Chapter 2

Dynamics of metal resistant copiotrophic bacteria and heavy metal content analysis of Torsa River

2. Dynamics of metal resistant copiotrophic bacteria and heavy metal content analysis of Torsa River:

Metals play an integral role in life processes of microorganisms. Some metals like Co, Zn, Ni, Cu, Mg, Mn, and Fe etc. are required as trace elements and therefore are essential, whereas others (Pb, Cd, Hg, Ag etc.) have no biological role and hence are non-essential. Essential metals often functions as catalyst for biochemical reactions, regulator for gene expression and are stabilizers of protein structure and functions (Costa and Klein, 1999). At higher concentration, essential metals exert toxic effects to microorganisms. Toxicity occurs through the displacements of essential metals from their native binding sites or through ligand interaction. Non-essential metals bind with greater affinity to thiol containing groups and oxygen sites than do essential metals (Hughes and Poole 1989; Poole and Gadd 1989). Toxicity results from alteration in the conformational structure of nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance (Poole and Gadd 1989). Bacteria, adapted in metal contaminated environment should have resistance mechanism to overcome 'high metal stress' condition.

Bacteria resistant to heavy metal(s), characterized so far, were isolated from metal contaminated environments, such as, zinc decantation tank of Belgium (Mergeay *et al.* 1978), low grade ore deposits of Belgium and Zaire (Mergeay 1991), metal working industries of Germany and Sweden (Mattsby-Baltzer *et al.* 1989), sewage contaminated water (Pickup *et al.* 1997), waste water treatment plant of Germany (Timotius and Schlegel 1987), anthropogenically nickel polluted or naturally nickel percolated eco-system (Stoppel and Schlegel 1995) and nickel hyper-accumulating rhizosphere (Baker and Brooks 1989) etc. There were several reports regarding isolation and characterization of metal resistant bacteria from ecosystem containing >1000 ppm Ni, Zn, Cu and Pb (Trajanovska *et al.*, 1997; Mengoni *et al.*, 2001; Héry *et al.*, 2003) The environment containing toxic quantity of heavy metal(s) has been generally found to exhibit poor microbial diversity, because the selective pressure tends to select only the resistant population possessing the genetic system to overcome the toxic effect of the metal ion. While isolating and quantifying the nickel resistant bacteria from an anthropogenically nickel polluted ecosystem, Stoppel and Schlegel (1995) concluded that metal resistant bacteria could not be isolated from the places not contaminated with heavy metals.

During bacteriological investigations of Torsa water, incidence and abundance of nickel, cobalt, copper and zinc resistant copiotrophic bacteria in the sampling months of January, February and March of the year 2001, tempted us to investigate the heavy metal content of the river water.

2.1. Investigation on Heavy Metal ion content of Torsa River water:

2.1.1. Introduction:

The term "heavy metal", although not rigidly defined, is generally held to refer to those metals having a density greater than five (Passow *et al.*, 1961). According to Kopp and Kroner (1968), element with atomic number greater than 20, excluding alkali metals, alkaline earths, lanthanides and actinides are termed as heavy metals. Murphy and Spiegel (1983) proposed that heavy metals are generally considered to be those giving a specific gravity greater than 4 to 5 and atomic numbers of 22- 34, 40- 51, 57- 83 or 89- 103. The latest classification is helpful in gaining insight into the toxic mode of action and biochemical activities of metal ions, because it is based on the reactivity of metal with available legends. Even at a very low concentration most of the toxic metals or 'bad ions' (Cadmium, Arsenic etc.) impart toxicity to organisms by binding irreversibly to the 'S' and 'N' atoms of macromolecules, mainly proteins and enzymes, and thereby destroying their function(s).

The major sources of heavy metals in rivers are, sewage, industrial effluents and runoff from chemically weathered soil, mining activity around the river, and other anthropogenic activities. After contamination, in natural water, heavy metals are absorbed by suspended particles and sedimented or can exist in the form of free ions and inorganic complexes (Stumm and Brauner, 1973). Concentrations of metals in aquatic ecosystem vary according to position, time, season, temperature, biological activities and ecology. It was observed that water bodies having low pH and less dissolved oxygen bears maximum chance of heavy metal contamination (Liyod, 1960; White *et al.*, 1963). Biological activity and chemical reactivity also depend upon the form in which these metals are present and their availability depends on specific conditions. Thus, the contents of heavy metals and their forms, states and chemical environments, bring obvious change in the respective flora and fauna.

