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Effect of heavy metal on survival of certain groups of indigenous soil microbial population

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ABSTRACT: Heavy metal pollution of soil is known to adversely effect microbial activities at elevated concentration. However, response of indigenous soil bacterial population to added heavy metal and metal combinations is poorly understood. In the present study salts of heavy metals like Cu, Cd, Cr, Hg, Mn, Ni, Pb and Zn were added in soil under laboratory conditions with different concentrations (50, 100, 150 and 200 µg/g of soil) and sufficient moisture. The microcosm were stored at 28 ± 1 °C for 28 days. Viable count of aerobic heterotrophs, asymbiotic nitrogen fixers and actinomycetes were determined at different time intervals (0, 7, 14, 21 and 28 days of incubation) using the plate dilution method. Aerobic- heterotrophic bacterial population were more sensitive to metal groups like Ni and Cd followed by Cu, Cd, Hg, Mn, Cr and Zn. Similarly a symbiotic nitrogen fixers showed higher sensitivity to metal groups like Cd, Pb, Hg followed by Cu, Cr, Mn, Ni and Zn. Actinomycetes were found most sensitive. Metal toxicity was higher for Pb, Mn, Ni followed by Cd, Hg, Cr and least to Cu and Zn. Toxicity of heavy metal was concentration as well as time dependent. Loss of microbial diversity is evident as we move towards higher concentration of heavy metal in soil. Further, experimentation is needed to understand the genetic diversity of the sensitive and metal tolerant microbial population and metal - microbe interaction under natural condition in soil. @JASEM

The impact and long- term ecological ramifications of pollution on the biosphere have resulted an increased interest to evaluate the interactions between pollutants, the environment, and the biota. The soil microbial population is under tremendous pressure due to contamination of soil by a variety of toxic substances such as pesticides, heavy metals and other organic pollutants of sewage sludge and wastewater and environmental origin (Mac-Grath *et al*., 1988, Chaudhary *et al*., 1996). Heavy metals at elevated concentration are known to effect soil microbial population and their associated activities, which may directly influence the soil fertility (Smith,1996). The concentration of a toxic metal that affects the growth and survival of different microorganisms varied greatly (Babich and Stotzky,1977). The magnitude of the microbial diversity makes it difficult to study the whole spectrum of population but certain important functional groups like ammonifying, nitrifying, nitrogen fixing, cellulytic and lignolytic microorganisms have been regularly studied. The relationship of micro-organisms, to heavy metal soil pollution, is complex and contradictory in case of sewage sludge application to the land (Smith, 1991). However, artificial contamination of soil of known physico-chemical characteristics with metal salts and enumeration of the surviving indigenous populations, in a short term study, may reveal the occurrence of microbes in a particular soil sample with intrinsic ability to tolerate metals. Our earlier observations revealed that heavy metal tolerance by a particular group of bacteria or an individual isolate in artificial media supplemented with heavy metal showed high tolerance level. (Ahmad *et al*., 2001, Hayat *et al*., 2002) where, conditions are totally different with natural condition of soil. It is expected that response of various soil microbial populations may possibly more accurately be assessed by studying the indigenous microbial populations in the soil amended with different concentration of heavy metals.

Therefore, the present study was undertaken, with the soil collected from the agricultural field and amended with a graded, known concentration of metal ions and its combinations in the lab conditions. The survival pattern of the common major groups of indigenous bacterial population (aerobic heterotrophs, asymbiotic diazotrophs and actinomycetes) was studied.

MATERIALS AND METHODS

Collection and preparation of soil sample: Soil samples, during the months of September-October 2001 were collected from agricultural farm of Mathura Oil Refinery, Mathura, U.P. India. This field is under cultivation and has received oil refinery treated wastewater for the last 15years. Before use, soil was sieved through a 1mm-pore size screen. Soil was mixed with chloride salts of heavy metals (Cu, Cd, Cr, Hg, Mn, Ni, Pb and Zn) at the concentrations of 50, 100, 150 and 200 µg/g of soil. Soil samples (100 g each) were then packed in sterile polythene bags and maintained at 50% water holding capacity and stored at 28±2°C for 35 days.

