* gene has been produced for the phytoremediation of mercury (Hg) which has become global problem in aquatic environments resulting from its industrial use in blanching operations as a catalyst, as a pigment in paints and in mining of gold. The genes *MerA* and *MerB* were isolated from mercury resistant bacteria which synthesizes the enzymes 'mercuric iron reductase' (*MerA*) and 'organomercurial lyase' (*MerB*) respectively. The transgenic poplar *MerA* expressing *MerA* gene, released 10 times more elemental mercury (Hg) than the untransformed plantlets. Transgenic plants *MerB* expressing *MerB* gene were significantly more tolerant to methylmercury and other organomercurials compared to the untransformed plants. The *MerB* plants (with *MerB* genes) effectively converted the highly toxic methylmercury to elemental mercury (Hg²⁺), which is about 100 times less toxic in plants. They were released from the plants by phytovolatilization.

The *MerA* and *MerB* double-transgenic poplar plant showed the highest tolerance to organic mercury (up to 10 µM) compared to the wild type plants (0.25 µM). (Rugh *et al.* 1998; Bizily *et al.* 2000).

4). Transfer and over-expression of two genes for production of enzymes γ -glutamyl cysteine synthetase and glutathione synthetase in transgenic Indian mustard (*Brassica* spp.) resulted in accumulation of higher levels of GSH and PC. They exhibited enhanced cadmium (Cd) tolerance and accumulation and also extracted more Cd, Cr, Cu, Pb, and Zn than wild plants (Zhu, *et al.* 1999).

5). Transgenic Indian mustard (*Brassica juncea*) was produced by introducing foreign genes.

a). A gene *APSI* encoding ATP-sulfurylase from *Arabidopsis thaliana* and its over-expression in *B. juncea* led to 2-3 fold higher accumulation of selenium (Se) in shoots and 1.5 fold higher accumulation in roots as compared to the wild type plants. (Pilon-Smits et al., 1999).

b). Transfer and over-expression of the gene *gshII* encoding the enzyme 'glutathione synthetase' (GS) from *E.coli* into *B.juncea* increased the cadmium (Cd) accumulation efficiency of the transformed transgenic plant to 3-fold high. (Zhu et al., 1999).

6). Transgenic plant of *Arabidopsis thaliana* have been produced by transfer of foreign genes with great value in phytoremediation technology.

a). The transfer of mercuric ion reductase gene (*merA*) from *E.coli* made the transgenic plant more tolerant to HgCl₂ and volatalized elemental mercury.

b). The transfer of mercuric ion reductase genes (*merB*) from *E.coli* made the plant more tolerant to methyl mercury and other organomercurials. The transgenic plant could grow on 10-fold higher methyl mercury than the wild type. (Rugh et al., 1996).

c). Transfer of both genes (*merA* & *merB*) from *E.coli* to *A.thaliana* made the plant to grow on 50-fold methyl mercury. (Bizily et al., 1999 & 2000).

d). Transfer of bacterial genes encoding arsenate reductase (*ars c*) and γ -glutamyl cysteine synthetase (γ -ECS) together from *E.coli* led to higher arsenic (As) tolerance and 4-17 fold higher arsenic hyperaccumulation in shoots. (Dhankher, et al., 2002).

e). Transfer and over-expression of gene *NiR cDNA* synthesizing the enzyme 'nitrate reductase' into *A. thaliana* led to 40 % increase in assimilation of nitrogen dioxide (NO₂).

7). Transgenic tobacco (*Nicotiana tabaccum*) plant was produced by transferring bacterial nitroreductase gene (pNITRED3). This increased the tolerance to the 2,4,6-trinitrotoluene (TNT) (recalcitrant military explosive) and also its 'detoxification' by the transgenic tobacco. (Hannink et al., 2001).

8). *Nicotiana glauca* is widely distributed, fast growing, high biomass producing and a herbivore-repulsive plant. Gisbert et al. (2003) used this plant to over-express wheat gene encoding PCS. The transgenic plant showed greatly increased tolerance to metals, such as lead (Pb) and cadmium (Cd), developing seedling roots 160 % longer than the wild types. Seedlings grown in the mined soils containing high levels of Pb (1572 ppm), accumulated double concentration of this heavy metal than the wild type.

9). Somatic cell hybrid produced between *Brassica juncea* (a high biomass Pb accumulator plant), and *Thlaspi caerulescens* (a known Zn and Ni hyperaccumulator), showed increased tolerance to Pb, Ni and Zn and the total amount of lead (Pb) phyto-extracted was much greater because of the high biomass produced. (Dushenkov et al. 2002).

10). Transporter protein and intracellular high-affinity binding sites mediate the uptake of the metals and other substances across the plasma membrane. Many 'metal transporter genes' have been cloned recently. (Datta & Sarkar, 2004). Maser et al., 2001 (quoted in Singh et al., 2006) have cloned genes of ZIP (Zn regulated transporter / Fe-regulated transporter like proteins) family e.g. *ZNT1* & *ZNT2*, from *Thlaspi careulescens*, which are highly expressed in roots of the accumulator plants.

Future Prospects

1). If a plant could be genetically altered to produce higher levels of endogenous organic acids such citric or malic acids, the PCs and MTs which works to bind metal cations to form their complexes and facilitate their bioavailability, uptake and transport, this can significantly improve phytoremediation process by phytoextraction.

2). It has been found that the amount of free histidine (which chelates & form complexes with nickel & facilitate their uptake and transport) increases in plant roots with their exposure to nickel in soil. By genetically modifying histidine metabolism, it might be possible to increase the capacity of nickel (Ni) bioaccumulation by plants.

3). Hyper-accumulating plants often accumulate a specific element only, thus limiting its applicability to phytoremediate sites which have multiple metal contamination. If the genes from various species known to produce selected enzyme that help bioaccumulation of specific metal is transferred to a particular species this plant can become a 'super-hyper-accumulator' capable of phytoremediating sites with multiple metal contamination.

4). Transporter proteins, isolated from hyper-accumulating species, such as Zn -Transporter Protein (ZnTP1) can only uptake zinc (Zn), but not the toxic metal cadmium (Cd). They are specific. Molecular study for alteration of such genes (*ZnT1*) for transport of other metals may be useful for enhancing phytoextraction.

5). The expression of the iron-transporter gene (*IRT1*) producing the iron-transporter protein (IRTP1) mediate the uptake of Na^+ and Cd^{2+} in yeast cells. Therefore, the transfer of such gene (*IRT1*) to plants may enhance the uptake of metal ions by the plant roots.

'Symbiotic Engineering' : Novel Biotechnology for Heavy Metal Bioremediation by Legume-Bacteria Symbiosis

A novel bioremediation system for heavy metals using the symbiotic relationship between leguminous plant and genetically engineered rhizobia bacteria has been suggested by Sriprang et al. (2002). It has been observed that rhizobium grow slowly for long times in soil, but if they infect a compatible legume they grow rapidly. Successful infection by even a single bacterium can lead to the formation of a 'nitrogen-fixing nodule' on the root of legume plant containing over 10^8 bacterial progeny. This special feature of symbiotic relationship gives clue for biotechnological transfer and expression of *MT* (metallothionein) genes that sequester heavy metals from contaminated soil. Once symbiosis with *MT* genes is established with legumes, the heavy metals will be accumulated in the nodules. This would be an alternative and more cost-effective method to remove heavy metals from soil.

Sriprang et al. (2002) developed this novel 'plant-bacterial bioremediation' technology for heavy metal removal by the introduction of the chimeric *MTL4* gene to *Meso-rhizobium haukii* sub-species *rengei* strain B3 which is a genetically engineered bacterium capable of establishing symbiotic relationship with a legume *Astralagus sinicus* (used as a green manure in rice fields in Japan & China), by eliciting the formation of nitrogen-fixing root nodules. The rhizobia undergo differentiation into a distinct cell type called as 'bacteriod', which is capable of fixing atmospheric nitrogen. Thus the legume *A. sinicus* would be used as a green manure to improve fertility in rice fields as well as to scavenge heavy metals from the soil. This was also a first report that a 'foreign gene' was expressed in bacteroids in the nodules.

III. Vermiremediation of Polluted Lands & Soils by Earthworms : Charles Darwin's Unheralded Soldiers of Mankind

A revolution is unfolding in vermiculture studies (rearing of earthworms – Charles Darwin's 'unheralded soldiers of mankind working day and night under the soil') for several environmental functions including soil remediation and land management. Earthworms have been used for land recovery, reclamation and rehabilitation of sub-optimal soils such as poor mineral soils, polder soils, open cast mining sites, closed landfill sites and cutover peats (Haimi, 2000; Lowe & Butt, 2003 and Butt et al., 2004). Within the soil environment, an earthworm's sphere of influence is known as the 'drilosphere system'. This incorporates the burrow systems, surface and

belowground earthworm casts, internal earthworm gut and process, the earthworm surface in contact with the soil, and associated biological, chemical and physical interactions, in addition to the soil microorganisms (Brown and Doube, 2004).