The rapid upsurge of heavy elements in the complex air-water-food system has concerned the environmentalists in India and consequently different rivers have been investigated in this light; e.g., Ghosh *et al.* (1976) estimated heavy metal in river Hooghly at Kolkata, Chattopadhyay *et al.* (1984) quantified heavy metals of Ganges River in Kanpur, Raina *et al.* (1984) measured the extent of metallic pollution of river Jhelum in Jammu and Kashmir, Verma (1990) monitored levels of heavy metals at Ghatshila (Bihar) in river Subernarekha, Sharma *et al.* (1993) determined distribution of heavy metal ion content of Beas River in Himachal Pradesh. It is therefore, very important to study the concentration of heavy metals in water bodies and in the recent past there has been increased emphasis on studies related to trace metal pollution of various rivers (Ranu et *al.*, 1991; Sharma *et al.*, 1993; Prebha and Selvapathy, 1997; Kaushik *et al.*, 2000 & 2001). The analysis of heavy metal ion content of water of Torsa River was undertaken since there has been no database available about the same. In the present study the concentration of heavy metals viz. Fe, Ni, Pb, Cd, Co, Zn, Cu and Mn were determined at three sampling stations to assess the suitability of the water for drinking and/ or irrigation purpose.

2.1.2. Materials and Methods:

2.1.2.1. Water sampling:

Water samples were collected from three sampling sites (SSI, SSII & SSIII), of Torsa River of northern West Bengal, India, once in every month between April 2001 to March 2002, except the month of July and August, because of heavy rainfall and flood during the monsoon season, water collection become very difficult. Sample sites I and II, were Hasimara and Falakata respectively, of the district Jalpaiguri, and III was at Ghugumari of the district Coochbehar, of the state of West Bengal, India (see figure 1.1.A of Chapter 1). From each sampling station, composite samples were collected in amber color glass bottles of 2L capacity. The samples were stored in iceboxes until brought to the laboratory.

2.1.2.2. Sample preservation:

Water samples were filtered through Whatman® 1 filter Paper (Whatman Int. Ltd., England) to remove suspended particles; acid digested using HNO_3 for minimizing the interference by organic matter; and was analyzed for estimation of heavy metals.

2.1.2.3. Reagents and Chemicals:

De-ionized double distilled water and analytical grades of metal salts ($MnCl_2$, $NiCl_2$ $6H_2O$, $CoCl_2$ $6H_2O$, $ZnSO_4$ $6H_2O$; $CuSO_4$ $7H_2O$, FeSO4 $7H_2O$ and CH_3COOPb $2H_2O$) were used to prepare 1M stock solutions. The stock solutions were diluted and were used to prepare standard curves.

2.1.2.4. Analysis of samples:

Deionized double distilled water was used for preparation of stock solutions and dilution purpose and was used as negative control during analysis. Quantitative estimation of heavy metal was done with atomic absorption spectrophotometer (Varian Spector AA 20 plus) using acetylene gas as fuel (at 89 psi) and air and nitrous oxide as supporting gases. AR grade purified metals and metal oxides were used for preparing various standards for calibration following Standard Methods (APHA, 1985).

2.1.3. Results and discussion:

Heavy metal ion analysis of the river water was done for quantitative estimation of Mn, Fe, Co, Ni,

Table 2.1. Heavy metal ion content of Torsa River water [The values are mean of three samples (one sample per sampling site)].

Sl. No.	Months (Apr.2001to Mar. 2002)	Mn	Fe	Co	Ni	Cu	Zn	Pb
		Metal ion concentration (ppb)						
1	January	25.04 (2.51)	561.31 (27.83)	11.09 (1.2)	5.26 (0.72)	135.83 (7.11)	691.32 (33.2)	174.16 (18.56)
2	February	17.40 (0.89)	232.16 (11.63)	13.31 (1.5)	1.44 (0.21)	20.57 (1.45)	612.49 (29.7)	145.21 (15.23)
3	March	–	1413.21 (7.92)	6.11 (0.81)	3.12 (0.31)	44.73 (4.62)	662.34 (32.26)	176.93 (17.55)
4	April	90.43 (4.76)	1732.35 (95.22)	5.72 (0.95)	20.30 (0.78)	57.20 (5.12)	109.06 (5.11)	123.23 (6.21)
5	May	--	--	2.22 (0.04)	2.37 (0.41)	13.19 (1.21)	63.01 (2.7)	87.03 (4.22)
6	June	88.23 (3.79)	3252.4 (148.32)	5.24 (1.11)	2.61 (0.12)	61.25 (2.71)	82.92 (7.2)	44.21 (2.11)
7	September	–	2311.58 (121.65)	6.05 (0.67)	5.65 (0.51)	32.70 (1.69)	41.84 (1.89)	42.13 (2.43)
8	October	25.93 (2.46)	1044.2 (89.34)	6.58 (1.1)	3.24 (0.23)	35.65 (1.93)	34.26 (1.32)	34.12 (1.98)
9	November	41.67 (4.88)	--	5.88 (0.59)	1.12 (0.08)	30.75 (1.77)	296.67 (14.31)	89.14 (4.18)
10	December	28.09 (2.52)	523.55 (26.42)	42.63 (4.76)	7.45 (0.32)	81.42 (4.21)	555.45 (24.67)	118.32 (5.95)
Maximum permissible limits (ppb)		500*	300*	50**	20*	1000*	3000*	500***

