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ANALYSIS OF MINES AND CONTAMINATED AGRICULTURAL SOIL SAMPLES FOR FUNGAL DIVERSITY AND TOLERANCE TO HEAVY METALS

SHAZIA IRAM[1], IFTIKHAR AHMAD[2] AND DORIS STUBEN[3]

[1]*Department of Environmental Sciences, Fatima Jinnah Women University, Rawalpindi, Pakistan.*
[2]*National Agricultural Research Center, Islamabad, Pakistan*
[3]*Institute of Mineralogy and Geochemistry, Forschungszentrum Umwelt (FZU)*
University of Karlsruhe, Germany.

Abstract

In the present investigation soil samples were collected from peri-urban agricultural soils irrigated by industrial and sewerage waste of Hudiara drain, Lahore, Pakistan and mine waste of Wiesloch, Germany for the analysis of fungal diversity and tolerance to heavy metals. Heavy metals analysis was done by X-ray florescence and ICP-MS. X-ray florescence showed that Zn, As and Pb concentration was higher in waste mine soil sample of Wiesloch, Germany. ICP-MS appeared to be more sensitive and showed that Fe, Mn, Cu, As, Sr, Mo, Cd, Sb, Ti, Zn and Pb were present in higher concentration in waste mine of Germany. Soils of peri-urban areas of Pakistan had lower concentration of heavy metals as compared to waste mine of Wiesloch, Germany. Diversity and frequency of fungi was analyzed using soil dilution method. Overall frequency percentage and diversity was higher in Pakistan soil than soil of Wiesloch, Germany. Different fungi viz., *Acremonium* sp., A*lternaria* sp., *Aspergillus niger*, *Aspergillus* sp., *Aspergillus nodulans, Aureobasidium* sp., *Chaetomium* sp., *Coniothyrium* sp., *Curvularia* sp., *Fusarium* sp., *Humicola* sp., *Monilia* sp., *Monocillium* sp., *Mortierella* sp., were isolated. *Aspergillus niger, Aspergillus flavus* and *Aspergillus nodulans* were selected and checked for tolerance to toxic metals ($CdCl_2$, $CuSO_4$, $NiCl_2$ and $ZnCl_2$) at different concentrations (1, 5, 10, 15, 20, 25, 30, 35, 40 ppm) by the measurement of radial growth. All the tested fungi showed tolerance to $ZnCl_2$ (25ppm) and $NiCl_2$ (12ppm) but no tolerance against $CdCl_2$ and $CuSO_4$.

Introduction

Biosphere pollution by chemicals and heavy metals such as cadmium, nickel, zinc, lead, copper etc., accelerated dramatically during the last few decades due to mining, smelting, manufacturing, use of agricultural fertilizers, pesticides, municipal wastes, traffic emissions, industrial effluents and industrial chemicals etc. The problem of environmental pollution due to toxic metals has begun to cause concern now in most major metropolitan cities. Toxic heavy metals entering the ecosystem may lead to geoaccumulation, bioaccumulation and bio magnification. Heavy metals like Fe, Cu, Zn, Ni and other trace elements are important for proper functioning of biological systems and their deficiency or excess could lead to a number of disorders (Ward, 1995). Food chain contamination by heavy metals has become a burning issue in recent years because of their potential accumulation in bio systems through contaminated water, soil and air.

Soil is a specie rich habitat containing all major groups of microorganisms. The soil microbiota is instrumental in the degradation and synthesis of organic compounds. It is also actively involved in the cycling of plant nutrients and in the weathering of primary minerals (Parkinson & Coleman 1991). Soil organisms consist of the micro flora (bacteria and fungi) and the soil fauna (protozoa and invertebrate groups such as nematodes, mites and earthworms) (Mueller & Bills, 2004). The effects of pollution on soil biodiversity can be amplified through the food chain and threaten sustainability of natural ecosystems (Bridges *et al.*, 2000; Amisu *et al.*, 2003).