Earthworms have been found to remove heavy metals, pesticides and lipophilic organic micropollutants like the polycyclic aromatic hydrocarbons (PAH) from the soil (Contreras-Ramos et. al, 2006; Sinha et. al, 2008). Vermiremediation (using chemical tolerant earthworm species) is emerging as a low-cost and convenient technology for cleaning up the chemically polluted / contaminated sites / lands in world.

It is envisaged that earthworm assisted bioremediation (vermiremediation) approaches might include –

- 1). Direct application of earthworms to contaminated soils (Schaefer, 2007).
- 2). Co-application of earthworms to contaminated soils with another organic material, such as compost (Ceccanti et al., 2006).
- 3). Application of contaminated media (soils) to earthworms as a part of feeding regime (Getliff et al., 2002)
- 4). Indirect use of earthworms through its digested (composted) materials (vermicompost). Worm vermicast is high in degrader microbes and thus high in catabolic activities (Alvarez-Bernal et al., 2006). Teotia et al., (1950) reported 32 million bacterial counts per gram of vermicast as compared to 6-9 million / gram in surrounding soils. Vermicompost works as a wonderful natural 'bio-adsorbent' material for various chemicals in the environment. It is rich in 'humic substances' which has high potential for retaining organic and inorganic pollutants. It forms stable complexes with diverse 'metallic ions'.

Vermiremediation is a self-promoted, self-regulated, self-improved & self-enhanced, low or no-energy requiring zero-waste technology, easy to construct, operate and maintain. It excels all 'bio-conversion' technologies by the fact that it can utilize organics that otherwise cannot be utilized by others. It excels all 'bio-treatment' technologies because it achieves greater utilization than the rate of destruction achieved by other technologies. It involves about 100-1000 times higher 'value addition' than other biological technologies. (Appenholf, 2003).

Natural Attributes & Adaptations of Earthworms to Perform the Role of Soil & Land Managers

Biology of Earthworms

Earthworms are long, narrow, cylindrical, bilaterally symmetrical, segmented animals without bones and measure few centimeters. Tropical worms generally are bigger & robust. An exceptionally big species about a meter long is reported from Victoria in Australia. The body is dark brown, glistening and covered with delicate cuticle. They weigh over 1400-1500 mg after 8-10 weeks. On an average, 2000 adult worms weigh 1 kg and one million worms weigh approximately 1 ton.

Earthworms harbor millions of 'nitrogen-fixing' and 'decomposer microbes' in their gut. Their body contains 65 % protein (70-80 % high quality 'lysine rich protein' on a dry weight basis), 14 % fats, 14 % carbohydrates and 3 % ash. (Gerard, 1960; Visvanathan et. al., 2005). Earthworms act as an aerator, grinder, crusher, chemical degrader and a biological stimulator wherever they inhabit (Dash, 1978; & Sinha et al., 2002). About 4,400 different species of earthworms have been identified, and quite a few of them are versatile bio-degraders.

Ecology of Earthworms

Earthworms are burrowing animals and form tunnels by literally eating their way through the soil. The distribution of earthworms in soil depends on factors like soil moisture, availability of organic matter and pH of the soil. They occur in diverse habitats specially those which are dark and moist. Earthworms are generally absent or rare in soil with a very coarse texture and high clay content or soil with pH < 4 (Gunathilagraj, 1996). Earthworms are very sensitive to touch, light and dryness. As worms breathe through their skin proper ventilation of air in soil medium is necessary. Water logging in the soil can cause them to come to the surface. Worms can tolerate

a temperature range between 5° C to 29° C. A temperature of 20 ° C to 25° C and a moisture of 60-75 % is optimum for earthworm function (Hand, 1988).

Feeding Behaviour

Researches into vermiculture has revealed that worms can feed upon wide variety of materials from earth. Aristotle called them '*intestine of earth*'. Earthworms love to feed upon 'cattle dung' which is preferred food for them. When given a choice between various foods the worms consumed 10 mg dry weight of dung per gram body weight per day together with smaller amount of leaf litter. In about 13 days *Allolobophora caliginosa* consumed 13.1 gram of dung while only 1.3 gram of grass leaves (Barley, 1959). However, firm leaves particularly the grass leaves are not eaten until they had decayed to a moist, brown condition. Worms have 'chemoreceptors' which aid in search of food.

The worms secrete enzymes proteases, lipases, amylases, cellulases and chitinases in their gizzard and intestine which bring about rapid biochemical conversion of the organic materials. They ingest the organic materials, pass it through its intestine, adjust the pH of the digested (degraded) materials, cull the unwanted microorganisms, and then deposit the processed cellulosic materials mixed with minerals and microbes as aggregates called 'vermicasts' in the soil. (Dash, 1978). Most earthworms consume, at the best, half their body weight of organic material in a day. *Eisenia fetida* is reported to consume organic matter at the rate equal to their body weight every day. (Visvanathan et al., 2005). Earthworm participation enhances natural biodegradation and decomposition from 60 to 80 %.

Resistant to Chemicals

Earthworms in general (specially *E. fetida*) are highly resistant to many chemical contaminants including heavy metals and organic pollutants in soil and have been reported to bio-accumulate them in their tissues. After the Seveso chemical plant explosion in 1976 in Italy, when vast inhabited area was contaminated with certain chemicals including the extremely toxic TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) several fauna perished but for the earthworms that were alone able to survive. Earthworms which ingested TCDD contaminated soils were shown to bio-accumulate dioxin in their tissues and concentrate it on average 14.5 fold. (Satchell, 1983). *E. fetida* was used as the test organisms for different soil contaminants and several reports indicated that *E. fetida* tolerated 1.5 % crude oil (containing several toxic organic pollutants) and survived in this environment. (OECD, 2000; Safwat et al., 2002).

Enormous Rate of Multiplication

Earthworms multiply very rapidly. They are bisexual animals and cross-fertilization occurs as a rule. After copulation the clitellum (a prominent band) of each worm eject lemon-shaped 'cocoon' where sperms enter to fertilize the eggs. Up to 3 cocoons per worm per week are produced. From each cocoon about 10-12 tiny worms emerge. Studies indicate that they double their number at least every 60 days. Given the optimal conditions of moisture, temperature and feeding materials earthworms can multiply by 2^8 i.e. 256 worms every 6 months from a single individual. Each of the 256 worms multiplies in the same proportion to produce a huge biomass of worms in a short time. The total life-cycle of the worms is about 220 days. They produce 300-400 young ones within this life period. (Hand, 1988).

Reinforce Decomposer Microbes in Soil

Earthworms promotes the growth of 'beneficial decomposer aerobic microbes' in soil and this they do by several ways – by improving 'aeration' through burrowing actions, by releasing 'chemical mediators' along their gut and body surface and indirectly through protozoa which they activate, which act at low concentrations on microbial metabolism, as vitamins or as chemical catalysts (Binet et al., 1998). Earthworms hosts millions of decomposer (biodegrader) microbes in their gut (as they devour on them) and excrete them in soil along with nutrients nitrogen (N) and phosphorus (P) in their excreta (Singleton et al., 2003). The nutrients N & P are further used by the microbes for multiplication and vigorous action. Edward and Fletcher (1988) showed that the number of bacteria and 'actinomycetes' contained in the ingested material increased up to 1000 fold while passing through the gut. A population of worms numbering about 15,000 will in turn

foster a microbial population of billions of millions. (Morgan & Burrows, 1982). Under favorable conditions, earthworms and microorganisms act 'symbiotically & synergistically' to accelerate and enhance bioremediation of soil.

Earthworms Species Mostly Involved in Soil Remediation

Certain species of earthworms such as *Eisenia fetida*, *Aporrectodea tuberculata*, *Lumbricus terrestris*, *L. rubellus*, *Dendrobaena rubida*, *D. veneta*, *Eiseniella tetraedra*, *Allobophora chlorotica* & *Pheretima Spp.* have been found to remove heavy metals, pesticides and lipophilic organic micropollutants like the polycyclic aromatic hydrocarbons (PAH) from the soil (Contreras-Ramos et. al, 2006). *E. fetida* has been used as the test organisms for different soil contaminants and several reports indicate that *E. fetida* tolerates 1.5 % crude oil while *L. terrestris* did not survive in 0.5 % of it (OECD, 2000; Safwat et al., 2002). Earthworms also inhibit the soil borne pathogens and work as a detoxifying agent for polluted soil. It reduces *Salmonella* population in soil to about 30 %.