--, not done; *, WHO Standards; **, USSR Standards, ***, Indian Standards

Cu, Zn and Pb. The heavy metal ion concentrations (mean of three samples; one sample per sampling site per month) of Torsa river water in different months are depicted in Table 2.1. Maximum and minimum zinc content of the river water was observed in the month of January (691.32 ppb) and October (34.26 ppb) respectively. The lead content of the river reached its maximum in the month of March and January (176.93 ppb and 174.16 ppb respectively) while minimum were recorded during September-October (34.12 - 42.13 ppb). Maximum quantities of dissolved Ni, Cu and Co ion content of the river were 20.3 ppb (in April), 135.83 ppb (in January) and 42.63 ppb (in December) respectively. The iron content of the river was recorded to be comparatively less during winter months (December to February), which reached its maximum concentration in the month of June (Table 2.1). The maximum Mn^{2+} ion content of Torsa River was also recorded in the month of April (90.43 ppb). The iron content of the river water was found to range between 232.16- 3252.4 ppb.

In all sampling months (except February 2002) the iron content of the river water was found higher than the maximum permissible limit as stated by WHO for consumption by human being. Copper is toxic to many aquatic lives, mainly to invertebrates and fishes, even at a concentration of 15 ppb (Lopez and Lee, 1977). In the present investigation, the copper content of the river water was recorded to range between 20.57-135.83 ppb through out the year except in May 2001. Toxicity of Cu to aquatic organisms varies with the chemicals, species present in water and other environmental parameters like temperature, pH, turbidity, and hardness. Agricultural runoff (from adjoining tea-gardens where copper fungicides are used frequently) and effluents from dolomite-excavated hillocks are suspected to be the probable source of copper ion, whereas, sewage is expected to be the source of other metal ions in Torsa. Considering the maximum permissible limit of heavy metal in the drinking water as stated by WHO, USSR and ISI (see Table 2.1), the water of Torsa cannot be regarded as heavy metal contaminated.

2.2. Dynamics of Nickel, Cobalt and Zinc resistant copiotrophic bacteria:

2.2.1. Introduction:

Bacteria, the simplest living entities, are ubiquitous and more abundant than any other life forms in the entire biosphere because of their diversity, rapid reproducibility, ratio of surface area to volume, which is high, and adaptability in any environmental niche. In contrast to the eukaryotic system, in general, the bacterial cells are small, enucleate and rely solely on diffusion for their livelihood (Beveridge, 1989), and hence are exposed to the elements of the biosphere. With the simpler physiology they efficiently respond to the changes of any of these elements/components through genetically programmed processes. This is necessary because, neither can they selectively acquire their essential nutrient components nor can they fling away toxic components (Beveridge, 1988).

The extreme heterogeneity of composition of natural water supports the sustenance of diverse microbial population, and further modifies and develops specialized ecological niches. Moreover, surface water also possesses necessary components to maintain an appropriate condition to support bacterial population not only for continuing their life processes there but also to protect them from otherwise highly changing situations.

Generally the concentrations of ions of heavy metals in river water are very low and allow unimpaired growth of normal microbial flora. Ecosystems polluted by toxic concentration of heavy metal(s) are inhabited by bacteria having genetic mechanism that confer resistance towards high concentration(s) of one or more than one metal ions(s). Metal resistant microorganisms have been mostly isolated and characterized from anthropogenically-metal-polluted sites (Schmidt and Schlegel, 1994; Stoppel and Schlegel, 1995; Trazanovska *et al.*, 1997; Singh and Kumar, 1998; Mengoni *et al.*, 2001). Schlegel *et al.* (1991) reported the occurrence of nickel-resistant bacteria in soil samples collected from the canopy of nickel-hyper-accumulating shrubs and trees. They proposed that the high population of nickel resistant strains in the plant rhizosphere resulted due to the 'nickel-cycle' that was operative in nickel-metallophytes. High proportion of nickel, cobalt, zinc and copper resistant bacteria were isolated from metal polluted domestic and industrial waste and soil samples (Schmidt and Schlegel, 1989). While studying the nature of bacterial flora of the soil having high lead content (260 mg/g), Trajanovska *et al.* (1997) reported the incidence and abundance of metal resistant Gram-positive and gram-negative bacterial flora. Similar observations were also reported by Héry *et al.* (2003) while isolating nickel resistant bacteria from neocaledonian soil that was rich in heavy metals. Pal *et al.* (2004) reported high percentage of recoverable metal resistant bacteria in serpentine soil containing high concentration of Ni (4136.7- 8033.4 mg/kg), Co (400- 533 mg/kg), and Cr (2760- 4436 mg/kg). The best characterized multiple metal resistant bacterial strains viz. *Alcaligenes eutrophus* CH34, *A. xylosooxidans* 31A, and *A. eutrophus* KT02, were also isolated from a metal decantation tank of a zinc factory in Liége, Belgium (Schmidt *et al.*, 1991).