Pakistan is an agricultural country having the world's largest canal irrigation system. Indus basin that covers 70% of irrigated area for crop production is the major source of water in the country. Owing to rapid increase in population and uncertain environmental conditions, this water is not adequate to cope with the crop water requirement and needs additional means to provide extra water for agricultural purposes. The main source of irrigation is canal and ground water but the quality of ground water is poor for the sustainability of agriculture system. To cope with the present demand, use of municipal sewage water that consists of domestic liquid waste, as well as industrial effluents is becoming a common practice. Hudiara Drain, which is a natural storm water channel, originates from Batala in Gurdaspur District, India and enters into Pakistan. As a result, organic wastes and toxic chemicals have badly affected aquatic life both in this drain and in the river Ravi.

Mining at Wiesloch has very old history from Roman times (2000 years). The intensification of mining operations, crushing, washing and smelting of the ore were performed close to the mining site and the amount of mining residues deposited during mining. The heaviest deposition of airborne metal particles occurs in the vicinity of mines, smelters and metal processing/heavy engineering works, which constitute the main emission sources. But many of the particles are so small that they can be carried to enormous distances by the wind. Mercury in particular, which largely occurs in gaseous form in the atmosphere, can be dispersed a very long way.

Environmental pollution with heavy metals is a global issue. It is present everywhere, though to different degrees and is specific to certain parts of the biogeosphere. Numerous organic and inorganic compounds, heavy metals, pollute the environment in particular. Living organisms are not able to prepare and adapt rapidly to a sudden and huge environmental load with different toxic substances, and therefore, the accumulation of certain elements, especially of heavy metals with mainly toxic effect, can cause undesirable changes in the biosphere with unforeseeable consequences (Djukic & Mandic, 2000).

In the past decade many countries have spent billions of dollars trying to clean up contaminated ground water and soil. Despite the large financial investment ground water clean up efforts are falling short of public expectations. The limitations of conventional ground water clean up technologies and the hazards of conventional soil clean up methods have spurred investigations into *In-situ* bioremediation, which uses microorganisms to destroy or immobilize contaiminants in place. Microorganisms are nature's original recyclers, converting toxic organic compounds to harmless products, often carbon dioxide and water. Conventional techniques commonly applied to remove heavy metals from waste water and contaminated soil includes chemical (precipitation, eutralization) or physical (ion exchange, membrane separation, electro dialysis and activated carbon adsorption) methods (Atkinson, 1998). Moreover these processes may be non–viable at low concentrations. Further these processes are expensive and not ecofriendly (Gadd & Griffith 1978; Volesky 1987).

Therefore researcher develops a feasible method to accelerate the process of decay and removal by encouraging the microbial and associated biota (flora and fauna), within the ecosystem, to degrade, accumulate and/or remove the pollutants from the identified sites. This process is known as bioremediation. Therefore we have studied filamentous fungi from waste mines and contaminated agricultural soil areas to assess their metal tolerance and metal removal potential from contaminated soils. Bioremediation is than a process that uses naturally occurring or genetically engineered microorganisms such as fungi and bacteria to transform harmful substances into less toxic or non-toxic compounds.

Bioremediation promote the growth of microorganism to degrade contaminants by utilizing those contaminants as carbon and energy sources. The bioremediation systems in operation today rely on microorganisms native to contaminated sites, encouraging them to work by supplying them with the optimum levels of nutrients and other chemicals essentials for their metabolism. Researcher are currently investigating ways to augment contaminated sites with non-narrative microbes including genetically engineered microorganisms specially suited to degrade the contaminants of concern particular sites. It is possible, that this process known as bioaugmentation could expand the range of possibilities for future bioremediation system.

Materials and Methods

Study areas and samples collection: Sampling was done from the well-defined depth of the selected sites and composite sample was formed by combining soil from several spots in the sample area, mixing thoroughly before analysis.

Three soil samples were collected from Wiesloch and Karlsruhe, Germany (Fig. 1). Mine soil and limestone soil was collected from Wiesloch, Germany and normal soil sample was collected from Karlsruhe, Germany. From the agricultural areas around the Hudiara drain, Lahore, Pakistan, 11 samples of soil contaminated by sewage and industrial waste water were collected (Fig. 2).

Isolation of fungi: Petri dishes filled with PDA medium were inoculated with 1 ml of soil suspension keeping 3 replicates of each treatment. The Petri dishes were placed in the incubator at an appropriate temperature. The same steps repeated with all the other soil samples (Usmani & Ghaffar, 1982).

