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Full Length Research Paper

Impact of heavy metals on the rhizosphere microflora of *Jatropha multifida* and their effective remediation

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The impact of heavy metals (arsenic, chromium, copper and magnesium) in the rhizosphere microflora of *Jatropha multifida* used for phytoremediation was studied. The pot culture experiment of *J. multifida* dealt with the biochemical characteristics of heavy metals contaminated soil amended with waste water biosludge and biofertilizer (*Azotobacter vinelandii*). Plant growth promoting rhizobacteria such as *Pseudomonas*, *Azotobacter* and *Rhizobium* were isolated, characterized and screened for their heavy metal tolerance. *Pseudomonas* was found to be the most tolerant followed by *Azotobacter* and *Rhizobium*. Amongst heavy metals, As was most toxic followed by Cr, Mg and Cu. Amongst different soil treatments, T4 (garden soil with heavy metal, biosludge, and biofertilizer) served the best treatment for plants and microbial endurance under metal contamination. The results advocate that the toxicity of heavy metals in soil can be restored with concomitant amendment of organic sludge and appropriate biofertilizer.

Key words: Heavy metals, rhizosphere, biosludge, biofertilizer, bioremediation.

INTRODUCTION

Today, heavy metal pollution has become one of the serious issues of concern amongst all environmental crises. Heavy metals are one of the major sources of environmental pollutants and exist in soil as free metal ions, soluble metal complexes, exchangeable metal ions, organically bound metals, precipitated or insoluble compounds like oxides, carbonates and hydroxides or they may form a part of silicate materials (Leyval et al., 1997). They arise in soil by repeated applications of sewage sludge, municipal wastes and animal slurries, activity of mining and smelting industries, impurities in fertilizers and decomposition of air pollutants by burning of fossil fuels and various industrial activities (Wang et al., 2001). Metals persist in soils and have a very slow leaching rate; hence, they tend to accumulate in soils making plants vulnerable to them.

Heavy metals such as Cu, Fe, Mn, Ni and Zn are essential for plant growth and are important constituents of many enzymes, whereas metals such as Al, As, Cd, Cr, Hg, Pb, Sb and Se are nonessential and toxic above certain threshold levels (Kennish, 1992; Davies et al., 2001; Panda and Choudhury, 2005). An increase in metal concentration also influences the soil microbial properties, especially respiration and enzymatic activity that serve as good indicators of metal pollution. Several studies have shown a negative relationship between heavy metal concentration and microbial activities, such as respiration (Bääth, 1989; Giller et al., 1989), mineralization (van Beelen and Doelman, 1997; Giller et al., 1989; van Beelen et al., 2001), nitrification (Yeates et al., 1994; Smolders et al., 2001), intracellular and extracellular enzymatic activities (Haanstra and Doelman, 1991; Yeates et al., 1994) and microbial community biomass and structure (Kelly and Tate, 1998). Earlier reports suggest that heavy metals inhibit the growth of specific microbial groups, especially nitrifiers and nitrogen fixers (Bääth, 1989). However, in some studies no correlation has been found between microbial parameters and heavy metal contamination (Kelly and Tate, 1998; Trasar-Cepeda et al., 2000).

Conventional technologies, such as precipitation, filtration, ion exchange, reverse osmosis, oxidation, reduction and membrane separation, are often

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Table 1. Analytical characteristics of biosludge.

Parameter	Concentration
Dry matter (g/kg)	191.1 ± 7.3
pH	6.7 ± 0.3
Electrical conductivity at 25°C (dSm/kg)	2.3 ± 0.2
Organic carbon (g/kg)	172.4 ± 6.7
Total nitrogen (g/kg)	21.7 ± 2.4
Total phosphorous (g/kg)	9.2 ± 1.7
Total potassium (g/kg)	3.2 ± 0.7

Mean ± standard deviation (n = 3).

inadequate to reduce heavy metal concentrations in the environment to acceptable regulatory standards. The chemical means of soil treatment through inorganic fertilizer addition cannot be encouraged as they would add more metal components in form of salts to the soil. However, bioremediation is well-known to be effective in eliminating the toxicity caused by metals. Bioremediation is the process whereby the organic wastes are biologically degraded under controlled conditions to an innocuous state or to levels below concentration limits established by regulatory authorities (Mueller et al., 1996). Microorganisms play a key role in controlling the speciation and cycling of metals in soil. Many bacterial strains contain genetic determinants of resistance to heavy metals such as mercury, silver, arsenic, bismuth, cadmium, chromium, nickel, lead and undoubtedly others. These resistance determinants are often found on plasmids and transposons which are exploited in bioremediation.