According to a school of thought it has been hypothesized that the toxic metal resistance systems arose soon after life began, in a world already polluted by volcanic activities and other geological sources (Misra, 1992). As with antibiotic resistance determinants, toxic heavy metal resistance determinants are pre-existent to recent human activities that create polluted environments (Silver and Phung, 1996). To our knowledge there exists a void of published document(s) reporting incidence and abundance of metal resistant bacteria from an ecosystem not polluted by heavy metals. Therefore, we have undertook a research program to look into the incidence and abundance of bacteria resistant to nickel, cobalt, zinc and copper from the waters of Torsa River (see Table 2.1) having heavy metal content below the range of toxicity.

2.2.2. Materials and Methods:

2.2.2.1. Sample collection:

Water samples were collected separately from three sampling sites, SSI, SSII and SSIII [Figure 1A of chapter 1] of river Torsa, once in every month between April 2001 to March 2002, except July and August. For the purpose of sampling, sterile glass containers with polypropylene caps were used and were brought into the laboratory in an ice bucket and were analyzed.

2.2.2.2. Bacterial cultures and media used:

The metal salts and bacterial strains used in this study are shown in Table 2.2 & Table 2.3 In order to enumerate total recoverable copiotrophic bacteria, 0.1 ml volume of diluted water samples were plated on nutrient agar (HIMEDIA) plates and were incubated overnight at 37 °C. In order to isolate metal resistant copiotrophic bacteria, nutrient agar plates containing 3mM $NiCl_2$/ 2mM $CoCl_2$/ 4mM $ZnSO_4$/ 3mM $CuSO_4$ were used. For the preparation of metal containing plates, analytical grade of metal salts were used to prepare 0.5 M stock solutions, which were filter-sterilized and added in nutrient agar before plating.

Table 2.2.1. Metal salts used in the media for the isolation of metal resistant bacteria and growth experiments.

Metal-salts	Chemical formula	Supplier (company)	Concentration used (mM)
Nickel Chloride	$NiCl_2, 6H_2O$	QUALIGEN, India	0.5- 14
Cobalt Chloride	$CoCl_2, 6H_2O$	QUALIGEN, India	0.5- 5.5
Cadmium Chloride	$Cd\,Cl_2, 2H_2O$	EMARK, India	0.5- 5.5
Copper Sulfate	$CuSO_4, 7H_2O$	SRL, India	0.5- 7
Zinc Sulfate	$ZnSO_4, 6H_2O$	QUALIGEN, India	0.5- 7.5

Table 2.2.2. Bacterial strains used as positive and negative control during characterization of metal resistant bacteria.

Bacterial strain	Properties and application(s)	Reference(s)
Escherichia coli strain XL1-Blue	*SupE44 hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi rel A*1 *lacF'* [*pro AB*$^+$ *lacI*^{q} *lacZ M*15 Tn*10* (tet^r)]; Used as negative control for characterization of metal resistant bacteria. MTC for metals: Ni (2.5 mM), Co (1.5 mM), Cu (2 mM), and (Zn 2.5 mM)	Bullock *et al.* (1987)
Alcaligenes eutrophus strain AE 104	Plasmid free metal sensitive bacterium used as negative control for characterization of metal resistant bacteria. MTC for Ni/ Co/ Cu/ Zn was less than 1.5 mM.	Mergeay *et al.* (1985)
Klebsiella oxytoca strain CCUG 15788	Contains *nir* determinants in the chromosome, conferring resistance towards 10 mM Ni^{2+}, 5 mM Cu^{2+} and 2mM Zn^{2+}. Positive control for characterization of metal resistant bacteria.	Stoppel *et al.* (1995)
Alcaligenes eutrophus * strain CH34	Contains *czc and cnr* determinants in the plasmids (pMOL 28 and pMOL 30), conferring resistance towards 3 mM Ni^{2+}, 5 mM Co^{2+} and 5mM Zn^{2+}. Used as positive control for characterization of metal resistant bacteria.	Mergeay *et al.* (1985)

*, Currently known as *Ralstonia metallidurans* CH 34 kindly provided by Prof. D. Nies, Martin-Luther University, Germany.