Identification and preservation of fungi: After incubation distinct colonies were counted and identified. The cultures were identified at generic level on the basis of macroscopic characters viz., colony morphology, colour, texture, shape, diameter and appearance of colony and microscopic characteristics viz., septation in mycelium, presence of specific reproductive structures, shape and structure of conidia and presence of sterile mycelium (Zafar *et al.*, 2006). Fungi were preserved on PDA slants for further research.

Heavy metal tolerance test: Potato Dextrose Agar (PDA) medium was prepared and amended with various amounts of heavy metals viz., $CdCl_2$, $CuSO_4$, $NiCl_2$ and $ZnCl_2$ to achieve the desired concentration of 1,5,10, 15, 20, 25, 30, 35 and 40mM.pH was maintained at 5.6 by adding 3 molar solution of NaOH. Media was autoclaved and cooled. Streptomycin 1ml was added in heavy metal and controlled media. An inoculum of test fungi was spotted in the center of metal and control plates without metal. The plates were incubated at $29 \pm 1^{o}C$ for 2-5 days to observe the growth of fungi on the spotted area. Colony morphology and radial growth diameter was measured (Malik & Jaiswal, 2000). The growth was monitored by culture from the point of inoculation or centre of the colony. Tolerance fungi were studied by the comparison among fungi grown in the presence of metals and in the absence of metals as control.

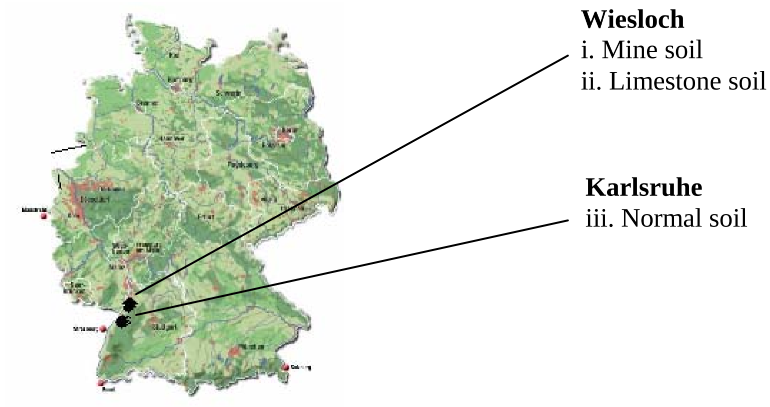

Fig. 1. Soil samples collection from Wiesloch and Karlsruhe, Germany.

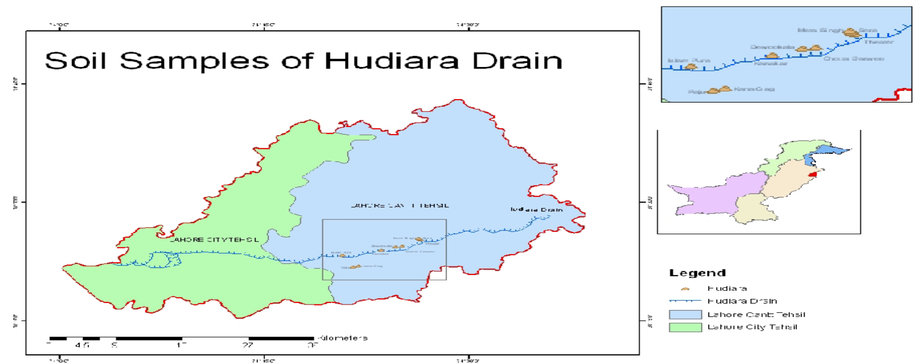

Fig. 2. Samples collection around the different locations of Hudiara drain, Lahore, Pakistan.

