Metal Contaminants Occurrence and Microbial Diversity From the Perspectives of Dual Tolerance by the Bacterial Isolates Along Altitudinal Zonation of River Teesta – a Pristine River System in Eastern Himalayas

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Abstract

The present study was conducted along River Teesta in Eastern Himalaya. Water and sediment samples were collected from six sampling points during the monsoon and winter seasons along the course of the river. The background information was collected by analysing physico-chemical parameters and the heavy metal concentrations. Culturable bacterial diversity using culture-based and molecular based 16S rRNA approaches characterized bacterial isolates to 5 major phyla, majority belonging Proteobacteria, Actinobacteria and Firmicutes followed by Bacteroidetes, Alpha-Proteobacteria, Beta-Proteobacteria, Gamma-Proteobacteria, Actinobacteria, Bacilli, Flavobacteriia, Deinococci, Sphingobacteriia and Cytophagia. The total of 245 characterized isolates belonged to 69 genera. Diversity indices were calculated for each site. There were variations in the community structure of culturable bacteria along the river stretch with some common and unique groups. Heavy metal tolerance and antibiotic resistance profiles showed some isolates to be tolerant to high levels of heavy metals and multiple antibiotic indicating a major concern. The antibiotic resistance diversified along the human impacted downstream sites. The present report on bacterial diversity and the associated metal and antibiotics tolerance is the first of its kind on Teesta river, the only major river system flowing through the state of Sikkim and parts of North Bengal.

Introduction

Microbial ecosystem is one of the key components of aquatic ecosystems playing important roles in various processes like biogeochemical cycling, microbial food webs, degradation of pollutants and energy flows (Guan et al. 2018). The discharge of wastewater from urban areas and industrial effluents has influenced the bacterial community composition, species abundance and their function thereby disrupting the nutrient cycling processes and negatively affecting the energy flow and loss of biodiversity. It is an established fact that the structure and function of aquatic ecosystems are facing potential threat posed by the changing global environment while the responsiveness of microbial community to the environmental variations still remains the topic of investigation and furthermore, microbial diversity in river systems is less commonly studied than the marine and lake ecosystems (Zeglin 2015). Spatial and temporal heterogeneity in bacterial diversity has been observed in various rivers driven by several environmental factors like temperature, nutrient uptake, organic matter concentration and composition, hydrology and various land use patterns (Gessner and Chauvet 1994; Valett et al. 1997; Dodds et al. 2000; Mulholland et al. 2008; Boyero et al. 2011). From the review conducted by Zeglin (2015) it was found that metal concentration in the river system was the most prominent drivers of microbial ecosystem whereas nutrients and land use were least defined. The same study also concluded that temporal variation (93%) was more prevalent than among stream variation (86%) or longitudinal variation (83%) in defining the composition and structure of microbiota in lotic systems. Rivers are contaminated not only with agents like pesticides, heavy metals or pharmaceuticals but also potentially allochthonous microorganisms (Breton-Deval et al. 2019). Therefore, it is also necessary to study the response of microbes at the structure and community level and throw insights on the response of microbes to the pollutants.

The Indian rivers are not just economically important but also considered to be sacred and holds important place in Indian tradition and culture (Semwal and Akolkar, 2006). In the recent past years, with fast growing population, upsurging industrialization and various agricultural practices there has been unregulated flow of waste water into the river thereby making it vulnerable to pollution. Studies have attributed anthropogenic activities resulting in heavy metal contamination of the riverine ecosystem (Singh et al. 2017; Patel et al. 2018; Zheng et al. 2018; Bhuyan et al. 2019). Elevated levels of toxic heavy metals like Hg, As, Pb, Cd, Cr, Zn and Cu have been reported from various Indian rivers (Hussain et al. 2017; Mahato et al. 2017; Singh et al. 2017; Patel et al. 2018; Rahman and Singh 2018). Heavy metals stress causes shift in bacterial community structure from native to the one dominated by more resistant and tolerant bacteria. In response to toxic concentrations of heavy metals, many aquatic organism including microorganisms can