Phytoremediation has recently become one of the concerned alternatives to the traditional methodology in restoring the polluted sites and degradation of contaminants in the rhizosphere (McCutcheon and Schnoor, 2003). Phytoremediation uses plants as filters for accumulating, immobilizing and transforming the contaminants to less harmful form and has now emerged as a promising strategy for *in situ* bioremediation (Vidali, 2001). Plants take up most mineral nutrients through the rhizosphere where microorganisms interact with plant products in root exudates that consists of a complex mixture of organic acid anions, phytosiderophores, sugars, vitamins, amino acids, purines, nucleosides, inorganic ions, gaseous molecules, enzymes, and root border cells (Dakora and Phillips, 2002). The rate of exudation is increased by the presence of essential microorganisms in the rhizosphere and promoted by the uptake and assimilation of certain nutrients (Gardner et al., 1983).

For effective phytoremediation, the plant should be non-edible and can be grown abundantly on wastelands. There have been many investigations using *Jatropha curcas* for phytoremediation (Debnath and Verma, 2008; Mangkoedihardjo et al., 2008; Zhang et al., 2008; Agamuthua et al., 2010) but very less literature is available on *J. multifida* in phytoremediation; although, it can withstand environmental stress. Amendment of biofertilizer, especially *Azotobacter,* has been found to be successful in treating contaminated soil as a consortium with phytoremediation. *Azotobacter vinelandii* has gained much importance in bioremediation (Piperidou et al., 2000) and mineral solubilization (Sashidhar and Podile, 2009) and nitrogen-fixation (Ravikumar et al., 2004). As a result of plant root and microbial interaction, organic and inorganic contaminants are immobilized and their chances of migration to the ground water are reduced.

In this study, we focus on some of the electrochemical, chemical and microbiological parameters to reveal the effect of heavy metals (As, Cr, Cu and Mg) on plant-soil-microbial interactions. The purpose of selecting these heavy metals was to understand their toxicity towards the microorganisms present in plant rhizosphere. Therefore, two essential elements (Cu and Mg) and two non-essential elements (As and Cr) were chosen for the investigation. This study was undertaken to link rhizospheric microbiological and soil biochemical parameters with soil quality conditions. Our intension was to create amicable surroundings for plants and microorganisms in soil with heavy metals by the improvement of soil conditions through organic wastes (biosludge) and biofertlizer (*A. vinelandii*), ultimately to enhance the phytoremediation process. The hypothesis was biofertilizer and biosludge interface would improve the soil biochemical and microbiological characteristics even in the presence of higher concentration of heavy metals.

MATERIALS AND METHODS

Experimental plot

The experimental pot cultures samples of *J. multifida* and garden soil were procured from plant nursery of VIT University, Vellore, Tamil Nadu, India. Plastic pots were chosen for the study with 10 kg soil and *Jatropha* plants of uniform length (± 60 cm) placed in each pot. The soil was collected from the VIT lawn and the biosludge was obtained from the waste-water treatment facilities located at VIT campus. The analytical characteristics of the biosludge under study are given in Table 1. The biosludge was dried at 45°C prior to its analysis. Total nitrogen, phosphorous and potassium in the biosludge was estimated using the standard methods (Lindsay and Norvel, 1978).

A. vinelandii, isolated at VIT University, was used as the biofertlizer. The biosludge was uniformly mixed with the soil before the soil was spiked with metal salts, such as $Na_2HAsO_4.7H_2O$, $K_2Cr_2O_7$, $CuSO_4.5H_2O$ and $MgSO_4.7H_2O$, that served the source for experimental heavy metals (As, Cr, Cu and Mg), at increasing concentrations ranging from 25 to 250 mg/kg for As and Cr and 500 to 3000 mg/kg for Cu and Mg, respectively. The experiment was designed as follows: T1 (garden soil with heavy metal); T2 (garden soil with heavy metal and biosludge); T3 (garden soil with heavy metal and biofertilizer); and T4 (garden soil with heavy metal, biosludge and biofertilizer). A separate pot with untreated soil was used as the control. The pots were watered daily with 500 ml of water per pot. In order, to prevent loss of nutrients and elements out of the pots, plastic trays were placed under each pot and the