Chemical analysis by XRF and ICPMS: XRF: A range of elements was determined by x-ray fluorescence spectrometry. The samples were dried by spreading it on a paper and exposing it by using a small oven. The dried samples were screened with a 2 mm mesh to remove large objects. The samples were ground and the soil sieve to reduce the particle size to less than 0.250 mm (or preferably to less than 0.125 mm), homogenized well and then sub-sample 3 to 5 grams of the dry, well-ground soil was placed in an XRF sample cup for analysis. In practice, data for Cu, Zn, As, Rb, Cd and Pb were taken was X-Ray counts were converted into concentrations by a computer program based on the matrix correction method (Kramar & Puchelt, 1981). The accuracy of determinations was checked by using certified reference materials.

ICPMS: ICPMS was used for the analysis of trace elements in soil samples. For total concentration, 2 g of air-dried soil was transferred into centrifuge tube. The seven fractions were carried out step by step. Standard reference samples (trace metals in drinking water HPS SOLL-W and HPS VF 4) were analyzed along with soil and mines samples. The concentrations of Al, Cr, Fe, Mn, Ni, Cu, Zn, As, Sr, Mo, Cd, Sb, Ti and Pb in each of the sample were determined. Blank values were negligible for all elements under consideration, which indicated the rather high purity of the reagents used.

Results and Discussion

Isolation of fungi and tolerance: Contaminations of soils with heavy metals have shown toxic impacts on plants, animals and human health. The extent of toxicity depends on the nature of the metals and soil characteristics, and the complex interactions between metals and the environment. Present study investigated the effectiveness of isolated fungi from contaminated agricultural and mine soil samples and isolated fungi treated with heavy metal at different concentration. Different fungi were isolated from collected soils samples (Table 1).

The number of fungi was more in Lahore samples as compared to mine samples (Fig. 1). *Aspergillus niger, Fusarium* sp., and *Mucor* sp., were present and dominant in all collected soil samples of drain and selected for resistance analysis. *Aspergillus niger, Fusarium* sp., *Mucor* sp., were able to resist 20mM concentration of $ZnCl_2$ and $NiCl_2$ (Figs. 2 & 3). Non of the fungi were able to tolerate the 25mM, 30mM, 35mM and 40mM concentration of $CdCl_2$, $CuSO_4$, $NiCl_2$ and $ZnCl_2$ (Mahapatra & Banerjee, 1996). *Aspergillus niger* showed the highest tolerance at 20 ppm followed by *Fusarium* sp., and *Mucor* sp.

Interactions between fungi and heavy metals were expatriated in many ways. Because of their resistance and detoxification to heavy metals, fungi are able to leach, absorb and transform heavy metals (Barea & Jeffries, 1996). Although heavy metals are toxic to fungal population and harmful to its processes, some special fungi have been applied to the treatment of industrial waste materials and the remediation of soils polluted by heavy metals. The application of fungi biomass and activity to evaluating the pollution situation and ecological risk of heavy metals in environment media is also significant (Chen *et al.*, 2002).

Filamentous fungi can also show high levels of metals and metalloids resistance, being this resistance associated to the capacity to accumulate these elements (Durán *et al*., 1999; Cánovas *et al*., 2003). The high incidence of heavy metal resistance detected in this work indicates the potential of fungi as bioremediation agents. Heavy metal contamination due to natural and anthropogenic sources is a global environmental concern. Release of heavy metal without proper treatment poses a significant threat to public health because of its persistence, biomagnification and accumulation in food chain (Comis, 1996). Microbial metal bioremediation is an efficient strategy due to its low cost, high efficiency and ecofriendly nature. Recent advances have been made in understanding metal-microbe interaction and their application for metal accumulation/detoxification (Rajendran *et al*., 2003).

Chemical Analysis by XRF and ICPMS: The concentration of heavy metals (As, Zn, Pb, Sb) and trace elements (Fe, Mn, Cu, Zn, As, Sr, Mo, Cd, Sb, Ti, Pb) were significantly higher in analyzed samples of Wiesloch as compared collected samples around the Hudiara drain (Tables 4 & 5). Most heavy metals are added during industrial activities, such as mining, coal and waste combustion and steel processing. Some decades ago there were several very large sources of metal emissions in the area of Wiesloch, Germany. For example, smelting plants annually released hundreds of tonnes of arsenic, lead, copper, zinc and other metals. Besides this, road traffic is also responsible for over a thousand tonnes of lead each year, a result of lead additives in petrol.