develop tolerance (Klerks and Weiss, 1987). Heavy metal tolerant bacteria have been isolated from polluted river system showing tolerance to copper, lead, nickel, cobalt, chromium, cadmium and mercury (Rajkumar et al. 2012; Sair et al. 2018). Studies have revealed that co-selection takes place in environmental bacteria with metal and antibiotics resistance (Wright et al. 2006; Kalayu and Ahemad, 2014). Bacteria in metal-contaminated environments have been reported to be a rich source for antibiotics resistance phenotypes than in control areas (Baker-Austin et al. 2006) and rivers are the major means of dissemination of antibiotic resistance genes which is a major public health concern.

The present study was undertaken to assess the spatial-temporal variation in the culturable bacterial diversity in surface water and sediment samples of the river Teesta, a pristine river system originating in the Eastern Himalayas which is subjected to various human intervention including several food, beverage and pharmaceutical companies and also to investigate the co-occurrence of heavy metal and antibiotic resistance among the isolates.

Materials And Methods

Study area and sampling

River Teesta, a prominent river of the Eastern Himalayan originates in the Teesta Khangse glacier located in north-eastern part of Sikkim in Northeast India. The total catchment area is 12,159 km² and is 414 km long with the greater part in India (151 km in Sikkim, 19 km along the border between Sikkim and West Bengal, 29 km in West Bengal), 94 km along the Indo-Bangladesh border, and the final 121 km in Bangladesh. In terms of socio-economic development Sikkim has flourished in the past decade with various food, beverage and pharmaceutical industries that have been established along the banks of the river. In addition, Teesta has been used for power generation and has five operational hydro projects located in Sikkim and West Bengal. This has led to the destruction of vegetation and natural habitats in these areas. The sampling points and site description are depicted in Fig.1 and Table 1. Water and sediment samples were collected in presterilized containers during monsoon and winter season in the year 2018-2019 from six sampling sites along the river with the altitude ranging from 5183m to 293m above mean sea level. Site T1 is a tributary site between site S2 and S3. The samples were brought to the laboratory on ice condition.

Background study of the sampling sites

The physico-chemical parameters and the heavy metal concentration in the river surface water and sediment samples were determined following the standard APHA (1998) protocols.

Bacterial isolation and enumeration

Isolation of bacteria from both the sediment and water samples was done by the standard spread plate technique. In brief, 1 gram of sediment sample was suspended in 10 ml of 0.85% saline and kept in shaking condition (150 rpm) for 1 hour. The samples were then serially diluted up to 10⁻⁵ and 0.1 ml from each 10⁻³, 10⁻⁴ and 10⁻⁵ dilutions were spread on Luria Bertani agar plates (composition in grams/litre: tryptone- 10, yeast extract- 5, NaCl- 10 and agar – 15). Similarly, water samples were diluted in 0.85% saline up to 10⁻⁴ fold dilution and subsequently 0.1 ml each from 10⁻², 10⁻³ and 10⁻⁴ were plated on Reasoner's 2A or R2A agar plates (composition in grams/ litre: acicase- 0.50, yeast extract- 0.50, proteose peptone- 0.50, dextrose- 0.50, starch soluble- 0.50, dipotassium hydrogen phosphate- 0.30, magnesium sulfate- 0.024, sodium pyruvate- 0.30 and agar- 15.0). All the media and glasswares were sterilized by autoclaving at 121°C for 20 minutes. The inoculated plates were incubated at 28 °C for 24-48 hours except for the samples from site S1 which was incubated at 10 °C for two weeks. After incubation, colonies were selected based on their characteristic color, morphology and texture and the selected colonies were purified by streak plate technique. The cultures were cryopreserved in the cryovials with 50% glycerol at -20 °C. Enumeration of total bacterial load was done by the standard

plate count (SPC) technique in the form of colony forming units (CFU). The bacterial load is expressed as CFU/ml in case of water samples and CFU/grams in sediment samples.