Physico-chemical characteristics of soil under study: The physico-chemical properties of soil were

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analysed as per method of Ghosh *et al*. (1983) and APHA, AWWA-WPCF (1985).

Heavy metal analysis: Well mixed, acid preserved soil sample was transferred to a beaker containing 5 ml of concentrated nitric acid and a few boiling chips. The sample was heated on a hot plate for complete digestion, as shown by light coloured, clear solution and filtered through whatman filter paper (No.42) (Vanloon and Lichwa, 1973). The filtrate was used for the determination of various metals, using a GBC 902 double beam atomic absorption spectrophotometer.

Enumeration of microbial population in soil: A standard spread plate method with modification was used (Cuppucino and Sherman, 1983). Total viable count of heterotrophic aerobic bacteria, actinomycetes and asymbiotic nitrogen fixers were evaluated on appropriate selective media (Herbort, 1990), as adopted earlier (Hayat *et al*., 2002). The composition (g/l) of media was as follows: Nutrient agar: (peptic digest of animal tissue 5 g; sodium chloride 5 g; beef extract 1.5 g; yeast extract 1.5 g; agar 15 g); Kennight medium (dextrose 1 g; potassium dihydrogen orthophosphate 0.1 g; sodium nitrate 0.1 g; potassium chloride 0.1 g; magnesium sulphate 0.1 g; agar 15 g); Jensen's medium (sucrose 20 g; dipotassium phosphate1 g; magnesium sulphate 0.5 g; sodium chloride 0.5g; ferrous sulphate 0.1 g; sodium molybdate 0.005 g; calcium carbonate 2g; agar 15 g). All the above microbiological media are sterilized by autoclaving at 121 ^{o}C for 15 min. The pH of each medium was adjusted according to bacterial groups.

Determination of survival of indigenous soil bacteria from soil amended with heavy metal: One gram of the soil samples amended with different concentrations of heavy metals was serially diluted in sterile normal saline solution. 0.1 ml of diluted sample was spread over the surface of plate count agar of the respective medium at 0, 7, 14, 21, and 28 days after incubation. The total viable count of each group of population was noted. Decrease in viable count in the heavy metal amended soil over control sample indicates the effect of heavy metals on metal sensitive population and survival of indigenous soil bacteria.

RESULTS AND DISCUSSION

Physicochemical and heavy metal characteristics of soil before amendments: The physico-chemical and heavy metal characteristics of the soil are presented in Table 1. All the parameters are below the permissible limit, recommended for soil as described by Krishna Murti and Vishwanathan (1991).

Table 1. Physico-chemical characteristics and heavy metal status of soil before metal amendments (all determinations in mgl^{-1} or as specified)

Physico-chemical characteristics		Heavy metal	
pH	7.60	Cadmium (ppm)	1.2
E.C. (dsm^{-1})	0.75	Nickel (ppm)	31.0
HCO_3^-	185	Lead (ppm)	15.0
Cl^-	35.50	Zinc (ppm)	49.2
Ca^{+2}	29.30	Chromium (ppm)	29.3
Mg^{+2}	19.23	Iron (ppm)	121.1
K^+	14.00	Magnese (ppm)	1.0
PO_4^{-3}	0.79	Copper (ppm)	14.3
Na^+	29.15		
NO_3^-	1.10		
Organic carbon(%)	0.39		
Organic matter (%)	0.67		
CEC meq/100g	3.30		
TDS	779.0		

Occurrence and survival of native bacteria in soil amended with heavy metals: Microbial systems for regulating trace-metal uptake can be important factors in competition with other microbes when the metal ions are either limiting (Kloepper *et al*., 1980) or present at toxic levels (Silver and Walderhuag, 1992). The understanding of microbial adaptation to the presence of metal in the soil is critical in determining the management and potential long-term effect of land receiving heavy metal contamination through wastewater/sludge.