2.2.2.3. Enumeration of nickel, cobalt, copper and zinc resistant copiotrophic bacteria:

For enumeration and quantification of recoverable metal resistant copiotrophic bacteria, 0.1 ml diluted water samples were directly plated on to nutrient agar plates containing 3mM $NiCl_2$/ 2mM $CoCl_2$/ 4mM $ZnSO_4$/ 3mM $CuSO_4$, and were incubated at 37 °C for 24- 48h. In all experiments, metal-sensitive-strains [*Alacligenes eutrophus* AE104 (Mergeay et *al.* 1985)] and *Escherichia coli* XL1Blue (Bullock *et al.*, 1987) were used as negative control; whereas two metal-resistant-strains, *Alcaligenes eutrophus* CH34 (Mergeay *et al.* 1985) and *Klebsiella oxytoca* CCUG15788 (Stoppel *et al.* 1995) were used as positive control.

2.2.2.4. Determination of MTC of selected nickel resistant isolates:

Isolates manifesting single colonies on the heavy metal containing plates were repeatedly single colony purified on respective metal containing plates. The cells of purified strains were spotted onto nutrient agar plates to prepare master plates and the same was used for replica plating on nutrient agar plates containing different concentrations of $NiCl_2$. The plates were incubated overnight at 35 °C and kept under observation. The maximum tolerable concentration (MTC) of the strains was referred to the highest concentration of metal salt present in the medium, which allows confluent growth after 48h of incubation.

Based on the gram staining (performed as described by Gerhardt *et al.*, 1994) and growth performances on nickel-plates, sixty metal-resistant strains were selected for further analysis. Selected isolates, showing MTC for nickel ≥3mM, were then clonally purified and were subjected to estimation for their MTC towards Co^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+}. For determination of MTC, dilution streaking of purified nickel resistant isolates was done on nutrient agar plates containing 1-10mM Co^{2+}/ Ni^{2+}/ Zn^{2+}/ Cd^{2+}.

2.2.2.5. Statistical analysis:

All statistical analyses were done using software package SPSS 10.0 for Windows.

2.2.3. Results and Discussions:

2.2.3.1. Isolation and quantification of metal resistant copiotrophic bacteria:

Water samples, one sample per sampling site of River Torsa per month, were collected from April 2001 to March 2002. Net recoverable Copiotrophic Bacterial Count (CBC) and fraction of it (considering CBC as 100 %) that withstood the challenge of 3 mM $NiCl_2$/ 2mM $CoCl_2$/ 4 mM $ZnSO_4$/ 3mM $CuSO_4$ in nutrient agar were enumerated from each sample. The values representing net CBC and percent CBC resistant to Nickel or Zinc per month are presented as arithmetic mean of three independent values obtained from the analysis of water samples from three sampling sites (Table 2.3). The maximum and minimum values for net CBC were obtained in the month of June (2.5 X 10^5 CFU/ml) and January (4.11 X 10^3 CFU/ml) respectively. The nickel or zinc resistant (Ni^r/ Zn^r) bacteria of Torsa river was recorded very high in the month of March (33.85% Ni^r and 26.41% Zn^r) followed by January (25.11% Ni^r and 24.58% Zn^r), whereas, in the month of September and October recovery of nickel or zinc resistant bacteria was negligible from the river. A minimum recovery of 0.1% nickel or zinc resistant bacteria was recorded in the month of September. A highest of 8.19% cobalt (Co^r) resistant bacteria was recorded during February 2002, whereas during September and October only 5-6 cobalt resistant bacterial colonies could be observed. While comparing with that of others, the recoverable copper resistant bacterial count in the river was recorded to be high throughout the year. A highest of 15.08 % copper resistant bacteria was enumerated during December 2001 and the lowest was recorded during October (1.56%) 2001 (Table 2.3).

Comparative bacteriological analysis clearly indicated that the metal-resistant bacterial population of Torsa is independent to that of total copiotrophic bacterial load. The seasonal fluctuation of metal resistant bacterial population in the river

Table 2.3. Net copiotrophic bacterial count and percent nickel, zinc, cobalt and copper resistant bacteria of Torsa River water in different sampling months (April 2001 to March 2002). [The values are mean of three samples (one sample per sampling site)].

