General Discussion and Summary

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In human history, urbanization has been at once a major result and an influential factor of socio-economic development. The ever-greater intensity of natural resource utilization dramatically modified the essential changes of the biosphere. There is a varying degree of change in the characteristics of climate in areas of maximum intensive economic development and profound alterations in the natural biologeochemical circulation of substances. The four heavy metals, Zinc, Cobalt, Cadmium, and Nickel are used in industrial countries and the growing industrial pockets of applications. Cobalt and Nickel are chemically related to Iron and are both used for the production of steel and for electroplating. Salts of these two metals have been used for centuries for the production of blue and green pigments respectively. Extensive uses of Cadmium, Zinc, Cobalt, and Nickel by man have consequently led to contamination of soil and freshwater habitats with the divalent cations of the four heavy metals.

Besides the anthropogenic contamination, heavy metal may leak from naturally occurring minerals into soil and freshwater habitats. In both cases it is not generally one cation that is present in toxic concentrations, but usually a major cation plus some accompanying ions, e.g. Zn^{2+} plus Cd^{2+} and Ni^{2+} plus Co^{2+} and CrO_4^{2-}. As a response to this challenge, multiple-ion resistant bacteria evolved which contain a variety of metal resistance determinants. It has been established by the earlier authors that from among the heavy metal resistant organisms, 'the Nickel resistant bacteria offer the unique opportunity to compare bacterial isolated from anthropogenically nickel polluted ecosystems with those isolated from naturally nickel percolated ecosystem' (Stoppel and Schlegel, 1995). They have stated their experience as- 'nickel resistant bacteria could not be isolated from normal arable soils in Germany and New Caledonia'. The search for nickel resistant bacteria from metal uncontaminated/ normal soil in Germany turned futile; and if such is the phenomenon taken to be granted –then similar investigations are needed to be carried out in other ecosystems before coming to a general conclusion. The general proposition forwarded by other authors regarding the evolution and origin of metal–resistance ".....it seems, however, more likely that toxic metal resistance systems arose soon after life began, in a world already polluted by volcanic activities and other geological sources. As with antibiotic resistance determinants, toxic heavy metal resistance determinants are pre-existent to recent human activities that created polluted environments....." (Silver and Phung, 1996). This study was undertaken to test this hypothesis.

The River Torsa has been chosen as a subject to test the hypothesis. Torsa traversing through an underdeveloped country Bhutan, flows through another largely underdeveloped part of the state of West Bengal, where Tea industries are only the major industries. The most characteristic development that took place in the catchments of Torsa was the swelling of rural populations. In the upper catchments of Torsa, within Bhutan, blasting of dolomite for the cement factories is the major mining activity. The conditions assumed before hand, therefore, might suite the condition of an ecosystem, least affected by the contamination of heavy metals. Therefore, the search of nickel resistant bacteria from the surface flow of Torsa was intricately associated with the water quality for validation of the hypothesis.

In order to assess the level of pollution, detailed physico-chemical and sanitary analysis of Torsa River was done for a period of one year (April 2001 to March 2002). The major characteristic features of Torsa were high pH, alkalinity, Mg-hardness, total ammonia 'N' content and total phosphate. The hardness value for Torsa was recorded high during dry seasons (November and December) which might be due to dolomite-mining upstream the river. A high correlation value between pH and total hardness (0.629) indicated soil erosion due to excessive mining of dolomites in its catchments. Higher phosphate content of Torsa was recorded in the month of February and March (66.5- 99.6 mg/l) and lowest was observed in October at two sampling sites. The total ammonia 'N' content of Torsa was recorded to be very high compared to the other rivers [Kaljani (0.1- 1.3 mg/l) and Teesta (0.5- 1.1 mg/l)] of the same geographical region. An elevated level of total phosphate 'P' content was a sign of pollution due to washing of clothes, and high total ammonia 'N' data in some months indicated sewage pollution. Low temperature and high aeration rate during winter was possibly the reason for increased amount of dissolved oxygen (7.8- 8.5 mg/l) in the river water. The BOD value of Torsa ranged between 0.6- 3.0 mg/l which was comparable to the BOD recorded in one of its tributary Kaljani, but was less than mountainous Bhagirathi where BOD ranged between 1.5- 6.9 mg/l (Gautam, 1990). As the river water was saturated with dissolved oxygen, decomposition of organic waste did not lead to high BOD. On comparing several parameters like chloride, ammonia 'N', phosphate 'P', BOD, COD, and DO content of Torsa with Yamuna, Cauvery, Ganges, etc. (Chakraborty *et al*., 1959; Dhanapakiam *et al*., 1999; Singh *et al*., 1999), it may not be branded as 'highly sewage contaminated' river. High MPN counts of total coliforms and fecal coliforms, pointed towards pollution of the river water by intestinal microflora. The ratio of fecal coliform to total coliform clearly indicated about the pollution by human excreta.