Aerobic heterotrophic bacteria: The plate viable count in control soil (without heavy metal) was in the range of 7.2 x 10^7 to 1.1x10^8 CFU/ g of soil. There was no significant inhibition in the viable count of aerobic heterotrophs for any metals at 50 and 100 μg/g of concentration up to 28 day of incubation (Table 2). On the other hand, treatment of Cu, Cd, Hg, Mn and Ni at 150 and 200 μg/g concentration led to significant decline in viable count or complete inhibition of growth after 21 day of incubation. This behaviour of indigenous soil bacteria was similar to our previous study where indigenous metal resistant aerobic- heterotrophs were directly isolated in significant number on nutrient agar plates supplemented with 200 and 400 μg/ml of metal concentration (Hayat *et al*. 2002). Because of the great diversity within this group of bacteria , plate viable count may not be affected at lower concentration of heavy metal but the diversity of the population is reduced.. Random selection of 50 colonies from different plates indicated that almost 75% of the strains were gram negative at higher metal concentration and remaining were gram positive or gram variable. Common gram positive bacteria at higher concentration was belonged to genus Bacillus (data not shown). At lower concentration morphological diversity was more as compared to at higher metal concentration. The predominance of gram-negative bacteria at higher concentration of metal is probably due to their higher level of intrinsic metal resistance than majority of the gram-positive bacteria. The basis of this difference might be due to the differences in the chemical composition of cell wall of gram-negative bacteria and gram- positive bacteria (Babich and Stotzky,1977).

Table 2. Survival of aerobic heterotrophic bacteria (CFU/gm) in soil amended with heavy metal under lab condition

Metal	Concentration metal added in soil (μg/gm)	Days after inoculation				
		0	7	14	21	28
		x10^7	x10^7	x10^6	x10^6	x10^4
	Control (0)	7.2 0	6.6	8.20	8.8	9.5
Cu	100	7.25	6.50	6.75	4.0	3.35
	150	8.10	6.75	4.65	4.95	Nil
	200	7.92	5.70	5.20	3.25	Nil
Cd	100	7.55	6.90	5.25	4.1	2.85
	150	7.50	6.85	3.25	.495	Nil
	200	7.25	6.30	4.15	.395	Nil
Cr	100	8.15	8.35	5.1	6.45	6.05
	150	8.0	7.8 0	3.65	5.20	1.7
	200	7.80	5.65	3.3	6.25	Nil
Hg	100	7.45	4.05	5.25	5.65	3.80
	150	7.10	4.0	5.25	4.15	Nil
	200	8.50	4.25	6.30	2.85	Nil
Mn	100	7.50	4.19	0.52	4.13	.29
	150	9.15	6.65	0.52	0.56	Nil
	200	7.9	0.68	.325	0.041	Nil
Ni	100	8.95	7.15	6.95	57.5	.835
	150	8.15	.925	.71	5.0	Nil
	200	7.55	.655	.059	Nil	Nil

Pb	100	7.40	6.81	.83	9.65	.510
	150	6.75	5.6	.73	7.20	.425
	200	7.25	.59	.006	Nil	Nil
Zn	100	9.2	7.65	6.9	62.5	6.66
	150	7.85	6.83	5.65	6.75	.825
	200	7.50	6.90	6.8 0	6.50	.525