Bacterial DNA isolation and 16S rDNA amplification

Genomic DNA was extracted using the Bacterial genomic DNA kit by HiMedia, India. The genomic DNA was amplified targeting the 16S rRNA gene using the universal bacterial primers 27F: 5'AGAGTTTGATCCTGGCTCAG 3' and 1492R: 5'TACGGYTACCTTGTTACGACT T 3' (Weisburg et al. 1991). The amplification was carried out on a GeneAmp PCR system 9700 (Applied Biosystems, USA). Each 30 µl reaction mixture consisted of 15.75 µl of millipore water, 3 µl of 10X buffer with 17.5mM MgCl₂, 3 µl of each forward and reverse primer of 10mM concentration, 2 µl of dNTP mixture, 0.25 µl of Taq polymerase and 3 µl of template DNA. The amplified PCR products were electrophoresed on 1.2% agarose gel and purified using QIA Quick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced at Macrogen Inc, Seoul, Korea.

Sequence alignment and phylogenetic analysis

Identification of the bacterial isolates were carried out by calculating 16S rRNA gene sequence similarity against all known procaryotic species in the Eztaxon server (Chun et al. 2007) which involves two steps. First step is the Basic Local Alignment Search Tool (BLAST) which is a sequence similarity search program was used to compare our query sequence to the sequence database of the National Centre for Biotechnology Information (Altschul et al. 1990) and secondly, pairwise sequence alignment to find out sequence similarity between the query sequence and hit sequences obtained in the first step. This algorithm uses 98.65% similarity threshold as species boundary. Before phylogenetic analysis, the sequences obtained from sanger sequencing were trimmed by the help of Molecular Evolutionary Genetics Analysis software, MEGA 7.0 (Tamura et al, 2011) and the sequences were aligned by using the clustal W inbuilt in MEGA 7.0 where the most similar sequences with best alignment score are aligned first followed by more distant groups and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with 1000 bootstrap replications to assess nodal support in the tree (Felsenstein, 1981). The nucleotide sequences were submitted to NCBI and the accession numbers were obtained.

Diversity analysis

With a view to compare the culturable bacterial diversity in both the water and sediment samples, samples were analysed both spatially and temporally in order to understand the effect of altitude gradient, human intervention and seasonal fluctuations. Diversity analysis was assessed by calculating Shannon-Weiner diversity index (H), Simpson's dominance index (D), evenness index (E) and richness index by Margalef.

Determination of MIC (Minimum Inhibitory Concentration) of the bacterial isolates against heavy metals

MIC of the bacterial isolates was determined against four heavy metals Pb, Zn, Cu and Cd by spot plate technique. Stock solutions of the metals were obtained from the metal salts of ZnSO₄. H₂O, Pb(NO₃)₂, CuSO₄.5H₂O and Cd(NO₃)₂ purchased from Sigma Aldrich to obtain stock solution of 10,00 ppm. Heavy metal containing plates were prepared by supplementing Luria Bertani agar with various concentrations of heavy metals ranging from 50 to 2000 ppm in case of Pb, Zn and Cu and Cd was used at the concentration ranging from 1-100 ppm. The isolates were grown overnight in Luria Bertani broth and 10µl of the culture corresponding to OD 0.5 at 600nm was spotted on the metal containing plates and incubated at 28 °C for 24 h. MIC was determined as the lowest concentration of metal that inhibited growth after 24 h.