Mechanism of Worm Action in Vermiremediation : The Uptake of Chemicals from Soil and Immobilization by Earthworms & Reinforcement of Microbial Action

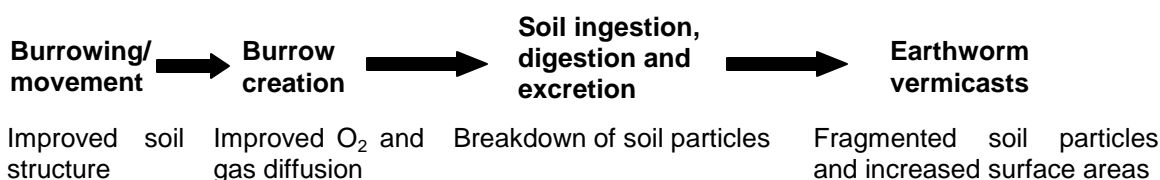
Earthworms have both 'abiotic' and 'biotic' effects on contaminated soils in the remediation process. Abiotic effects are burrowing actions and the resulting burrows acts as inputs points and preferred pathways for water and particle movement, nutrient flow and aeration. This also results into mechanical breakdown of soil particles exposing greater surface areas for biotic action. During burrowing worms ingest and digest large amount of contaminated soils. By digestion the size of the soil organic matter containing contaminants is reduced significantly thus exposing more surface area of contaminated soil for microbial action and remediation. The biotic effects are proliferation of degrader microbes (bacteria, fungi and actinomycetes) by the earthworms by their excretion in contaminated soil which includes urine, intestinal mucus, glucose and other nutrients. There are also direct biotic effects of earthworms in the form of 'feeding behaviours' upon contaminants fates in soil. Studies indicate that earthworms increase their oral intake of soil particles when driven by 'hunger stress'. There were total petroleum losses (TPH) in contaminated soil where earthworms were not provided with any food (Schaefer et al., 2005).

Hydrophobic organic contaminants are taken up by the earthworms in two ways-

- 1). By passive diffusion from the soil solution through the worms outer membrane;
- 2). By intestinal re-sorption of the compounds from the soil while it passes through the gut (by digestion) and then their degradation by enzymatic activity called 'Cytochrome P 450' system. This enzymatic activity have been found to operate particularly in *Eisenia fetida* which survive the benzo(a)pyrene concentration of 1,008 mg/kg of soil (Achazi et al,1998).

Earthworms uptake chemicals from the soil through passive 'absorption' of the dissolved fraction, through the moist 'body wall' in the interstitial water and also by mouth and 'intestinal uptake' while the soil passes through the gut. The passive diffusion is driven by the difference between the pore water in soil and within the earthworm's tissues (Jager et al, 2003). The accumulation increases when the concentration of PAHs in their surrounding soil water or in their food increases (Belfroid et, al. 1995). Johnsen et al., (2005) also reported that the earthworms may take PAHs up through absorption by the body surface and also by feeding and ingestion, since PAHs sorb to the soil organic detritus, which the worms feed on.

Earthworm Actions in Improvement of Soil Structure & Properties



Increased porosity		Release of organic residues, enzymes & plant growth hormones	Increased water holding capacity of soil
Improved aggregation	Improved moisture	Transport & dispersal of beneficial microorganisms,	
Release of bioavailable plant nutrients	drainage and dispersal	elimination of harmful microorganisms	Higher soil organic matter
Organic matter turnover	Improved nutrient movement	Higher organic matter turnover	Enhanced bioavailable NPK & micronutrients with increased C:N ratio
Improved fertility		Addition of high energy water soluble organic compounds	Improve soil pH
Beneficial microorganism promotion and dispersal	Beneficial microorganism promotion and dispersal	Beneficial microorganisms promotion & proliferation	Beneficial microorganism promotion & multiplication

Earthworms apparently possess a number of mechanisms for uptake, immobilization and excretion of heavy metals and other chemicals. They either 'biodegrade' or 'bio-transform' the chemical contaminants rendering them harmless.

1). Biodegradation of Chemical Contaminants by Stimulating Soil Microbial Activity

Several soil microorganisms especially bacteria and fungi biodegrade several categories of chemicals including hydrocarbons in soil. Ma et al., (1995) found that earthworms biodegrade organic contaminants like phthalate, phenanthrene and fluoranthene indirectly through enhancing microbial activities. Earthworms hosts millions of biodegrader microbes in their gut and excrete them in soil along with nutrients nitrogen (N) and phosphorus (P) in their excreta (Singleton et al., 2003). The nutrients N & P are further used by the microbes for multiplication and vigorous action. Edward and Fletcher (1988) showed that the number of bacteria and 'actinomycetes' contained in the ingested material increased up to 1000 fold while passing through the gut. A population of worms numbering about 15,000 will in turn foster a microbial population of billions of millions. (Morgan & Burrows, 1982).

Singleton et al. (2003) studied the bacterial flora associated with the intestine and vermicasts of the earthworms and found species like *Pseudomonas*, *Paenibacillus*, *Azoarcus*, *Burkholderia*, *Spiroplasm*, *Acaligenes* and *Acidobacterium*. Some of them, such as *Pseudomonas*, *Acaligenes* and *Acidobacterium* are known to degrade hydrocarbons. Morgan and Burrows (1982) reported that the internal fluid of cocoons of *E. fetida* contains bacteria *Nocardia*, *Psuedomonas* & *Acaligenes*. *Rhodococcus*, and *Azatobacter* have been found in the burrows of *L. terrestris*. *Rhodococcus* can use anthracene, phenanthrene, pyrene and fluoranthene as sole source of carbon and energy. Some fungi such as *Pencillium*, *Mucor* and *Aspergillus* have also been found in the intestine of earthworms and they degrade PAHs. Biodegradation of PAHs occur when microorganisms break the aromatic rings of benzene and produce aliphatic compounds that readily enters the tricarboxylic acid cycle (metabolic activity) operating in living cells. *Cunniinghamella elegans* and *Candida tropicalis* have been reported to degrade PAHs. (Kanaly & Harayama, 2000). Degradation products of PAHs are, however, not necessarily less toxic than the parent compounds. *Acaligenes* can even degrade PCBs and *Mucor* dieldrin (Johnsen et al., 2005; Contreas – Ramos et. al., 2006).

With the passage of time, contaminants become entrapped and sorbed onto and into soil mineral and organic matter fractions, rendering them inaccessible, and thus unavailable for microbial action. Significantly, the worm activity in the drilosphere release these residues of contaminants bound with the soil fractions for rapid microbial actions and bioremediation (Gevao, et al., 2001).

2). Biotransformation of Chemical Contaminants in Soil

Some metals are bound by a protein called 'metallothioneins' found in earthworms which has very high capacity to bind metals. Ireland (1979) found that cadmium (Cd) and lead (Pb) are particularly concentrated in chloragogen cells in *L. terrestris* and *D. rubidus*, where it is bound in the form of Cd-metallothioneins & Pb-metallothioneins respectively (i.e. bio-transformed) with small amounts deposited in waste nodules. The chloragogen cells in earthworms appears to mainly accumulate heavy metals absorbed by the gut and their immobilization in the small spheroidal chloragosomes and debris vesicles that the cells contain.

Some Important Studies on Earthworm Assisted Bioremediation

Potential of earthworms for remediation of chemically contaminated soils have been studied by several authors. Hickman and Reid (2008) has made a very good study on the subject and have reviewed a number of papers.

I). Removal of Heavy Metals from Soil

Mining activities unleash large amount of different heavy metals and contaminate vast land areas. They routinely gives out arsenic, cadmium, lead, mercury, nickel and beryllium. Earthworms can tolerate high concentrations of heavy metals in the environment.

1). Hartenstein et al., (1980), studied and reported that earthworms can bio-accumulate high concentrations of metals including heavy metals in their tissues without affecting their physiology and this particularly when the metals are mostly non-bioavailable. Studies indicate that earthworms can take up and bio-accumulate heavy metals like cadmium (Cd), mercury (Hg), lead (Pb) copper (Cu), manganese (Mn), calcium (Ca), iron (Fe) and zinc (Zn). They can particularly ingest and accumulate extremely high amounts of zinc (Zn) and cadmium (Cd). Cadmium is very mobile and may be readily incorporated into soft and non-calcareous tissues of earthworms. Cadmium levels up to 100 mg per kg dry weight have been found in tissues. Of all the metals Cd and Pb appears to accumulate in most species of earthworms at greater level. Earthworms collected from the roadsides and mining sites show higher amounts of heavy metals than those from the other sites. Thus earthworms can also work as a 'bioindicator' of heavy metal contamination in soil.

2). Ireland (1983) reported that the earthworms species *Lumbricus terrestris* can bio-accumulate in their tissues 90 -180 mg lead (Pb) / gm of dry weight, while *L. rubellus* and *D. rubida* it was 2600 mg /gm and 7600 mg /gm of dry weight respectively. Zinc (Zn), manganese (Mn), and iron (Fe) were shown to be excreted through the calciferous glands of earthworms.

3). Contreras-Ramos et, al.,(2005) also confirmed that the earthworms reduced the concentrations of chromium (Cr), copper (Cu), zinc (Zn) and lead (Pb) in the vermicomposted sludge (biosolids) below the limits set by the USEPA in 60 days.