drained-out water collected was put back in the respective pots. Soil moisture content was adjusted regularly by mass to about 60% of water-holding capacity with deionized water. Each treatment of plant consisted of three replicate for statistical purpose. This study was conducted after a year of the earlier mentioned experimental set up. Soil was collected from the rhizosphere of *J. multifida* from different pots for analysis. For chemical analysis, the soil was collected, air-dried, passed through a 2 mm sieve and stored at 4°C to minimize microbial activity. For biological analysis, fresh samples were collected each time.

Soil analysis

Soil pH and electrical conductivity were measured potentiometrically with pH meter and conductivity meter, respectively. The available nitrogen (NH_4^+, NO_3 and NO_2^-) was determined by the method of Subbiah and Asija (1956) using Kjeldahl nitrogen analyzer. The nitrogen available for mineralization was estimated using alkaline $KMnO_4$, which oxidized and hydrolyzed the organic matter present in the soil. The liberated ammonia was condensed and absorbed in boric acid, which was titrated against a standard acid. Organic carbon was analysed by the method of Walkely and Black (1934). The chloroform fumigation-extraction method by Vance et al. (1987) was used to determine the microbial biomass carbon in soil. With this method, a direct measurement of carbon and other nutrients containing microbial biomass was carried out. Overnight fumigation of chloroform was made to kill all the microorganisms in soil sample, after which the amount of microbial biomass carbon in the sample was measured. Microbial dehydrogenase activity was measured by the reduction of 2,3,5-triphenyltetrazolium chloride (INT) to a red-colored iodonitrotetrazolium formazan (INTF) that was estimated by colorimetric method as described by Mathew and Obbard (2001).

Metal analysis

Total heavy metals present in the soils were determined through atomic absorption spectroscopy (AAS). Wet oxidation of the soil was employed using diacid mixture of $HClO_4$-HF (Hossner, 1996). Bioavailable heavy metals were analyzed using DTPA (Diethylene-triamine-penta-acetic acid) (Lindsay and Norvell, 1978).

Microbiological analysis

Microbial enumeration of all soil treatments was achieved by standard procedures and the bacteria isolated were confirmed with Bergy's manual. Heavy metal tolerance by microorganisms was tested using agar diffusion assay method. Solutions of different concentrations of heavy metals (As, Cr, Cu and Mg) were prepared from 0 to 1000 µg/ml through 5, 10, 20, 50, 100, 200, 500 and 700 µg/ml using the respective metal salts $Na_2HAsO_4.7H_2O$, $K_2Cr_2O_7$, $CuSO_4.7H_2O$ and $MgSO_4.7H_2O$. Metal solutions were saturated in the wells dug on the culture medium in the Petri plate and the tolerance index was determined by studying the respective zones of inhibition.

RESULTS AND DISCUSSION

Many factors such as moisture, oxygen, organic matter, macro, micro and trace elements, temperature, pH, conductivity, soil texture, and a few others like agronomic practices are known to influence the microbial flora in the rhizosphere soil. The soil sample collected from the rhizosphere of *Jatropha* plants, cultivated on heavy metal spiked soil with and without biosludge and biofertilizer amendments were investigated for electrochemical, chemical and microbiological parameters. Biosludge applications had led to an increase in organic carbon, available nitrogen, microbial biomass carbon and dehydrogenase activity including other microbiological characteristics.

Soil pH is one of the significant factors affecting heavy metal uptake by plants as stated by Eriksson (1989). In this study, results showed that the pH was alkaline in the control soil and soils with lower concentrations of heavy metals in all the four treatments. With increase in metal concentration, there was a decrease in pH and vice versa. Studies have shown that a slight fluctuation in pH is inhibitory to microorganisms rather than to higher plants. The pH varied from 8.51 to 8.70 in control soil, whereas in heavy metal contaminated soil it varied from 7.60 to 8.10. With higher pH, the soil gets alkaline and the bioavailability of metal ions is reduced. Lower pH is optimal for metal availability but is adverse to the vegetation (Hutchinson et al., 2003). The electrical conductivity of control soil in all the four treatments varied from 0.20 to 0.21 mS/cm, whereas it varied in the range of 0.13 to 0.64 mS/cm in soils with heavy metals. Alternatively, conductivity increased with increase in metal concentration which may be due to the fact that conductivity represents the concentration of soluble salts in the soil (Doran and Parkin, 1996).