Sl. No.	Months	Net recoverable copiotrophic bacteria (CFU/ml)	%Nickel resistant bacteria (CFU/ml)	%Zinc resistant bacteria (CFU/ml)	% Cobalt resistant bacteria (CFU/ml)	%Copper resistant bacteria (CFU/ml)
1	January	4.11 X 10^3 (1040.83)	25.11 (3.80)	24.58 (2.58)	7.88 (2.33)	9.88 (0.78)
2	February	4.77 X 10^3 (2750.15)	16.39 (8.99)	21.40 (0.71)	8.19 (1.67)	6.46 (2.18)
3	March	9.41 X 10^3 (6502.3)	33.85 (42.87)	26.41 (3.99)	1.87 (0.65)	7.24 (3.12)
4	April	15.01 X 10^3 (1000)	13.81 (0.722)	6.42 (0.31)	5.03 (0.98)	4.34 (0.89)
5	May	4.1 X 10^4 (28988.5)	5.22 (5.58)	2.10 (1.77)	4.76 (3.66)	11.19 (1.34)
6	June	2.5 X 10^5 (18520.6)	2.10 (1.21)	2.09 (2.78)	1.78 (1.21)	4.62 (1.77)
7	September	2.8 X 10^4 (15044.38)	0.1 (0.15)	0.1 (0.17)	NS	2.86 (2.11)
8	October	67 X 10^3 (72020.83)	NS	NS	NS	1.8 (1.56)
9	November	6.1 X 10^4 (43408.91)	2.24 (0.71)	2.34 (0.48)	0.44 (0.21)	4.71 (0.59)
10	December	5.2 X 10^4 (42188.95)	3.70 (2.95)	12.96 (2.77)	4.43 (0.76)	15.08 (6.88)

NS, not significant (value <0.03); values in the parenthesis are standard deviations.

water has provoked to think about the reason behind the phenomenon. Virtually all physico-chemical parameters were used as independent variables in correlation studies with the fluctuating percentage of metal resistant bacteria, but the analyses did not yield any satisfactory result; nevertheless when trace metal ion content were used, some high correlation values were observed.

2.2.3.2. Determination of multiple metal resistant profiles of selected nickel resistant strains:

Amongst all metal resistant bacteria isolated from river Torsa, strains showing MTC of nickel ≥3 mM were tested for gram negativity. Gram-negative nickel resistant strains were purified and their MTC towards Ni, Co, Cd and Zn were determined (Table 2.4). Compared to that of *Achromobacter xylosoxidans* 31A (Schmidt and Schlegel, 1994) and *Hafnia alvei* 5-5 (Stoppel and Schlegel, 1995) (MTC for Ni^{2+} is 30 –40 mM), the nickel resistant strains of Torsa showed MTC towards nickel ranging between 3.0 –12 mM, that enabled them to be regarded as low to moderate -level-nickel-resistant strains. All nickel resistant strains of Torsa showed 1.0– 4.5 mM MTC towards cobalt, 1.0– 6.0 mM MTC towards zinc and 0.5– 4.0 mM MTC towards cadmium.

2.2.3.3. Correlation between Metal content and Metal resistant bacterial population:

The percentage of nickel or zinc resistant copiotrophic bacteria, and, individually different metal ion content of the river in different sampling months (Table 2.1), was used as variables in correlation and regression analysis by SPSS package. Both nickel and zinc resistant bacterial population showed very high Pearson correlation (r) values with respect to zinc and lead ion content of the river [0.74 (correlation significant at 0.05 level) - 0.94 (correlation significant at 0.01 level)], while the other metal ion content(s) used as variable(s) produced insignificant correlations. Surprisingly, the zinc and lead content of the Torsa river water in 10 different sampling months produced high correlation value [0.876 (correlation is significant at the 0.01 level)]. In regression analysis, the data entered as independent and dependent variables, the maximum value of r^2 (the coefficient of determination) was observed when the variable pair was zinc content of the river and percentage of zinc resistant bacteria (r^2 = 0.885). The copper resistant bacterial population showed positive-correlation value of 0.5, 0.534 and 0.68 with the lead, zinc and cobalt content respectively. Total copiotrophic bacteria resistant to 2mM $CoCl_2$, showed a maximum value of 0.678 with the lead content of the river water. In order to visualize the high correlation, the nickel and zinc resistant CBC was plotted against zinc and lead content of the river water in respective months and are represented in Figure 2.1.

The importance of heavy metal resistance in ecological studies was recognized only recently (Mergeay, 1991). Till date, no information is virtually available about the incidence and abundance of metal resistant bacteria in an environment that is not considered as contaminated, if not said 'polluted', by heavy metals. Based on what is known about the effects of zinc and lead ions on biological system, the government-sponsored research in UK has proposed safe limit(s) of them not exceeding 50 and 0.5 ppm respectively, in rivers used by people for bathing and water sports (http://www.water.org.uk/static/files_archive/1Zinc_ _Water_UK.pdf; & http://www.water.org.uk/static/files_archive/1Lead_ _Water_UK.pdf). The zinc content of River Torsa (34- 691.3 ppb), though observed to be much lower than the maximum permissible limit for consumption recommended by WHO and APHA (APHA 1985), was observed to be higher than the average zinc concentration (5- 45 ppb) of the various other rivers world wide (Shiller and Boyle 1985).