Potential aerobic asymbiotic nitrogen fixers Most of the free nitrogen fixing bacteria are capable of growing on nitrogen free solid medium belong to genera, *Azotobacter, Azomonas, Derxia,* some species of *Pseudomonas***,** and *Klebsiella* (Alexander, 1984). All the above bacteria are primarily gram-ve, therefore, may have certain common characteristics of metal tolerance due to their similar cell wall composition, also the majority of the nitrogen fixers are good extra-cellular polysaccharide producers which may further protect the cell from the toxic effect of heavy metal (Geesey and Jang, 1990). In contrast to aerobic heterotrophs, this group of bacteria could not survive for 28 days even at 100 µg/g of heavy metals like Cd, Hg & Pb, while other heavy metal (Cu, Cr, Mn and Ni) inhibited growth after 21 or 28 days of incubation at 150 µg/g of concentration. Similarly, Pb, at 200 µg/g inhibited microbial growth after 14 day of incubation followed by Ni, Hg, Mn, Cr, Cd and Cu and Zn (Tables 3). Higher sensitivity to toxic metals may be due to lower diversity among asymbiotic nitrogen fixers compared to heterotrophic groups. Morphological diversity of asymbiotic nitrogen fixers revealed the presence of three (I, II &III) major groups of bacteria. At elevated metal concentration in soil, the group-I bacterial population was found predominant. Morphological, cultural, and certain biochemical characteristics of these bacteria are given in table 4.

Table 3. Survival of asymbiotic N_2-fixing bacteria (CFU/gm) in soil amended with heavy metal under lab condition

Metal	Concentrations (µg/gm)	Days after inoculation				
		0	7	14	21	28
		10^6	10^5	10^5	10^4	10^3
	Control (0)	4.55	8.25	7.7	11.9	62.5
Cu	100	3.85	8.1	6.45	7.75	20 .
	150	4.75	4.85	5.02	5.4	Nil
	200	3.95	3.75	2.85	4.7	Nil
Cd	100	4.12	6.90	7.7	8..50	Nil
	150	4.50	5.05	5.15	5.55	Nil
	200	3.68	3. 85	4.3	3.70	Nil
Cr	100	4.30	7.9	7.7	30.5	8 .0
	150	3.55	4.4	4.1	52.5	Nil
	200	4.20	2.25	3.3	28.5	Nil
Hg	100	3.8	6.75	6.5	29.0	Nil
	150	3.76	4.0	0.79	10 .0	Nil
	200	4.23	0.92	0.54	Nil	Nil
Mn	100	4.50	7.50	5.65	6.65	8.95
	150	4.85	6.85	6.65	.835	Nil
	200	4.45	.795	.0625	Nil	Nil
Ni	100	4.65	6.90	5.65	6.50	9.35
	150	4.95	.775	.0685	Nil	Nil
	200	4.65	.665	.0535	Nil	Nil

Pb	100	4.65	7.65	.580	.0435	Nil
	150	5.65	.835	.0625	Nil	Nil
	200	4.85	.00735	Nil	Nil	Nil
Zn	100	48.4	73.0	62.5	8.50	7.30
	150	53.5	68.5	77.0	7.65	5.85
	200	51.5	69.5	69.5	.565	Nil

Table-4. Morphological and biochemical diversity of of asymbiotic diazotrophic bacteria.

ıaracteristics	'oup-I	'oup-II	'oup- III
'am reaction	e	e	e
:ll shape	/oid	ɔd to ovoid	ɔd - coccoid
ılony morphology	ırge with irregular ırgin	hitish , bluish white :ge round colonies	ınall, watery, semi-ınslucent or transparent ıuciligenous
gmentation	ack- brown	:llow-green, some ıorescent	ɔ pigmentation
ılysaccarides production dole production trate utilization trate reduction	100% strains	100% strains	90% strains
ıtalase test 'ease test	0		
ıntative identification	chroococcum	:otobacter sp-1	ɔn-Azotobacter nitrogen ‹ers