Table 2 represents the organic carbon and available nitrogen of experimental soil amended with biosludge and biofertilizer. T1 and T3 showed less organic carbon and available nitrogen content as compared with T2 and T4. Satisfactory nitrogen content in T4 and T3 might be due to *A. vinelandii* amendment which acted as a free-living nitrogen-fixer and enhanced the nitrogen content in soil. With the amendments of biosludge, organic carbon is added to the soil whose interaction with microorganisms supplements other nutrients and makes them available to plants (Barajas-Aceves, 2005). Hence, increased organic carbon enhanced the availability of nitrogen and other nutrients as the results reveal. In addition, high concentration of heavy metals in soil is detrimental to its indigenous microflora, particularly in soil where the organic matter content is low (Brookes et al., 1986; Giller et al., 1989).

Application of As, Cr, Cu and Mg to soil has lead to their reduction from the pots either through stabilization or immobilization (Table 3). The bioavailable heavy metals were lower in biosludge amended treatments (T2 and T4). This may be because heavy metal polluted soils display a large heterogeneity of metal distribution among soil constituents. Total heavy metals varied in a range of 15.4 ± 1.7 to 2600.70 ± 121.7 mg/kg within T1 to T4, whereas the range of variation in bioavailable heavy metals was1.21 ± 0.09 to 397.38 ± 23.5 mg/kg. Clear distinctions

Table 2. Organic carbon and available nitrogen in the experimental soils.

Metal/ concentration in soil (mg/kg)		Parameter	Treatment			
			T1	T2	T3	T4
Control	0	Organic C	0.41 ± 0.03	1.26 ± 0.03	0.53 ± 0.01	1.22 ± 0.03
		Available N	23.10 ± 1.3	69.49 ± 2.1	34.32 ± 2.0	78.10 ± 4.3
As	25	Organic C	0.45 ± 0.05	1.4 ± 0.01	0.56 ± 0.01	1.42 ± 0.03
		Available N	19.22 ± 2.2	49.31 ± 3.0	20.56 ± 1.9	54.32 ± 3.7
	250	Organic C	0.57 ± 0.0	1.36 ± 0.1	0.58 ± 0.01	1.35 ± 0.04
		Available N	18.20 ± 1.0	43.80 ± 3.4	26.40 ± 1.3	46.80 ± 2.9
Cr	25	Organic C	0.45 ± 0.05	1.26 ± 0.02	0.59 ± 0.01	1.65 ± 0.01
		Available N	17.40 ± 2.1	52.20 ± 3.2	22.40 ± 0.9	66.80 ± 4.0
	250	Organic C	0.43 ± 0.0	1.43 ± 0.02	0.43 ± 0.01	1.40 ± 0.01
		Available N	9.80 ± 0.4	29.80 ± 2.0	17.60 ± 1.8	35.20 ± 2.3
Cu	500	Organic C	0.52 ± 0.01	1.31 ± 0.01	0.50 ± 0.1	1.29 ± 0.04
		Available N	23.80 ± 1.0	68.80 ± 3.1	31.00 ± 1.6	75.00 ± 3.5
	3000	Organic C	0.52 ± 0.02	1.53 ± 0.06	0.51 ± 0.02	1.49 ± 0.04
		Available N	14.60 ± 1.1	35.80 ± 2.4	25.80 ± 2.3	49.40 ± 3.2
Mg	500	Organic C	0.43 ± 0.01	1.22 ± 0.03	0.40 ± 0.02	1.16 ± 0.03
		Available N	23.40 ± 1.8	62.40 ± 3.4	33.80 ± 2.4	74.80 ± 4.5
	3000	Organic C	0.51 ± 0.03	1.36 ± 0.05	0.50 ± 0.01	1.32 ± 0.05
		Available N	16.21 ± 2.0	31.44 ± 3.2	23.80 ± 1.7	46.60 ± 3.2

Organic carbon (%); available nitrogen (mg/kg); mean ± standard deviation (n = 3).