2.3. Conclusion:

The nickel and zinc resistant copiotrophic bacterial load in Torsa waters showed high correlation with zinc and lead content of the river. The average zinc and lead ion content of the river water in different sampling months (Table 2.2) bears a strong positive correlation (r = 0.876) between each other. In the regression milieu, coefficient of determination (r^2) is a more meaningful measure than r, for the former tells us the proportion of variation in the dependent variable explained by the explanatory variable(s)

Table 2.4. MTC (Maximum Tolerable Concentration) profile of selected Gram-negative metal resistant isolates of Torsa River towards four heavy metals, cobalt, nickel zinc and cadmium.

Sl. no	Name of the Isolates	Maximum Tolerable Concentration (MTC) (mM)				Sl. no	Name of the Isolates	Maximum Tolerable Concentration (MTC) (mM)			
		Co	Ni	Zn	Cd			Co	Ni	Zn	Cd
1	Vas 2N8	3.5	4.0	3.0	2.0	31	BB 27	4.5	4.0	1.5	1.0
2	Vas 3N8	2.5	4.0	3.0	1.0	32	BB 32	3.5	3.0	1.5	1.0
3	BB 1A	2.5	6.5	4.5	1.5	33	BB 38	2.0	3.5	1.0	1.0
4	BB 30	2.5	5.0	3.5	1.5	34	BB 40	2.0	3.5	1.0	1.0
5	BB 37	2.5	6.5	2.5	1.5	35	BB 44	2.0	3.0	1.5	2.0
6	1 NiCo43	2.5	4.0	4.0	1.0	36	BB 50	2.0	3.0	1.0	1.5
7	2 NiCo43	3.0	4.0	3.0	1.0	37	BB 60	3.5	4.0	1.5	2.5
8	3 NiCo43	3.0	4.0	3.0	1.0	38	BB61	2.0	4.5	1.5	1.0
9	4 NiCo43	2.5	4.0	4.0	1.0	39	NiVa 50	1.0	4.0	3.0	1.0
10	5 NiCo43	2.5	4.0	4.0	1.0	40	NiVa 51	3.0	7.5	5.5	1.5
11	6 NiCo43	3.5	5.5	2.0	1.5	41	NiVa 53	4.0	5.0	2.5	1.0
12	7 NiCo43	2.5	4.5	4.0	1.5	42	NiVa 54	2.0	3.5	3.0	1.0
13	9 NiCo43	2.5	4.5	3.5	1.0	43	NiVa 55	2.0	3.5	3.0	0.5
14	10 NiCo43	3.5	4.0	3.5	1.5	44	NiVa 56	2.0	3.5	4.5	0.5
15	14 NiCo43	3.5	4.0	3.5	1.5	45	NiVa 57	1.0	3.0	1.5	0.5
16	17 NiCo43	3.5	4.0	3.0	1.5	46	NiVa 60	4.0	3.5	4.5	1.5
17	6 CoNi34	3.5	4.0	4.0	1.5	47	NiVa 61	4.0	5.5	2.0	1.5
18	7 CoNi34	3.0	4.5	3.5	1.0	48	NiVas 111	3.0	5.0	1.0	0.5
19	BB 2	3.0	3.0	3.0	1.0	49	NiVas 112	2.5	3.0	1.0	0.5
20	BB 4	3.0	3.0	3.0	1.0	50	NiVas 113	1.0	12.0	1.0	0.25
21	BB 6	3.0	3.5	3.5	0.5	51	NiVas 114	1.0	12.0	1.0	0.25
22	BB 7	3.5	3.0	3.0	0.5	52	Vco 32	3.0	3.0	2.0	1.5
23	BB 10	3.0	3.0	3.0	0.5	53	Vco 33	3.0	3.0	2.0	1.5
24	BB 13	3.0	3.0	3.0	1.0	54	Vco 34	3.0	3.0	1.0	3.0
25	BB 16	4.0	3.5	2.5	1.0	55	VCd 41	1.0	3.5	1.0	4.0
26	BB 17	4.0	5.0	5.5	1.0	56	VCd 42	3.0	3.0	6.0	4.0
27	BB 19	4.5	5.0	5.0	1.5	57	VCd 45	3.5	3.0	5.0	4.0
28	BB 20	3.5	3.0	2.0	1.5	58	VCd 46	1.0	3.0	5.0	4.0
29	BB 25	4.0	3.0	2.5	1.5	59	VCd 55	1.0	3.0	5.0	4.0
30	BB 26	2.0	4.0	2.5	1.0	60	5CoNi 34	4.0	6.0	2.0	1.0