4). Malley et. al., (2006) also studied bioaccumulation of copper (Cu) and zinc (Zn) in *E. fetida* after 10 weeks of experiment.

Table- 13
Concentration of Cu and Zn in *E. fetida* Tissues Initially and After 10 Weeks

	Cu (mg/Kg) ±SD	Zn (mg/Kg) ±SD
Initial sample	17.29±2	108.22±4
Control	20.34 ±4	127.54±8
Dosage 1	104.58 ± 47	137.52±8
Dosage 2	158.95 ± 10	132.03±16
Dosage 3	213.07 ± 22	138.51±18

Source: Malley et al., (2006)

Fate of Heavy Metals Bioaccumulated in Earthworms

Some metals are bound by a protein called 'metallothioneins' found in earthworms which has very high capacity to bind metals. The chloragogen cells in earthworms appears to mainly accumulate heavy metals absorbed by the gut and their immobilization in the small spheroidal chloragosomes and debris vesicles that the cells contain.

Vermiremediation of Cadmium (Cd) Contaminated Soil

Potential sources of cadmium include metallurgical alloying, ceramics manufacture, electroplating, inorganic pigments, textile printing, chemical industries and mine drainage. Of the total cadmium use 90% is utilised in electroplating, pigments, plastics stabilizers, alloying and batteries. Various technologies to treat cadmium include chemical precipitation, ion exchange, evaporative recovery and vermiremediation. Vermiremediation of cadmium has advantages over other methods since it is natural way of remediating the contaminated soil and is inexpensive.

Earth worm exhibit a distinct metabolic pathway that allows the bio-accumulation of cadmium (Cd) to yield body burdens in excess of 1/1000th of total dry body weight, a most impressive figure by any standard. Earthworms transform the cadmium using metal-binding protein namely the two isoforms of metallothionein. Chemical analysis of recombinant protein showed that although both isoforms bind equimolar amounts of cadmium (6 mol) wMT-2 is more stable during proton competition. Furthermore, isoform-specific transcript analysis demonstrated that only wMT-2 is responsive to cadmium in a dose and temporal manner. The specific sequestration of cadmium to wMT-2 protein was confirmed in situ using polyclonal antisera. The latter also provided the means for mapping the cellular and intracellular distribution of metallothionein, thus yielding a holistic insight into its involvement in cadmium transit during absorption, storage, and excretion.

II). Removal of Polycyclic Aromatic Hydrocarbons (PAH's)

Polycyclic aromatic hydrocarbons (PAHs) refer to a group of over 100 chemicals of which anthracene, benzo(a)pyrene, naphthalene, pyrene are common. They are formed by the incomplete combustion of coal, oil and wood. They are emitted from petroleum refineries and coal, oil, and cokefired power plants. PAHs are also produced during production of asphalt and aluminum, pulp and paper. Some PAHs readily volatilize and contaminate land and soil. Food grown on such soils may contain PAHs.

PAHs are identified as priority pollutants and are causing great concern with respect to human health and environment. They are inherently 'recalcitrant hydrocarbons', and the higher molecular weight PAHs are very difficult to remediate.

1). Ma et. al., (1995) studied the influence of earthworms species *L. rubellus* on the disappearance of spiked PAHs phenanthrene & fluoranthene (100 µg / kg of soil) and found that the losses of both PAHs occurred at a faster rate in soils with earthworms, than the soil without worms. After 56 days (8 weeks), 86 % of the phenanthrene was removed. They also studied the influence of *E. fetida* on the soil contaminated with three PAHs compounds and came with very encouraging results (Table 14).

Table - 14
Removal of Soil PAH by Earthworms (*E. fetida*) and by Microbial Degradation
(Contamination < 100 mg/kg of Soil With 10 Worms / 50 gm of Soil for 11 Weeks)

PAH	Soil With Earthworms		Soil Without Earthworms (Microbial Action)
	Amount Decrease	% Removal	% Removal
1. Anthracene	2 fold	51 %	23 %
2. Benzo(a) pyrene	1.4 fold	47 %	13 %
3. Phenanthrene	Complete	100 %	77 %

Source : Ma et, al. (1995)

2). Eijsackers et al. (2001) reported that the concentration of Phenanthrene decrease steadily when the worms are added. After 50 days, only very low concentration of Phen was detected (< 0.5 mg / kg of soil). And after 11 weeks no Phen was detected as it was < 0.03 mg / kg of soil.

3). Parish et al., (2005) studied the bio-accumulation of PAHs in earthworms from contaminated soils obtained from disused manufacturing gas plant and found that earthworms readily accumulated 3 to 4 ring PAH compounds but not above that.

4). Contreras-Ramos et.al, (2006) studied the uptake of three PAHs viz. phenanthrene, anthracene and benzo(a)pyrene at different concentrations by *E. fetida* and measured the PAHs concentrations in the soil and in the tissues of earthworms exposed to the PAHs for 11 weeks. 10 earthworms per 50 gms of soil (equivalent to 200 worms per kg of soil) was added and sufficient moisture was maintained. The concentration of anthracene decreased by 2-fold after addition of earthworms and the average removal was 51 % which was only 23 % by microbes alone when the earthworms were not added to the soil. On an average the concentration of benzo(a)pyrene decreased by 1.4- fold and the average removal was 47 % which was only 13 % by microbes when earthworms were not present. Phenanthrene was completely removed (100 %) by earthworms when the amount of the chemical was < 100 mg/kg of soil, while only 77 % was removed by microbes in absence of earthworms. However, no earthworms survived when the chemical was added at the rate of 150 mg/kg of soil. Earthworms have been found to bio-accumulate the PAH compounds in the fatty deposits in their body (Contreras-Ramos et al., 2006).

Table – 15
Removal of PAHs from Contaminated Soil With & Without Earthworms
(After 11 Weeks : mg / kg of soil)

PAHs	Amount Added (mg per kg of soil)	Without Earthworm	With Earthworm (10 Worms in 50 gm Soil)
Anthracene	200	170	43
	500	409	211
	1000	592	422
Benzo(a)pyrene	50	42	19
	100	81	44
	150	133	79
Phenanthrene	50	24	0
	100	72	0
	150	100	12

Source: Contreras-Ramos (2006)

5). In relation to the study of Contreras – Ramos (2006), co-workers Alvarez-Bernal et, al., (2007) further studied dissipation of soil associated phenanthrene (500 mg / kg of soil), anthracene (350 mg / kg of soil), and benzo(a)pyrene (150 mg / kg of soil) when vermicompost (made from farmyard waste by *E. fetida*) was added to the contaminated soil @ 308 mg of vermicompost to 250 mg of soil for 100 days. Greatest dissipation of the three PAHs was in the vermicompost and it was more rapid within the initial 30 days. Losses were almost 100 % of the initially spiked PAHs.

III). Removal of Petroleum and Crude Oil Hydrocarbons

Large tracts of land are being contaminated by oil drilling activities and oil refineries. *E. fetida* has been found to tolerate 1.5 % crude oil containing several toxic organic pollutants (OECD, 2000; Safwat et al., 2002).

1). Schaefer (2001) studied and concluded that increased microbial catabolic activity due to the presence of *Eisinia fetida* was responsible for the loss of 91 % (1074 mg / kg of soil to 96 mg / kg) of crude oil contamination in 56 days of treatment.

2). Tomoko et, al., (2005) studied the addition of earthworms species *E.fetida* with varying organic wastes to an oil contaminated soil and found that worms significantly decreased oil contents in comparison to the control.

3). Ceccanti et. al., (2006) studied biochemical processes, including microbial enzyme activity and CO₂ evolution, which occurred during bioremediation treatment of oil refinery sludge with-

- 1). A mixture of microorganisms, enzymes and nutrients;
- 2). Vermicompost only;
- 3). Vermicompost with earthworms (*E.fetida*: 10 per kg of soil) for 3 months.

They concluded that the reduction of the total petroleum hydrocarbon (TPH) of the oil refinery sludge was the greatest in the treatment with earthworms (reduction of 53.3 %) followed by compost only (reduction of 39.5 %).

4). Schaefer and Filser (2007) reported their another work which used the three differing earthworm species to effect TPH losses in crude oil contaminated soils. They noted that there were more degradation products in the soils containing earthworms, indicating degradation of longer chain aliphatics (C₂₉ - C₃₆) into shorter chain aliphatics (C₁₉ - C₂₉). Prindle et. al., (2007) also studied that within a complex mixed gasoline mixture, larger *n*-alkanes were more readily degraded than the smaller *n*-alkanes.

5). Martin-Gil et, al., (2007) studied the use of earthworms *E. fetida* and vermicomposting in the treatment of high molecular weight hydrocarbons 'asphaltenes' from the Prestige Oil Spill. About 80 % vegetable waste (potato peelings etc.) was added to 20 % heavily fuel oil contaminated soil and then vermicomposted in treatment vessel. Earthworms were added at the density of 330 gm / sq. meter of treatment vessel for 6 months. Earthworms mineralized the asphaltenes thus eliminating it from the system.