can be sited between the total and bioavailable metals in different treatments. The variations in total heavy metals may be due to the complexes formed by both soluble and insoluble ionic states of metals in soil, whereas in bioavailable heavy metals it may be due to the metals that are readily available to plants and microorganisms in soluble ionic states. The availability of metals in the soil depends on the nature of the chemical association between a metal with the organic residual and soil matrix, pH, concentration of the element in the soil and the ability of the plant to uptake a particular element. Additionally, the strong interaction between pH and metal effect is well-known; since the solubility of heavy metals and their effect on soil microorganisms increases at lower pH conditions (Knight et al., 1997). This supports our findings as discussed before, with increase in metal concentration there was a decrease in pH. Moreover, the microorganisms play a significant part in making the essential metals available to plants. A number of authors have agreed that the presence of organic matter increases DTPA extractability of metals (Ortiz and Alcañiz, 2006).

Soil microbial population in the rhizosphere soil contaminated with different concentrations of As, Cr, Cu and Mg amended with biosludge and biofertilizer were enumerated quantitatively. In general, the control garden soil showed pronounced microbial flora in comparison to the heavy metal contaminated soil. T4 showed the best growth for all microbial communities recording individual average of $14x10^3$ CFU/g of soil for As, $22x10^3$ CFU/g of soil for Cr, $32x10^3$ CFU/g of soil for Cu and $26x10^3$ CFU/g of soil for Mg, respectively. T2 illustrated supporting growth for microorganisms due to biosludge addition in all the metal concentrations ranging from $24x10^3$ to $23x10^4$ CFU/g of soil. T3 showed good *Azotobacter* community due to its amendment as a biofertilizer. Amongst all the treatments, T1 represented minimal microflora with individual average of $12x10^3$ CFU/g of soil for As, $14x10^3$ CFU/g of soil for Cr, $19x10^3$ CFU/g of soil for Cu and x CFU/g of soil for Cr, $19x10^3$ CFU/g of soil for Cu and

Table 3. Total and bioavailable heavy metals in the experimental soils.

Metal/concentration in soil (mg/kg)		Parameter	Treatment			
			T1	T2	T3	T4
As	25	Total HM*	16.61 ± 1.3	15.42 ± 1.7	18.27 ± 2.1	15.90 ± 1.8
		Bioavailable HM	2.08 ± 0.4	1.21 ± 0.09	1.96 ± 0.3	1.32 ± 0.09
	250	Total HM	159.91 ± 9.1	162.17 ± 8.2	143.74 ± 8.7	148.00 ± 7.2
		Bioavailable HM	42.71 ± 4.7	27.80 ± 2.2	40.12 ± 4.1	25.99 ± 1.9
Cr	25	Total HM	17.16 ± 1.6	15.75 ± 2.1	16.20 ± 1.8	18.39 ± 2.7
		Bioavailable HM	1.93 ± 0.5	1.32 ± 0.5	1.84 ± 0.2	1.80 ± 0.07
	250	Total HM	167.53 ± 12.2	159.04 ± 9.7	168.66 ± 10.2	160.10 ± 8.9
		Bioavailable HM	39.83 ± 2.3	26.22 ± 3.0	37.96 ± 2.7	25.54 ± 2.5
Cu	500	Total HM	391.10 ± 24.3	398.58 ± 31.4	390.30 ± 37.1	389.24 ± 29.9
		Bioavailable HM	62.64 ± 4.6	38.98 ± 3.2	57.37 ± 4.6	36.95 ± 3.5
	3000	Total HM	2600.70 ± 121.7	2558.84 ± 142.4	2595.15 ± 145.3	2434.40 ± 154.1
		Bioavailable HM	212.49 ± 19.1	166.11 ± 10.6	225.98 ± 22.4	157.12 ± 13.7
Mg	500	Total HM	347.10 ± 23.4	342.95 ± 35.6	349.33 ± 29.1	339.71 ± 31.4
		Bioavailable HM	66.97 ± 4.6	45.53 ± 3.5	61.23 ± 7.3	43.28 ± 4.6
	3000	Total HM	2421.60 ± 108.6	2318.35 ± 167.7	2389.10 ± 159.5	2479.00 ± 137.5
		Bioavailable HM	397.38 ± 23.5	226.79 ± 26.7	378.54 ± 27.5	229.27 ± 19.6

*Heavy metal (mg/kg); mean ± standard deviation (n = 3).

interface reduced the heavy metal toxicity. This is due to certain additive effects or interactions exhibited by soil microorganisms in adverse conditions (Chaperon and Sauvé, 2008). Due to sufficient organic matter in T4, conditions in soil improved which helped in microbial proliferation. The stated results confer that amongst all the four heavy metals, As is the most toxic followed by Cr, Mg and Cu (As > Cr > Mg > Cu).