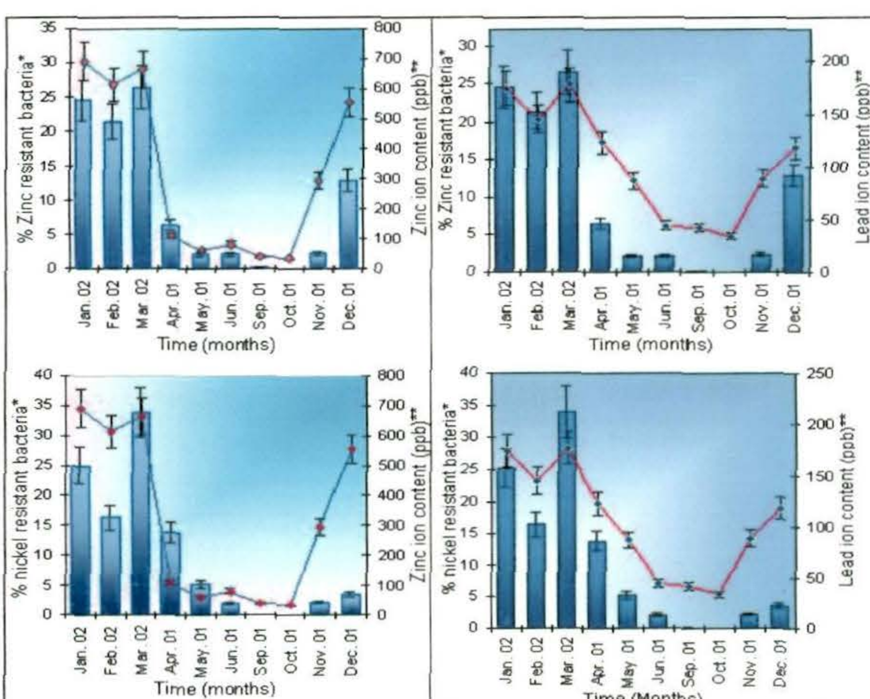

Figure 2.2. Data on percent recovery of nickel and zinc resistant bacteria in different sampling months plotted along with data on either lead or zinc ion content of the water in the same sampling month: a diagrammatic representation.
*, average value calculated from the data of metal resistant bacteria of three sampling sites; **, average value scored from the data of metal ion content of three sampling sites; bar represents the percentage of metal resistant bacteria.

and therefore provides an overall measure of the extent to which the variation in one variable determines the variation in the other. The latter does not have such significance. The values of r^2, derived from regression analysis, the variable pair constituting zinc resistant copiotrophic fraction and the zinc content of the Torsa River was 0.885. The variable pair constituting nickel resistant copiotrophic fraction and zinc content of the river yielded r^2 value of 0.547. The values of r^2 derived in this case means that about 89% and 55% of the variation(s) in zinc resistant and nickel resistant bacterial population respectively are explained by the zinc content of the Torsa River and therefore indicate the direct causality between them.

The assumption was further supported by the result of induction studies, carried out in the laboratory, on some purified nickel or zinc resistant Torsa isolates, where cells induced with 325- 650 ppb Zn^{2+} was found to have their lag phase significantly reduced against the challenge nickel or zinc concentration of 3 mM. This phenomenon indicates the fact that the fraction of river water copiotrophs that possess such inducible nickel or zinc resistance genetic system (requiring ppb level of zinc for induction) would get the selective growth advantage over metal-sensitive copiotrophs. In fact, the recovery of nickel or zinc resistant copiotrophs on metal-containing nutrient agar plates was poor in months when zinc content of the river was found below the inducible concentration (for nickel or zinc resistance), while in sampling months that recorded high Zn^{2+}content (555.46- 691.32 ppb) the recovery was high (Table 2.3). Although a high correlation value between lead content and nickel/ zinc resistant bacterial population was scored, but nickel or zinc resistance in bacterial isolates could not be induced by pre-exposing the cells in Pb^{2+} containing medium (Bhadra *et al*, 2005a). The lead ion content of the river therefore may not have any biological significance in inducing nickel and zinc resistance. The high correlation score (0.87) between lead and zinc ion content of the river may be explained by the dissolution of these two metals from the same geological source.

2.4. Summary of the chapter:

Heavy metal content analysis of river Torsa of India did not indicate any alarming level of toxicity for human consumption. High correlation between Pb and Zn ion content of the river was recorded. The variation in recoverable nickel, cobalt, copper and zinc resistant copiotrophic bacterial count and the variation of metal ion content of the river water were subjected to correlation analysis using software package SPSS. Both lead and zinc ion content of the river showed high correlation with the bacterial population resistant to nickel and zinc; whereas copper and cobalt ion content showed less correlation with zinc and lead content of the river. Growth studies conducted with some exemplary nickel resistant strains revealed that pre-exposure of the cells to ppb level of Zn^{2+}, comparable to the indigenous zinc ion concentration of the river, could induce the nickel resistance. A minimum concentration of 5- 10 μM Zn^{2+}(325-650 ppb) was found effective in inducing the nickel resistance of the isolates.