Actinomycetes: Soil actinomycetes are the filamentous gram +ve bacteria representing several morphological types, which includes isolated genera like *Streptomyces, Nocardia, Pseudonocardia, Micromonospora* and some unidentified isolates.. This group of soil bacteria are considered to play very important role in maintaining soil properties although ,they are poor competitor than other soil bacteria. (Alexander, 1984). The data presented in table 5 and 6 revealed inhibition of growth even at 50 µg/g concentration of Mn, Ni and Pb after 28 day of incubation. However, Cd showed toxicity at100 µg/g of soil, Cr, Zn and Hg were effective at 150 µg/g and Cu was effective only at 200 µg/g of soil. In general it can be stated that actinomycetes were less tolerant than gram negative heterotrophic and nitrogen fixing bacterial population . These observations are comparable with our previous findings (Hayat *et al*., 2002).

Table 5 Survival of soil actinomycetes (CFU/gm) in soil amended with heavy metal under lab condition

Metal	Concentrations (µg/gm)	Days after inoculation			
		0	7	14	21
		$x10^6$	$x10^6$	$x10^4$	$x10^3$
	Control (0)	3.75	3.95	8.85	38.5
Cu	100	3.90	3.85	8.45	7.0
	150	4.15	0.65	4.25	3.5
	200	3.50	0.145	1.56	Nil
Cd	100	3.75	3.50	3.90	Nil
	150	4.55	0.58	2.80	Nil
	200	4.05	0.280	1.30	Nil
Cr	100	4.75	3.80	1.25	4.0
	150	4.25	0.76	6.0	Nil
	200	3.85	.0305	Nil	Nil
Hg	100	3.76	4.25	2.75	6.01
	150	4.80	.0765	1.60	Nil
	200	4.12	.0405	Nil	Nil

Table 6. Survival of actinomycetes (CFU/gm) in soil amended with heavy metal under lab Condition

Metal	Concentrations (µg/gm)	Days after inoculation				
		$0x10^6$	$7x10^6$	$14x10^5$	$21x10^4$	$28x10^3$
	Control (0)	5.54	5.65	8.35	4.75	9.66
Mn	50	4.80	3.55	7.55	5.55	Nil
	100	4.65	2.65	.650	.655	Nil
	150	5.12	.85	.066	Nil	Nil
	200	5.00	0.76	.043	Nil	Nil
Ni	50	5.35	4.35	6.55	4.85	Nil
	100	4.95	5.65	6.50	5.50	Nil
	150	5.30	4.85	6.50	0.48	Nil
	200	5.23	4.25	4.75	0.68	Nil
Pb	50	4.95	5.75	4.25	0.43	Nil
	100	5.45	4.25	5.50	0.450	Nil
	150	4.54	0.46	.068	Nil	Nil
	200	4.65	0.75	.069	Nil	Nil
Zn	50	5.35	5.50	6.50	7.50	5.50
	100	4.85	4.50	5.65	6.85	5.65
	150	4.50	0.65	0.78	0.56	Nil
	200	5.65	0.85	0.56	0.43	Nil

In the present investigation, toxicity of the heavy metals was concentration and time dependent for each group of soil organisms. No specific pattern of toxicity was observed against these diverse microbial groups. However, in general aerobic heterotrophic populations were more sensitive to metal groups like Ni & Cd followed by Cu, Cd, Hg, Mn, Cr and least to Zn . Similarly asymbiotic nitrogen fixers showed higher sensitivity to metal groups like Cd, Pb, Hg followed by Cu, Cr, Mn, Ni and least to Zn. Further, to know the exact level of tolerance and adaptability by specific groups or individual organism, long-term experiment using sterile soil amended with various heavy metals alone as well as in combinations is to be carried out.

Conclusions: The preliminary findings indicated that native soil bacterial population might have adapted to changed soil conditions, as the test soil received oil refinery effluent continuously as irrigant for the last 15 years. Similar study using soil of different level of metal pollution and control should be studied to draw a long-term impact of heavy metal pollution on genetic diversity of soil microbial populations to explore the possible metal-metal-microbe interaction and their possible impact on soil health.

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