IV). Removal of Agrochemicals

Large areas of farmland soils all over the world, but especially in the areas of high use of 'agrochemicals' in the wake of 'green revolution' has been badly contaminated. Over 130 formulations of different pesticides, fungicides and herbicides are known today. Aldrin, dieldrin, DDT, chlordane, endrin, heptachlor, mirex and toxaphene are persistent organic pollutants (POPs) which can remain in the soil even up to 20 years.

A study found that after only one application of the relatively persistent pesticide, 'aldrin' to soil, more than 34% was found to be present in the soil 5 years later. Most of the remaining 66% must therefore have remained in soil, either as unchanged aldrin or in the form of other closely related chemicals formed by the decomposition of aldrin. Several studies have found definite relationship between 'organochlorine pesticide' residues in the soil and their amount in earthworms, with an average concentration factor (in earthworm tissues) of about 9 for all compounds and doses tested. (Davis, 1971). Studies indicate that the earthworms bio-accumulate or biodegrade 'organochlorine pesticide' residues in the soil.

1). Ramtek and Hans (1992) isolated microbes from the gut of earthworm *Pheretima posthuma* treated with hexachlorocyclohexane (HCH) and noted significant subsequent HCH degradation. The HCH degrader microorganisms in the worms gut gradually increased over a 5 week period, replacing other gut microflora, indicating the potential for specialized gut growth by earthworms in order to degrade organic chemicals.

However, it still remains a question whether –

- a). The microbes are indigenous to earthworms guts.
- b). Earthworms gut microflora comes from the surrounding soil and plant remains.
- c). Specialised feeding determines distinctive gut flora.

2). Bolan & Baskaran (1996) studied the effect of earthworm species *Lumbricus rubellus* & *Allobophora callignosa* vermicast on the sorption and movement of herbicides C¹⁴-metsulfuron methyl, C¹⁴ – atrazine, C¹⁴ – 2,4 dichlorophenoxyacetic acid (2,4 - D) in soil. Worm vermicasts sorbed higher amount of herbicides from the contaminated soil than the control soil due to the higher levels of organic carbon & more finer size of fractions in worm worked contaminated soils

(due to grinding actions of earthworms and selective feedings). Increased agrochemical sorption due to worm activity was also studied by other authors for C¹⁴ – atrazine and C¹⁴ – matachlor in organic rich earthworm burrow linings. (Hickman & Reid, 2008).

3). Gevao et, al., (2001) applied earthworms (*Aporrectodea longa*) @ 5 worms per 2 kg of soil contaminated with non-extractable pesticides (C¹⁴ - isoproturon, C¹⁴ – dicamba and C¹⁴ – atrazine) residues in soil for 28 days. They found that due to earthworm physical activities (burrowing actions), a greater degree of previously bound pesticides residues in soil was released as compared to those without worms. When the study was applied to freshly added pesticides in soil, it was noted that the non-extractable residues of C¹⁴ - isoproturon, C¹⁴ – dicamba and C¹⁴ – atrazine were higher by factors 2, 2, and 4 respectively in the soil without worms. Thus, not only the earthworms restricted the formation of bound fraction of pesticides, but also enhanced the release and mineralization of bound pesticides residues. This is very significant action from the bioremediation point of view.

V). Removal of Polychlorinated Biphenyls (PCBs)

PCBs are a group of oily, colorless, organic fluids belonging to the same chemical family as the pesticide DDT. They constitute a family of chemicals with over 200 types, and are used in transformers and power capacitors, electrical insulators, as hydraulic fluids and diffusion pump oil, in heat transfer applications, as plasticizers for many products. PCBs are categorized as unusually toxic and 'persistent organic pollutant' (POPs). They were produced at about 100 million pounds per year during the 1960s and 70s but now severely curtailed due to its potential adverse effects on the human health and the environment.

Singer et, al., (2001) studied the role of earthworm species *Pheretima hawayana* in mixing and distribution of PCB-degrader microorganisms when added to 'Aroclor 1242' contaminated soil (100 mg / kg of soil) over 18 weeks period. Ten (10) earthworms per 0.6 kg of contaminated soil was added. The contaminated soil treated with earthworms resulted in significantly greater PCB losses (average 52 %) when compared to the soil without earthworm treatment which was 41 %. Further, the soil treated with earthworms had more uniform distribution of PCB due to earthworm bioturbation and mixing process. The authors concluded that PCB losses from contaminated soils were partly due to burrowing activities of worms, thus allowing more infiltration of microorganisms and about 10-fold greater gas exchange and diffusion. Also, the deposition of 'nutrient rich' vermicast in the burrows maintained a more metabolically active degrader microbial community.

VI). Removal of Endocrine Disrupting Chemicals (EDCs)

EDCs are relatively recent discoveries with the development of new analytical instruments. There are some 45 such industrial chemicals so far identified. They have been reported in sewage and sewage sludge and the soils contaminated by them. Unfortunately our modern Sewage Treatment Plants (STPs) fail to remove the EDCs from the treated sewage. They are causing serious concern with respect to human health as they can disrupt the functions of important hormones in human body.

Studies by Markman et al. (2007) have shown that the earthworms can bio-accumulate significantly high concentrations of endocrine disrupting chemical (EDCs) (dibutylphthalate, dioctylphthalate, bisphenol-A and 17 *b*-estradiol) in their tissues. Tiger worms (*E. fetida*) living in sewage percolating filter beds exhibited relatively high concentrations of EDCs. Those in garden soil were similarly affected. This occupies great significance as the EDCs in environment (soil and water) pose serious risk to human health and their removal using earthworms might be possible.

VII). Removal of Other Toxic Organic Compounds

- 1). Sternerson (1984) highlighted the potential of earthworms in the degradation of man-made chemicals such as TNT, DDT & DDE via detoxication enzymes.
- 2). Renoux et, al., (2000) that the presence of earthworms *Esinia andrei* either metabolized trinitrotoluene (TNT) or promoted TNT degrader microorganisms

Table : 16
Vermiremediation of Different Chemical Contaminants in Soil Performed by Some Earthworm Species

Chemical Compound	Earthworm species involved	Effect
Herbicides		
Atrazine, 2,4-D and Metsulfuron methyl	<i>L. rubellus</i> and <i>A. caliginosa</i>	Casts increased sorption of compounds
Atrazine and Metachlor	<i>L. terrestris</i>	Increased sorption of compound to burrow linings
Atrazine	<i>A. giardi</i>	Burrows and casts increased compound sorption
Insecticides		
Endosulfan	<i>M. posthuma</i>	Gut microflora promoted as specific degraders
Hexachlorohexane (HCH)	<i>P. posthuma</i>	Gut microflora promoted as specific degraders
HCH and DDT	<i>Pheretima posthuma</i>	Released previously bound residues
Crude oils and petrochemicals		
Oil refinery sludge	<i>E. fetida</i>	Promotion of microbial numbers, in combination with compost increased total petroleum hydrocarbon (TPH) loss.
Crude oil	<i>E. fetida</i>	Increased crude oil degraders and thus degradation
Oil	<i>E. fetida</i>	Increased oil losses
Hydrocarbon drilling fluids and drill cuttings	<i>E. fetida</i> fed contaminants with food mixture	Increased TPH losses
PAHs		
Phenanthrene and Fluoranthene	<i>L. rubellus</i>	Increased rate of loss
Phenanthrene and Fluoranthene	<i>E. fetida</i>	Increased PAH availability via consumption
Anthracene, Phenanthrene and Benzo(a) pyrene	<i>E. fetida</i>	Increased losses
Anthracene, Phenanthrene and Benzo(a) pyrene	<i>E. fetida</i> & vermicompost	Increased rate of losses

Chlorinated aromatics

Aroclor-1242	<i>P.hawayana</i>	Increased loss due to burrowing and microbially rich casts, effective mixing of compound
Aroclor-1242	<i>P.hawayana</i>	Effective mixing of compound and distribution of microorganisms, some sorption effects, increased microbial activity.
Aroclor-1248	<i>E. fetida</i>	Potential digestive activity and gut populations aided in losses, high bioaccumulation

Other

2,4,6-trinitrotoluene	<i>E.andrei</i>	Metabolise compound or promote degradation
di-(2-ethylhexyl) phthalate	<i>L. terrestris</i>	Hydrolyzing abilities

Source: Hickman & Reid (2008)

Experimental Study of Vermiremediation of PAHs Contaminated Soils at Griffith University, Australia

The soil was obtained from a former gas works site in Brisbane where gas was being produced from coal. The initial concentration of total PAHs compounds in the soil at site was greater than 11,820 mg/kg of soil. The legislative requirements for soil PAHs concentration is only 100 mg/kg for industrial sites and 20 mg/kg for residential sites.