The heavy metal ion (Mn, Fe, Co, Ni, Cu, Zn and Pb) content of the river water was compared with the maximum permissible limits as stated by WHO, USSR and Indian Standards. Heavy metal did not indicate any alarming level of toxicity. 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. The iron content of the river water was found to range between 232.16- 3252.4 ppb. The maximum Mn^{2+} ion content of Torsa River was also recorded in the month of June (90.43 ppb). On the basis of the metal ion content data, it can be concluded that the river water is safe for human use.

The total recoverable copiotrophic bacterial count (CBC) and the metal-resistant CBC of the river water were estimated in every sampling month. The data showed that the nickel or zinc resistant (Ni^r/ Zn^r) bacteria of Torsa river was 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 from the river water was negligible. A minimum recovery of 0.1% nickel or zinc resistant bacteria was recorded in the month of September. A highest of 8.19% cobalt resistant (Co^r) 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 other metal resistant bacterial count, 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 estimated during December 2001 and the lowest was recorded during October 2001. In order to find out the reason behind the month-wise fluctuation of metal resistant bacteria, the percentage of metal resistant bacterial population of every sampling month were used as variable and were subjected to correlation analysis with the data of all physico-chemical parameters of respective months. Surprisingly, the zinc and lead content of the river water showed high correlation with the nickel and zinc resistant bacterial count which indicated the effect of micromolar quantity of these metal ions on the percentage recovery of metal resistant bacteria. Under laboratory condition, few exemplary nickel-resistant Torsa isolates when pre-incubated in a medium supplemented with zinc (keeping concentration equal to that of the river water), the growth advantage of the isolates in nickel challenged medium was noted. Although a high correlation value between lead content and nickel/ zinc resistant bacterial population was scored, but similar advantage of growth in nickel challenged medium was not observed by preexposing the cells in Pb^{2+} (the concentration equal to that of river water) containing media. 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 might be reasoned by dissolution of these two metals from the same geological source(s).

Studies related to induction of nickel resistance, in some of the Torsa isolates, yielded interesting observations on the phenotypic expression of resistance. Nickel resistance of some Torsa isolates, NiVa 61, 5CoNi 34, 6NiCo 43, BB37 and BB1A, was found to be fully induced when exposed to 5-20 µM (325 –1300 ppb) zinc sulfate. Undoubtedly, these nickel resistant strains have in them the potential genetic material for enriching the genomics of nickel and zinc resistance in prokaryotes. Furthermore, the property of induction of metal resistance by ppb level of bio-available metal ions in the environment could be genetically manipulated for constructing microbe-based biosensors.

Molecular techniques involving, PCR amplification, DNA-DNA hybridization, DNA-sequencing, and sequence analysis were used to identity the nature of nickel resistant genetic system(s) present in Torsa isolates. The results of the experiments showed that only few isolates harbored well-known nickel resistant determinants (*cnr*, *nir* and *czc*), while of other isolates were presumed to contain either novel or diverse nickel resistant determinant(s). Under low stringency condition, the restricted genomic DNA of two Torsa isolates, NiVa 51 and BB1A, generated weak hybridization signal with *nir*-specific probe, suggesting a *nirA* homologous region in the genomic DNA of these isolates. The genomic DNA and PCR product (generated with *cnr-ncc* specific primer) of another isolate, NiVas 114, produced faint hybridization signal with *cnr*–specific probe.

The PCR products of NiVas 114, which hybridized with *cnr* specific probe, were cloned in pGEM® T-easy vector and were sequenced. The nucleotide sequence of the PCR products showed only 41-46% identity with the probe DNA sequence. Four ORFs were predicted from the two sequences. The translated products predicted from the ORFs showed no significant identity with any of the known cation-efflux proteins. Structure-function analysis, of the amino acid sequence of an ORF (pBC1.5-ORF1), predicted to be an integral membrane protein. The novel ORF was characterized but it is too early to predict its role in efflux function. Gene targeting or gene disruptions studies in these isolates would enable to study the gene function thus enriching the genomics of bacterial metal resistance.

The phenotypic characterization of nickel resistant isolates from Torsa induced the present author to go by polyphasic approach. Molecular taxonomy including 16S rRNA gene sequence analysis was used to denote the proper taxonomic position of the isolates. The isolate NiVa 51 and NiVas 114 turned out to be novel species of *Serratia* and *Enterobacter* respectively, for which the name(s) *Serratia ureilytica* and *Enterobacter indica* were proposed.

Finally, it may be concluded that nickel resistant bacteria could be isolated from natural fresh water habitat even if it is not contaminated by heavy metal to the level of toxicities. The discovery of novel induction phenomenon of nickel resistance by ppb level of metal ions –itself spoke of the presence of diverse genetic system. The search has opened up avenues for future academic research as well as application in the field of biosensor development.