Table 1. Type of fungi isolated from heavy metal contaminated soil samples of Pakistan and Germany.

Fungi	Hudiara Drain, Lahore, Pakistan											Mines, Wiesloch Germany		
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	M1	M2	M3
Aspergillus flavus	-	+	-	+	+	+	-	+	-	+	-	-	-	-
Acremonium sp.	-	+	-	+	-	+	-	-	-	-	-	-	-	-
Alternaria sp.	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Aspergillus niger	+	+	+	+	-	+	+	+	+	+	+	-	-	-
Aspergillus sp.	+	+	+	-	+	+	+	+	-	-	+	-	-	-
Aspergillus nodulans	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Aureobasidium sp.	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Chaetomium sp.	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Coniothyrium sp.	-	-	+	-	+	-	+	-	-	+	-	-	-	-
Curvularia sp.	-	-	-	+	-	-	-	-	-	-	+	-	-	-
Fusarium sp.	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Humicola sp.	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Monilia sp.	-	-	+	-	-	+	-	-	-	-	-	-	-	-
Monocillium sp.	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Mortierella sp.	-	+	-	-	-	+	-	+	-	-	+	-	-	-
Mucor sp.	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Penicillium sp.	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Phoma sp.	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Phytophtora sp.	+	-	-	-	-	-	-	-	-	+	-	-	-	-
Rhizopus sp.	-	-	-	-	-	-	+	-	-	-	-	-	-	-

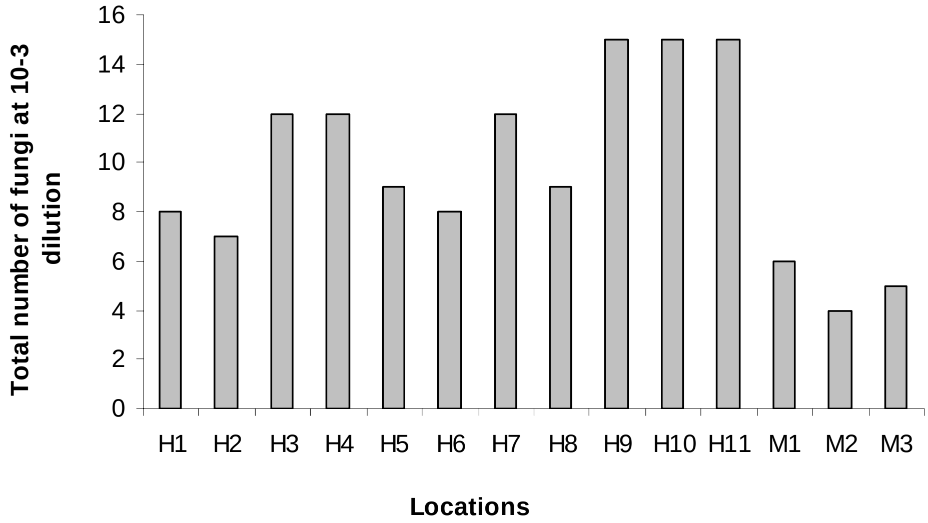

Fig. 3. Total number of fungi isolated from the contaminated soils of Pakistan and Germany.