10 Kg of contaminated soil was taken in each of the four 40 litre black HDPE containers and made into Treatments Sets 1,2, 3 and 4. The first remained as control and no treatment was done. In Treatment 2 approximately 500 earthworms (mixed species of *E. fetida*, *Perionyx excavatus* and *Eudrillus euginae*) of varying ages and sizes were added to the soil. (Worms were contained in about 2 kg of primary feed materials bought from Bunning Hardware). To this was added 5 kg of semi-dried cow dung as secondary feed material. In Treatment Set 3, about 5 kg of kitchen waste organics were added as secondary feed material to the 500 worms. In Treatment Set 4, only 5 kg of organic compost was added to the contaminated soil and no worms. This was set up to assess the effect of only microbial action on the contaminated soil as any organic compost is known to contain enormous amount of decomposer microbes.

In all the four treatments enough water was added time to time, to maintain the moisture content between 70-80 % and were allowed to stand for 12 weeks. They were kept under shade thoroughly covered with thick and moist newspapers to prevent any volatilization or photolysis of the PAH compounds in the soil.

Thus, Treatments Sets 2 and 3 had total of 17 kg materials (10 kg contaminated soil + 2 kg of primary feed materials with worms + 5 kg of secondary feed material added additionally). Treatment Set 4 had 15 kg (10 kg soil + 5 kg compost). Due to addition of feed materials (cow dung and kitchen waste) and compost in the contaminated soil significant dilution of PAH compounds are expected to be made and was taken into consideration while determining the impact of earthworms and the microbes in the removal of PAH compounds.

The results have been shown in tables 17 & 18 and figures 23 & 24.

Table - 17

Removal of Some PAH Compounds from Contaminated Soil by Earthworms Provided With Different Feed Materials

(10 Kg Contaminated Soil + 500 Worms* in 2 kg Feed Materials With Additional Feed Materials Cow Dung (5kg) & Kitchen Waste (5kg) for 12 Wks) and compost (5Kg).

Extracted PAH Compounds	(Set 1)	(Set 2)	(Set 3)	(Set 4)
	Treatment - 1 Only Soil (CONTROL) Initial Value (mg/kg)	Treatment - 2 Soil + Worms + Cow Dung After 12 weeks (mg/kg)	Treatment - 3 Soil + Worms + Kitchen Waste After 12 weeks (mg/kg)	Treatment 4 Soil + Compost (NO WORMS) After 12 weeks (mg/kg)
1. Benzo (a) anthracene	3945	929	1128	2476
2. Chrysene	2694	897	456	1589
3. Benzo (b) flouranthene	3067	318	101	1087
4. Benzo (k) flouranthene	315	31	64	189
5. Benzo (a) pyrene	726	83	162	367
6. Dibenzo (a,h) pyrene & Benzo (g,h,i) pyrene	1072	179	492	497
Total Measure	11, 819	2,437	2,403	6,205

Sinha et al. (2008): *The Environmentalist* (UK), Springer Publication

* Mixed species of worms (*E.fetida*, *Perionyx excavatus* & *Eudrillus eugeniae*) were used

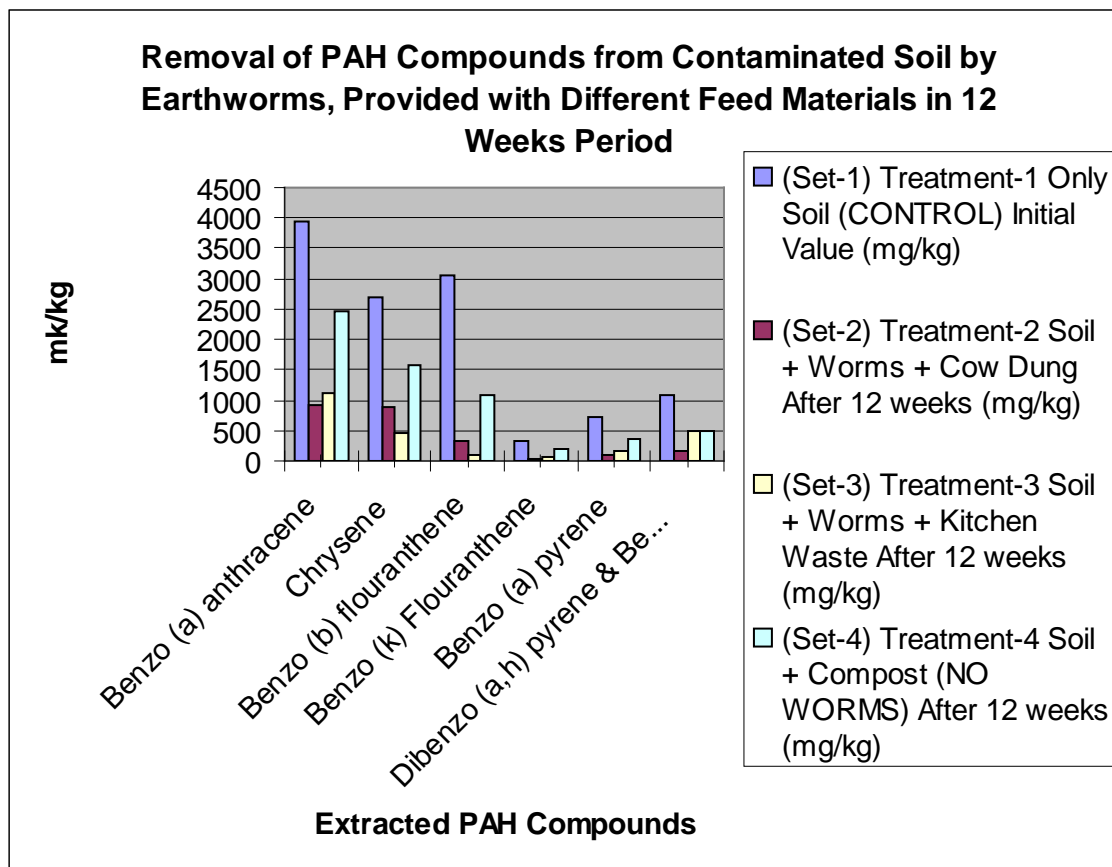


Table - 18
Percent Removal of Some PAH Compounds from Contaminated Soil by Earthworms Provided With Different Feed Materials
 (10 Kg Contaminated Soil + 500 Worms* in 2 kg Feed Materials With Additional Feed Materials Cow Dung (5kg) & Kitchen Waste (5kg) for 12 Wks)

Extracted PAH Compounds	(Set 2)	(Set 3)	(Set 4)
	Treatment - 2 Soil + Worms + Cow Dung After 12 weeks	Treatment - 3 Soil + Worms + Kitchen Waste After 12 weeks	Treatment - 4 Soil + Compost (NO WORMS) After 12 weeks
1. Benzo (a) anthracene	76 % (58 %)	71 % (56 %)	37 % (6 %)
2. Chrysene	67 % (49 %)	83 % (68 %)	41 % (12 %)
3. Benzo (b) flouranthene	90 % (72 %)	97 % (82 %)	65 % (47%)
4. Benzo (k) flouranthene	90 % (72 %)	80 % (65 %)	40 % (10%)
5. Benzo (a) pyrene	89 % (71 %)	78 % (63 %)	49 % (24 %)
6. Dibenzo (a,h) pyrene & Benzo (g,h,i) pyrene	83 % (65 %)	54 % (39 %)	54% (30 %)
	Av. = 79 % (61 %)	80 % (65 %)	47.5 % (21 %)

Sinha et al. (2008): *The Environmentalist* (UK), Springer Publication

(%) Values within bracket are those after taking the dilution factor (due to mixing of feed materials) into account. This is just in 12 weeks period.

Figure 1: Percent removal of PAH from contaminated soil by earthworms, provided with different feed materials.

Figure 2: Percent removal of some PAH compounds from contaminated soil by earthworms provided with different feed materials, after taking the dilution factor (due to mixing of feed materials) into account.

Findings and Results

Results confirm the decisive role of earthworms in PAH removal which can be by both activities – bioaccumulation, and by promoting microbial activities. When microbial activity in the soil is enhanced alone (without aided by earthworms) by adding microbe rich organic compost to the soil (Set 4) the removal rate of PAHs are not very significant. This indicates that earthworms acts in a different manner and contributes decomposer microbes for hydrocarbons which otherwise is normally not available in soil or in ordinary compost. Singleton et al. (2003) has reported some 'uncultured' bacterial flora tightly associated with the intestine of the earthworms. Some of them, such as *Pseudomonas*, *Acaligenes* and *Acidobacterium* are known to degrade hydrocarbons.