The bacteria isolated were *Pseudomonas*, *Azotobacter* and *Rhizobium.* Maximum resistance to heavy metals is exhibited by *Pseudomonas*, followed by *Azotobacter* and *Rhizobium* (Figure 1). Tolerance to Cr was found up to 200 µg/ml by *Pseudomonas* and *Azotobacter* and 300 µg/ml by *Rhizobium*, while tolerance to As was almost equal in the three bacteria but less. *Pseudomonas* and *Azotobacter* were able to tolerate higher concentrations of Cu and Mg. This study infers that comparatively higher concentration of Cu and Mg did not affect microbial growth to a larger extent but those of As and Cr had an adverse effect.

Microbial biomass carbon and dehydrogenase enzyme assay are the direct measures for the microbial load in soil. Measurement of microbial biomass and dehydrogenase activity is essential to investigate the functions of microbial communities. These approaches have the resolution to get a comprehensive view of various stages of microbial community changes due to anthropogenic disturbances or sustainable farming systems. In this investigation, T2 and T4 were rich in organic matter because of biosludge amendment; otherwise may be due to positive influence of microbial interactions and microbial biomass carbon in soil (Figures 2 and 3). The more the interaction, the more the microbial biomass carbon noted. Markedly, Cu and Mg at 500 mg/kg showed the best results for biomass carbon and dehydrogenase activity amongst all the treatments with heavy metals. Maximum dehydrogenase activity was 3.18 µg INTF/g in the control soil of T4 followed by 2.93, 1.16 and 1.10 µg INTF/g in T2, T3 and T1, respectively. Similar results were found in the case of microbial biomass carbon. Higher concentration of heavy metals significantly reduced the microbial biomass carbon and dehydogenase activity in the soil.

The changes in biomass carbon are much faster and greater than total soil organic carbon (Jenkinson and Ladd, 1981). Biomass carbon as percentage of soil organic carbon decreases with an increase in heavy metal concentration (Barajas-Aceves, 2005). Population of rhizosphere microorganisms are also reported to increase in relation to increasing inputs of composted

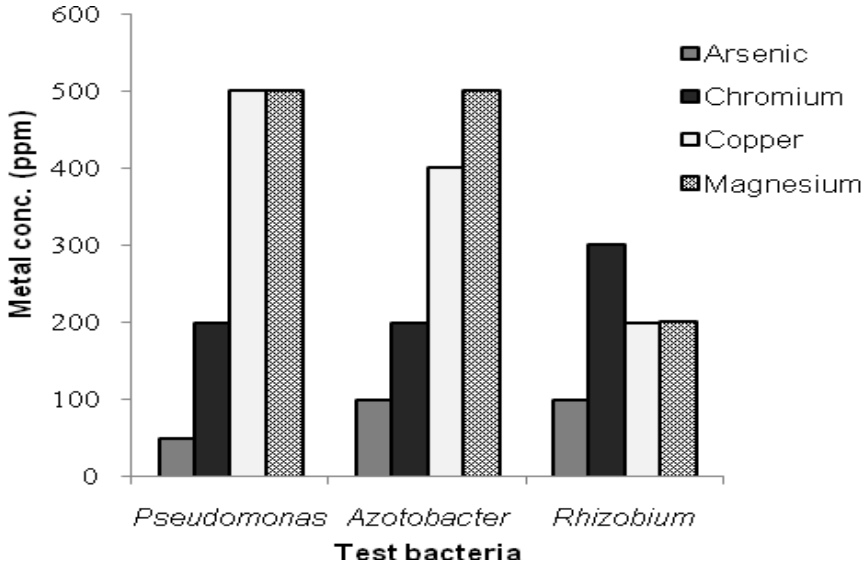

Figure 1. Bacterial heavy metal tolerance index. Statistical chart showing the trend of heavy metal resistance by *Pseudomonas*, *Azotobacter* and *Rhizobium.*