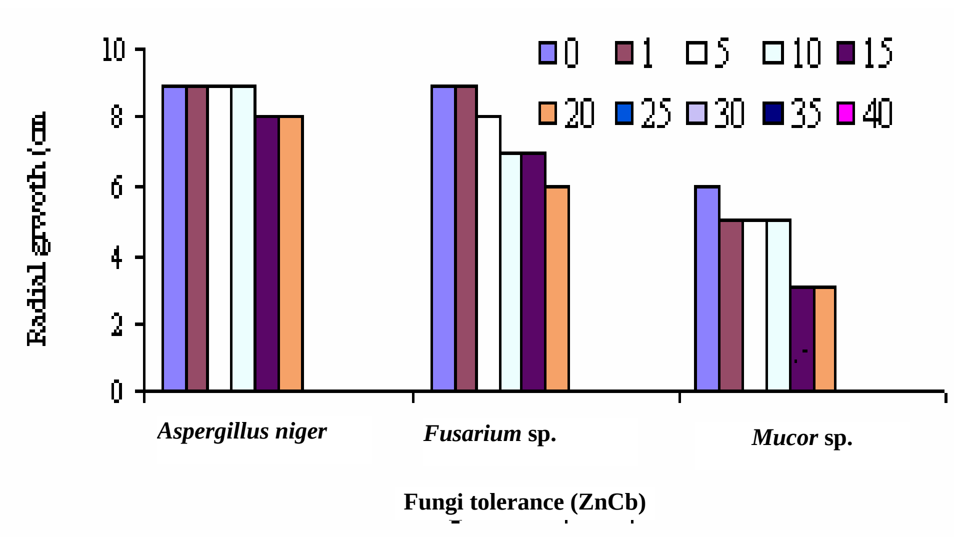

Fig. 4. Radial growth (cm) of fungi against $ZnCl_2$ (0-40ppm).

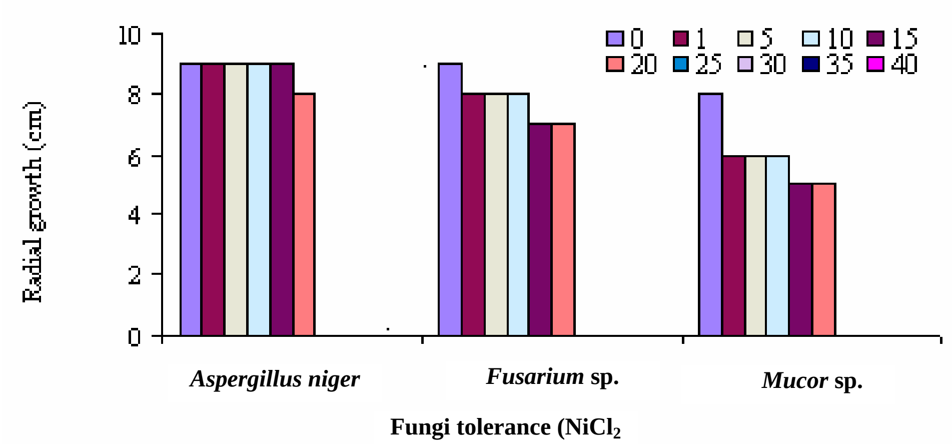

Fig. 5. Radial growth (cm) of fungi against $NiCl_2$ (0-40ppm).

Table 2. Scale of resistance of selecteds fungi against $ZnCl_2$ (0-40ppm).

$ZnCl_4$ Conc. (ppm)	*Aspergillus niger*	*Fusarium* sp.	*Mucor* sp.
0	++++	++++	+++
1	++++	++++	+++
5	++++	++++	+++
10	++++	+++	+++
15	++++	+++	++
20	++++	+++	++
25	-	-	-
30	-	-	-
35	-	-	-
40	-	-	-

*Maximum growth = 8-9cm (++++), Moderate growth = (7-5cm) +++, Slightly growth = (4-3cm) ++, Very slightly growth = 2-1 (+), No growth = 0cm.

Table 3. Scale of Tolerance of selected fungi against $ZnCl_2$ (0-40ppm).

Conc. Ni C12 (ppm)	*Aspergillus niger*	*Fusarium* sp.	*Mucor* sp.
0	++++	++++	++++
1	++++	++++	+++
5	++++	++++	+++
10	++++	++++	+++
15	++++	+++	+++
20	++++	+++	+++
25	-	-	-
30	-	-	-
35	-	-	-
40	-	-	-

*Maximum growth = 8-9cm (++++), Moderate growth = (7-5cm) +++, Slightly growth = (4-3cm) ++, Very slightly growth = 2-1 (+), No growth = 0cm.

Table 4. Analysis of heavy metals by xray inflorescence.

Heavy metals	Hudiara drain		Permissible or recommended limit
	Mean St. Dev.	Mean St. Dev.	
Cu	45 ± 31.58	47 ± 21.7	750 (USA 1993)
Zn	168.8 ± 207.3	2800 ± 2759	1400
As	13.91 ± 4.3	6498 ± 10799	20
Rb	125.55 ± 14.98	70 ± 58.8	120
Ag	2.182 ± 1.662	6.33 ± 3.79	9
Cd	2.82 ± 5.71	8.33 ± 7.51	20
Sb	4.727 ± 1.737	87 ± 131.0	10 (Germany 1980)
Pb	32.55 ± 9.05	1408 ± 1947	150

Table 5. Trace elements analysis by ICP-MS in the samples of Pakistan and Germany (mg/kg).