Providing the earthworms with additional feed materials (in the form of cow dung and kitchen waste – both organics) must have played important role in raising worm activity and in its reproductive behavior, and also in stimulating the soil microbial activity. There was not much significant difference in the impact of two types of feeds. Ma et al. (1995) showed an increase in PAH loss from polluted soil when the worms were denied of any additional source of food. They concluded that earthworms increase oral intake of soil particles when driven by 'hunger stress' and consequently ingested more PAHs polluted soil. While this may be a temporary phenomenon in short time (9 weeks of study) it can never be a long-term strategy because any bioremediation treatment of polluted soil is time-taking – in months and years. Worms would starve and die by then. And, adding organic feed materials to the polluted soil has several other advantages. It promotes microbial activities in soil and when the worms ingest them and excrete – the excreted products are nutrient rich organic fertilizers.

Coupling Vermiremediation With Phytoremediation for Cleanup & Reclamation of Marine Sediments

The reclamation of chemically contaminated marine sediments are becoming a global environmental problem because every year high volumes of 'dredged sediments' are produced world-wide which needs treatment. Dredged sediments are characterized by 'high salt', asphytic content, and both 'organic and inorganic' contaminants such as hydrocarbons, nutrients, heavy metals, chemicals & synthetic substances. As the traditional treatments involved very high cost, some alternative biotechnological treatment for reclamation was suggested (Bianchi et. al., 2009 a & b). It was coupled with phytoremediation involving marine grasses *Paspalum vaginatum* & *Tamarix gallica* (salt tolerant species) and earthworm species *Eisenia fetida*. After one year of treatment the physical, chemical and biological conditions of the sediments were fit enough to allow grasses to grow and worms to survive. When grown alone the plants would barely survive in the contaminated sediments although they were reported to be bioaccumulating heavy metals like copper (Cu), zinc (Zn), lead (Pb) and arsenic (As) in high concentrations (Bianchi et. al., 2009; Bech et. al., 2002; Conesa et. al., 2006). The worms alone would not perform very well without the plants but their presence made a big difference. Through excretory action they transformed the sediments into a 'soil-like' material for the plants to grow and the plants provided the organic materials for feeding the worms.

Earthworms Vermicompost: A Wonderful Bioadsorbent Material for Chemical Pollutants in Environment

Vermicompost is rich in 'humic substances' (mainly humic and fulvic acids) which has high potential for retaining organic and inorganic pollutants from soil. On the surface of humic materials, electrostatic forces domain the adsorptive processes. This kind of 'adsorption' is attributed to a great number of 'peripheral and hydrophilic' chemical groups, mainly -COOH, -SH and -OH (Pereira and Arruda, 2003).

Vermicompost have been found to degrade triazines herbicides e.g. simazine, terbutylazine, cyanazine and prometryn in soils and completely remove them (Delgado-Moreno and Pena, 2007). Landgraf et al., (1998) reported that herbicide 'metribuzin' was effectively adsorbed and degraded by vermicompost. Fernandez-Bayo et al., (2007) reported that sandy and clay soils contaminated with insecticide 'imidacloprid' which are 'hydrophobic' molecules are effectively retained by the vermicompost which have 'hydrophilic' chemical groups. This is attributed to the 'humic and lignin contents' present in the vermicompost. Contreras-Ramos et al. (2008) reported efficient removal of polycyclic aromatic substances e.g. phenanthrene, anthracene and benzo(a)pyrene from contaminated soils mixed with vermicompost.

Advantages of Vermiremediation Technology

Earthworms Improves Total Quality of Soil in Terms of Physical, Chemical & Biological Properties

There are several advantages in using earthworms for bioremediation of chemically contaminated soils. They have been shown to both 'retard the binding of chemical compounds with soil particles' and also 'increase compound availability' for microbial action while also enhancing the population of degrader microbes within the system. Earthworms have the potential to be employed not only in the recovery of contaminated soils as a part of bioremediation strategy, but also in the subsequent improvement of that soil and the land as a whole, for other beneficial uses.

Significantly, vermiremediation leads to total improvement in the quality of soil and land where the worms inhabit. Earthworms significantly contribute as soil conditioner to improve the physical, chemical as well as the biological properties of the soil and its nutritive value. They swallow large amount of soil everyday, grind them in their gizzard and digest them in their intestine with aid of enzymes. Only 5-10 percent of the chemically digested and ingested material is absorbed into the body and the rest is excreted out in the form of fine mucus coated granular aggregates called 'vermicastings' which are rich in NKP (nitrates, phosphates and potash), micronutrients and beneficial soil microbes including the 'nitrogen fixers' and 'mycorrhizal fungus'. The organic matter in the soil undergo 'humification' in the worm intestine in which the large organic particles

are converted into a complex amorphous colloid containing 'phenolic' materials. About one-fourth of the organic matter is converted into humus. The colloidal humus acts as 'slow release fertilizer' in the soil.

During the vermi-remediation process of soil, the population of earthworms increases significantly benefiting the soil in several ways. A 'wasteland' is transformed into 'wonderland'. Earthworms are in fact regarded as 'biological indicator' of good fertile soil (Neilson, 1951). One acre of wasteland when transformed into fertile land may contain more than 50,000 worms of diverse species. Bhawalkar & Bhawalkar (1994) experimented and concluded that an earthworm population of 0.2 – 1.0 million per hectare of polluted land / wasteland can be established within a short period of three (3) months.

Environmental – Economics of Vermiremediation Technology : Converting 'Wasteland' into 'Wonderland'

Vermiremediation technology by earthworms is a self-promoted, self-regulated, self-improved, self-driven, self-powered & self-enhanced, low or no energy requiring zero-waste technology, easy to construct, operate and maintain. Any vermiculture technology involves about 100-1000 times higher 'value addition' than other biological technologies. Obtaining earthworms from vermiculture farms would be one-time cost in any vermiremediation technology as the earthworms multiply rapidly creating huge army of worms which further promote and enhance the process.

Vermiremediation of chemically polluted / contaminated soils / lands would cost about \$ 500 - 1000 per hectare as compared to \$ 10,000 - 15,000 per hectare by mechanical excavation of contaminated soil & its landfill disposal. Of considerable economic and environmental significance is that the worm feed used in vermiremediation process is necessarily an 'organic waste' product. This means that it would also lead to reuse and recycling of vast amount of organic wastes which otherwise end up in landfills for disposal at high cost. And what is of still greater economic and environmental significance is that the polluted land is not only 'cleaned-up' but also 'improved in quality'. Earthworms improves the fertility of soil by adding its excreta – the vermicast (as vermicompost) which contains rich nutrients (NKP and micronutrients), enzymes and growth hormones and beneficial nitrogen-fixing and decomposer microbes. The soil becomes lighter and porous rich in biological activities and the productivity is increased to several times. Earthworms not only convert the 'wasteland' into 'wonderland' it itself becomes a valuable asset. Vermiculture is a flourishing industry all over the world today.

CONCLUSION

(I). Microbial Remediation of Polluted Lands

Microbial bioremediation has developed from the laboratory to a fully commercialized technology over the last 30 years in many industrialized nations. Microbes have been mostly used to treat industrial waste streams, with the organisms either 'immobilized' on to different support matrixes or in a 'free-living' state, enclosed in treatment tanks or other kinds of reactor vessels. Subsequently the metal-loaded microbial biomass is either disposed appropriately (in secured hazardous waste landfills or incinerated), or treated to 'recover' the metals for re-use in industries.

Microbial technology has gained popularity for site remediation as it treats the soil contaminants in site itself, and significantly cuts down the excavation costs of soil. It is intrinsic process done *in-situ* and relies on the naturally occurring biological process carried out by native and indigenous microorganisms. It can be applied to sites with high water table and does not destroy the site that is to be treated (detoxified). Microbial remediation can handle all types of soil pollutants starting from rare metals to radionuclides. Native microbes in any contaminated site is 'acclimatized' and are capable of transforming the toxic metals to their oxides or hydroxides.

Bioremediation can be both '*in-situ*' and '*ex-situ*'. In the '*ex-situ*' bioremediation which is above ground treatment, soils have been excavated and washed or sediments have been extracted from subsurface and then decontaminated. This becomes more costly treatment. Recently, 'genetically engineered' microbes have been used for '*ex-situ*' bioremediation.

Bioremediation by microbes works either by transforming or by degrading contaminants to less toxic / hazardous chemicals or innocuous substances. In case of metal contaminated sites, the microbes interact with metals and transform them from one chemical form to another by changing their 'oxidation state' through addition or removal of electrons. In some microbial bioremediation strategies, the solubility of the transformed metal increases, thus increasing the mobility of the contaminants and allowing it to be more easily flushed out of the environment. In other strategies, the opposite occurs. The transformed metals precipitate out of the solution, leading to their immobilization. Both strategies of bio-transformation of metals either 'immobilize' the contaminants in place or 'accelerate' their removal.

Chemical treatments for the removal of heavy metals from contaminated sites/materials are chemical extraction with acids and / or chelating agents for soil treatment and precipitation for groundwater cleaning. In industries, the metals contained in acid-drainage waters are most of the time precipitated using lime. Such treatments are expensive, and results into formation of large quantities of metal-hydroxides. Bioremediation of contaminated sites by microbes is very cost-effective and environmentally friendly as compared to the chemical treatment which requires excavation or pumping of contaminated materials from the polluted site, followed by the addition of reducing chemicals. It leads to considerable destruction of the site treated and the land may become useless for any future use.