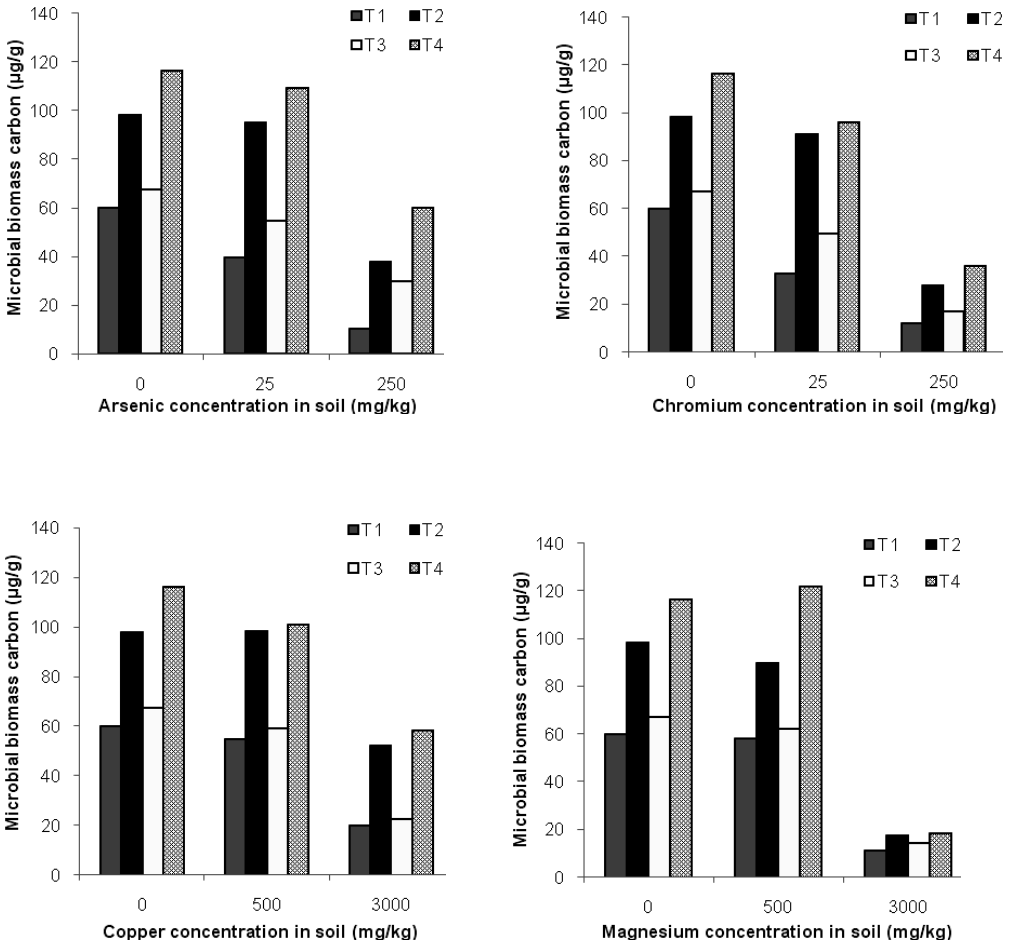

Figure 2. Microbial biomass carbon in soils amended with and without heavy metals (arsenic, chromium, copper and magnesium).