	Hudiara Drain, Lahore				Wiesloch Germany			
	Mean	**± S.D**	**Min**	**Max**	**Mean**	**± S.D**	**Min**	**Max**
Al	12640	± 1212	11504	13916	4986	± 6236	576	9396
Cr	99.84	± 14.72	86.64	115.71	80.7	± 41.1	51.7	109.7
Fe	85366	± 6374	79887	92362	183902	± 139427	85312	282491
Mn	1385	± 139.0	1247.2	1525.3	1602	± 1635	446	2758
Ni	841	± 1276	94	2315	170	± 184	40	300
Cu	79.41	± 5.68	75.33	85.90	107.89	± 5.92	103.70	112.07
Zn	273.9	± 53.3	218.9	325.2	11303	± 2822	9308	13299
As	98.85	± 9.38	88.74	107.28	35768	± 46787	2685	68851
Sr	114.90	± 16.51	105.34	133.97	1039	± 625	597	1481
Mo	9.94	± 2.43	7.48	12.33	37.5	± 37.3	11.1	63.51
Cd	1.085	± 0.614	0.554	1.757	37.75	± 5.31	34.00	41.51
Sb	1.148	± 1.641	0.200	3.043	897	± 1112	111	1683
Ti	4.807	± 1.442	3.150	5.779	7609	± 10651	78	15141
Pb	73.63	± 11.72	63.29	86.36	5996	± 2536	4203	7789

The chemical contaminated soil sample around the Hudiara drain contains higher concentration of Al, Cr and Ni as compared to other samples. The high levels of metals due to anthropogenic sources and growing population is also the main source. The heavy metals with enrichment levels exceeding the normally expected distribution in soil give rise to concern over the suitability of the soils for growing crops (Alloway, 1995). High levels of these elements are observed in some pockets only, very close to some of the industries manufacturing steel, pesticides, chemicals, pharmaceuticals etc., which indicates that the source of these elements could be the industrial effluents. In Pakistan around Lahore many industries are manufacturing pesticides and chemicals. The waste water of industries is going to field and supplying the irrigation water. In plants, heavy metals like cadmium, lead and nickel are highly toxic at relatively low concentrations. Heavy metal toxicity is the result of complex interaction of major toxic ions with other essential or non-essential ions (Agarwal, 1999). Dyeing and printing of textile being the major industries at Lahore, Pakistan and as textile processing require a good amount of water, these industries are invariably situated near riverbanks where transportation facilities also exist. As a result, wastewater discharge from these textile units carry the effluents to the soil and cause degradation of water quality in the area and further contamination of the soil. Generally, the growers are unaware of ion toxicity, which could be introduced into the food chain by vegetables and crops irrigated with effluents. These toxic ions may retain in soil or leach out through the soil and may contaminate ground water along with the soil itself and finally enter the food chain and cause health hazard in animals and plants. Human exposure to these metals through ingestion of contaminated food or uptake of drinking water can lead to their accumulation in humans, animals and plants (Khan, 2006a).

The present study proposed that the characteristics of bioremediation technology, detoxification and mineralization of the pollutant to biomass, CO_2 and H_2O make it an attractive, environmentally sound and potentially cost effective alternative to conventional treatment, which rely on incineration, volatilization immobilization of the pollutants. Conventional treatments may simply transfer the pollution, creating new waste such as incineration residues and not eliminate the problem. However the general acceptance of the bioremediation as a treatment technology requires the demonstration of its effectiveness, reliability and predictability and its advantages over conventional treatment technologies. The successful implementation of a bioremediation process and the demonstration of its effectiveness require an interdisciplinary, systematic monitoring and evaluation strategy at each level. Before any decision to use a particular treatment process is made, a detailed site characterization need to be conducted, which will provide balance information on the chemical nature of the contamination.

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