Some risks and hazards of environmental biotechnology working with microbes are associated with three properties of microorganisms. They are :

- 1). The potential of a few strains to cause diseases thus behaving as pathogens);
- 2). The potential for undetected genotypic or phenotypic changes to alter a tested and approved microbial process; &
- 3). The ubiquity of organisms which can contaminate the system.

Less hazardous microorganisms that would fulfill the same objectives as the 'undesirable pathogen' can be discovered & selected from microbial diversity, or created by genetic engineering. Biotechnological researches for several years have employed selection techniques to screen wide genotypes of related organisms for non-pathogens as well as more complex methods by inducing mutations and selection of desirable strains. Genetic engineering is also used to remove the useful genetic components responsible for bioremediation properties from the otherwise pathogenic organisms and incorporate them into non-pathogenic organisms such as *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* and use them for bioremediation.

(II). Plant remediation of Polluted Lands

Phytoremediation technologies are based on the principles of bioengineering & environmental biotechnology that a 'living plant' can be considered as a 'solar driven pump', which can extract, concentrate, degrade, volatilize or vaporize, soluble toxic substances from the soil, water or air, through their natural water & mineral uptake, transport, partitioning, assimilation & transpiration mechanisms and systems. It is also cost-effective, economically viable, ecologically compatible and socially acceptable and safer methods for management of polluted lands & soil as compared to the physical and chemical methods (as there is no risk from exposure to the toxic substances at waste and spill sites and remediation of polluted soil in world may involve a cost up to US \$ 300 billions by other methods). (Raskin et al., 1997).

It is a cheap alternative to the SUCK, MUCK and TRUCK cleaning approach to contaminated sites. The technology capitalizes on the synergistic relationship among plants, microbes, water & soil that have evolved naturally over millions of years.

The technology can be implemented *in-situ* or *ex-situ* to cleanup diverse organic contaminants, e.g. petroleum hydrocarbons, gas condensates, solvents, crude oil, pesticides, herbicides, explosives, chlorinated compounds and polyaromatic hydrocarbons as well as typical inorganic toxicants like heavy metals, metalloids, radionuclides etc.

By planting 'hyper-accumulator' plants in metal contaminated soils & industrial waste sites, several heavy metals can be removed by harvesting the above-ground parts. The precious

metals can be recovered back from the plant parts (leaves & roots) through solvent extraction method for reuse in industries thus preventing the mining of these metals and the consequent environmental problems. Growing hyper-accumulator plants in contaminated soils can reduce soil erosion, increase soil organic matter and restore fertility. The technology is 60 to 80 % less costly as compared to the conventional physical & chemical methods of soil & water decontamination. Furthermore, the physio-chemical methods used for soil remediation render the land useless as a medium for plant growth as they also remove all biological soil organisms such as the useful microbes e.g. nitrogen fixing bacteria & mycorrhizal fungi, and also the earthworms – all necessary for maintaining soil fertility.

In addition to pulling out the toxic contaminants from the soil to metabolize, concentrate (bioaccumulate) or evaporate, the phytoremediation techniques involve extensive pull out and evaporation of water from the plant covered sites. This high consumption and recycling of water can also prevent wash out of pollutants and slow down the possible migration of toxic compounds through the soil and into the groundwater.

Researches into environmental biotechnology promises to enhance the phytoremediation efficiency of known phytoremediator plants. A genetic combination of 'fast growing', 'high biomass yielding' & 'high tolerance & hyper-accumulation' of toxic metals in plant shoot is best suited for the purpose of phytoremediation. It should also be easily cultivated and harvested.

These days several commercial organizations are employing phytoremediation technology for environmental clean up and restoration. Phytotech Inc. of US is using Indian mustard (*Brassica* spp.) to remove lead (Pb) from soil and sunflower (*Helianthus annuus*) to remove uranium (U) and cesium (Cs) from aqueous waste stream. Phytoworks Inc. of US is focusing on remediation of organics and mercury (Hg) by using transgenic plants metabolizing mercury. Earthcare Inc. is working on phytoremediation of organic contaminants.

The novel bioremediation system called 'symbiotic engineering' involving advantages of both the bacterium rhizobium and the leguminous plants using many useful genes like *AtPCS*, *MTL4* (metallothionein gene) and *IRT1* (iron regulated transporter gene) may provide another valuable bioremediation tool.

However, there are some limitations of all phytoremediation technologies. These are -

1. The contaminated sites / water bodies may be too toxic for plants to survive and work;
2. In case of phytoremediation, if the contaminants are below the root zone, it may not be extracted or stabilised;
3. Time required for phytoremediation of contaminated sites may be too long, often going in years.

(iii). Vermiremediation of Polluted Lands by Earthworms

Through a combination of direct (bioaccumulation of chemical contaminants) and indirect (bioadsorption of chemical contaminants by worm vermicast) action, proliferation and promotion of chemical degrader & catabolic microorganisms in soil, and through their own biological, physical and chemical actions on soil, the earthworm assisted vermiremediation process has been shown to be suited to a wide range of soils contaminated with organic and inorganic chemicals.

Earthworms have great potential in removing hydrocarbons and many other chemicals from contaminated soil, even the PAHs like benzo(a)pyrene which is very resistant to degradation. They are extremely resistant to toxic PAHs and tolerate concentrations normally not encountered in the soil. It is important to note that nearly 80 % removal (60-65 % if the dilution factor is taken into account) of seven important PAH compounds was achieved in just 12 weeks period and that too with only about 500 worms (of both mature and juvenile population) in 10 kg of soil (50 worms/kg of soil). And this was during the winter season in Brisbane (March – May 2006) when the biological activities of worms are the lowest ebb. Increasing the number of worms per kg of contaminated soil to about 100 mature adult worm /kg of soil, and the time of remediation up to 16 weeks could have completely (100 %) removed the PAH compounds (Sinha et al., 2008).

Many soils contain abundance of pores with diameters of 20 nm or less. Such pores are too small to allow the smallest bacterium (1 µm), protozoa (10 µm) or root hairs (7 µm) to penetrate and attack the chemicals. A chemical contaminant residing in such fine pores in soil is thus completely protected from attack by a microbe in the soil for biodegradation action. In other words such chemical contaminants are not 'bio-available' for any biological action. Earthworms play a very important and critical role here by enlarging the pores through continuous 'burrowing actions' in the soil, thus allowing the microbes to enter into the pores and act on the contaminants. It also stimulates the population of decomposer microbes to several folds for enhanced biodegradation action. The 'gizzard' in the earthworms helps to grind the food very thoroughly with the help of tiny stones swallowed by the worms into smaller particles 2-4 µm in size. This grinding action may serve to make PAHs or any other chemical contaminants sequestered in the soil 'bio-available' to decomposer microbes for degradation.

Vermi-remediation may prove very cost-effective and environmentally sustainable way to treat polluted soils and sites contaminated with diverse chemicals in just few weeks to months. Moreover, this is an '*in-situ*' treatment on site. Comparing the cost incurred in *ex-situ* mechanical treatment by excavation of contaminated soils and their transport to treatment sites or for safe disposal in secured landfills (as hazardous wastes), this technology is most economic. The greatest economic and environmental benefit is that the remediated soil is 'recovered' for 'reuse' i.e. the land becomes suitable for productive works – agriculture, horticulture or for construction activities.

With the passage of time, the remedial action of worms becomes greatly intensified. As the worms multiply at an enormous rate it can quickly achieve a huge arsenal for enhanced degradation of chemicals in much shorter time. One limitation might be the huge numbers of earthworms that are required for vermiremediation – about 10 worms per 50 gm of soil (Contreras-Ramos et. al, 2006) and maintaining sufficient moisture for normal functioning of the worms. But this should not be a limiting factor in the view that worms multiply at rapid rate by 2^8 i.e. 256 worms every 6 months from a single individual and each of the 256 worms multiplies in the same proportion to produce a huge biomass of worms in a short time. To maintain adequate soil moisture the pond or lake water, rainwater or even 'recycled wastewater' can be used.

Tribute to the Earthworms

Earthworms as 'ecosystem engineers' are justifying the beliefs and fulfilling the dreams of the great visionary scientist Sir Charles Darwin as 'unheralded soldiers' of mankind and 'friend of farmer's. Darwin wrote a book in which he emphasized that '*there may not be any other creature in world that has played so important a role in the history of life on earth*'.

One of the leading authorities on earthworms and vermiculture studies Dr. Anatoly Igonin of Russia has said –

'Nobody and nothing can be compared with earthworms and their positive influence on the whole living Nature. They create soil and everything that lives in it. They are the most numerous animals on Earth and the main creatures converting all organic matter into soil humus providing soil's fertility and biosphere's functions: disinfecting, neutralizing, protective and productive'.

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