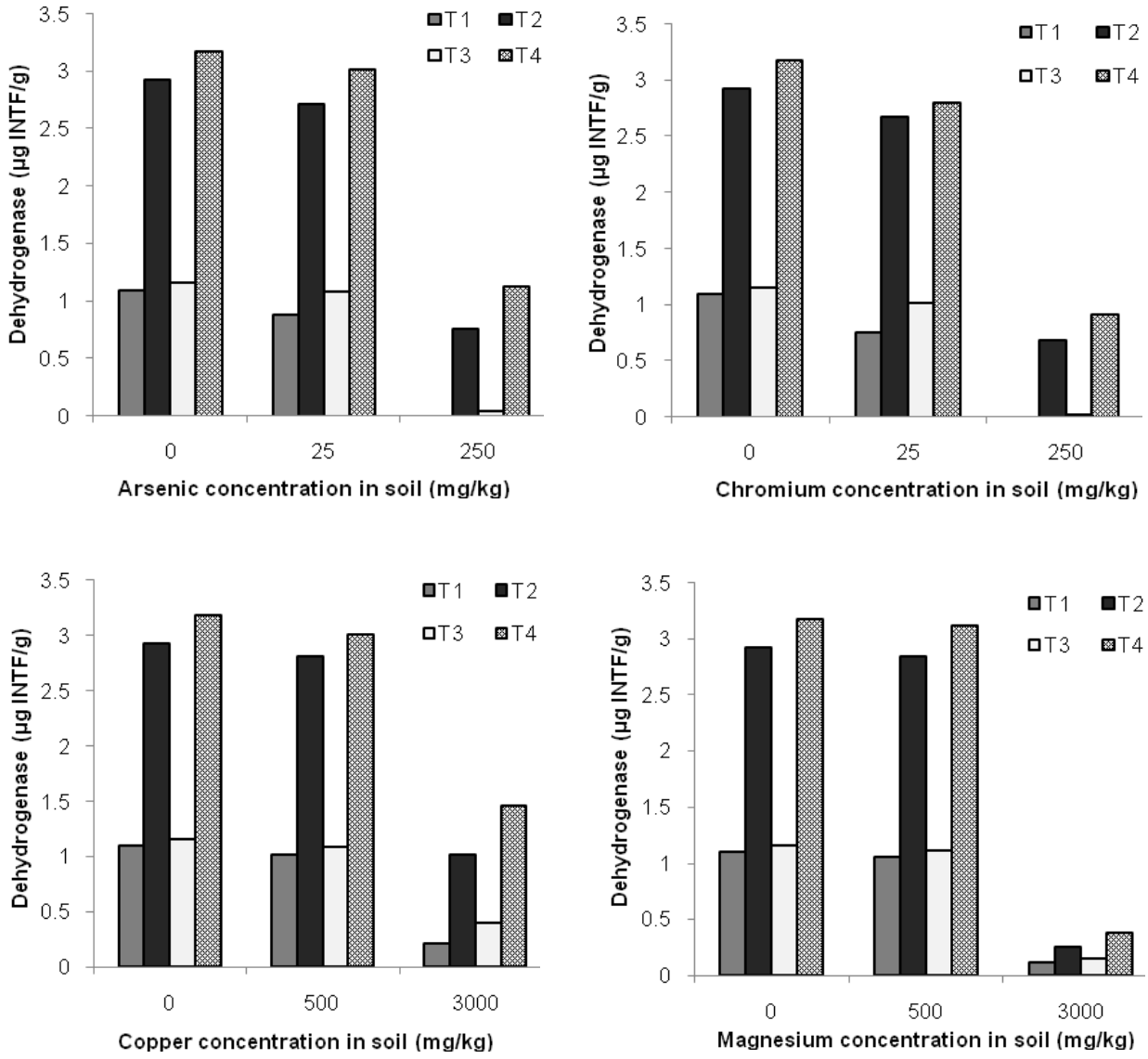

Figure 3. Dehydrogenase activity in soils amended with and without heavy metals (arsenic, chromium, copper and magnesium).

organic matter in soil. The population of microbial communities was found to reach its lowest in all the treatment trials due to the increase in metal concentration.

Biosludge or organic waste application is one of the most effective treatments in soil amendments which increase the soil microbial biomass carbon. Interestingly, there was increased microbial diversity in the control soils, with the highest metal availabilities and the lowest microbial biomass carbon compared with biosludge amended soil. This is because elevated metal concentrations can exert a selective pressure on the microbial communities increasing the numbers of metal tolerant or resistant strains compared to soil ameliorated by biosludge application with reduced metal bioavailability (Gil-Sotres et al., 2005). Additionally, the interaction between microorganisms, plant roots and amendments might have a greater impact on both the increase of nutrient uptake and migration of metal uptake (Smith, 1994).

With the current outcomes of the pot culture experiments of *J. multifida*, it can be ascertained that through phytoremediation and bioaugmentation of essential microorganisms it is possible in reclaiming heavy metal contaminated soils and wastelands. In addition, at conditions of low organic matter in soil, biosludge and biofertilizer act as suitable concomitant to enhance the microbial activity for bioremediation. Furthermore, when the bioremediation technology is applied for any ecosystem restoration, the by-products

such as water and carbon dioxide are non-toxic and are harmless to the environment and living organisms.

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