Antibiotic susceptibility test for the isolates

The antibiotic susceptibility of the bacterial isolates was conducted using agar diffusion technique on Mueller-Hinton agar (MHA) plates following the guidelines of the Clinical Laboratory standard Institute (CLSI) 2013. In the present study total 15 antibiotics was used which are as follows Meropenem (10mcg), Imipenem (10mcg), Erythromycin (10mcg), Ciprofloxacin (5mcg), Nalidixic acid (30mcg), Netillin (30mcg), Novobiocin (30mcg), Neomycin (30mcg), Tigecycline (15mcg), Lincomycin (15mcg), Rifampicin (5mcg), Polymyxin B (300U), Co- Trimoxazole (25mcg), Ceftazidime (30mcg), Trimethoprim (5mcg) using standard antibiotic discs (HiMedia, India). The multiple antibiotic resistance (MAR) index for the test isolates was calculated as a/b where a represents the number of antibiotics the isolates is resistant to and b represents the total number of antibiotics used against the isolate (Krumperman 1983). A MAR index value of >0.2 indicates that the isolate is multiple antibiotic resistant whereas a MAR index value of $< \text{ or } = 0.2$ indicates a very low or negligible antibiotic resistance.

Statistical analysis

PAST 4.07b software was used for conducting all the statistical analysis including the calculation of diversity indices and preparing graphs. Pearson's correlation was employed to understand the relationship between various physico-chemical properties and the culturable microbial taxa in both the water and sediment samples.

Results And Discussion

Physico-chemical parameters of river water and sediment samples

The mean values along with the standard deviation of the various physico-chemical parameters are presented in the Table 2. The temperature of the river water ranged from 2°C to 19 °C where the lowest temperature was recorded at site S1 during winter. The mean values of all the parameters were found to be below the permissible limit of BIS 2012. However, BOD was slightly higher at site S3 followed by site S5 and T1. The determined DO at S1 is lower than the other sites because of the high altitude with low atmospheric oxygen.

The estimation of Pb, Zn, Cu and Cd were carried out in both the water and sediment samples. The mean concentration of the metals for the analysed samples are presented in Table 3. The concentration of Pb in the water samples from the sites S4, S5 and T1 slightly crossed the permissible limit of BIS during both monsoon and winter seasons whereas other heavy metals were found to be below the threshold range of BIS. In the sediment samples, the concentrations of heavy metals were found to be several orders of magnitude higher than the water samples. The mean concentration of Pb in the downstream sites S4, S5 and T1 ranged from 12.5 -27.4 ppm which surpassed the limit of BIS which is 11 ppm. The concentration of Zn ranged from 1.8 ppm to 85.6 ppm and the concentration was found to exceed the BIS limit of 16 ppm in the monsoon season as compared to winter season. this elevated levels of heavy metals in the downstream sites could be the effect of industrialization.

Mann-Whitney U test, was employed due to skewness of data to find out for any seasonal variations in the heavy metal concentrations in monsoon and winter season. The test revealed no significant differences in the heavy metal concentrations in monsoon and winter season for water samples. In case of sediment samples we see significant difference in concentration of Zn ($t_{df(10)} = 7.477, p<0.01$) and Cu ($t_{df(10)}=3.533, p<0.01$) between the two seasons which showed that both the concentration of Zn and Cu decreased significantly in winter season at various sites . However, no significant differences were observed in concentrations of Pb and Cd between the two seasons.

Total viable count

Out of the water and sediment samples, rich bacterial load was shown by the sediment samples which was expressed as CFU/g than the water samples depicted in Table 4. The mean bacterial load was highest in site S4 and lowest in S1

in both the water sample and sediment respectively. Less bacterial load at S1 could be explained by the high altitude of S1, cold and oligotrophic environment. From ANOVA, the variation in bacterial load between different sites was highly significant in both the water sample ($F_{df(5)} = 35.16, p < 0.01$) and sediment ($F_{df(5)} = 5.42, p < 0.01$) as well. However, the t-test showed no significant seasonal variation in the mean bacterial load in both the water samples ($t_{df(10)} = 1.954, p = .079$) and sediment samples ($t_{df(10)} = -0.889, p = .395$). In the present study, bacterial load was higher in the downstream sites which could be explained by the waste water discharge close to the sites thereby increasing the organic matter which supports bacterial growth. On the basis of colony morphology total 129 and 116 bacterial isolates were obtained both from water and sediment samples during monsoon and winter season respectively.

Culturable bacterial diversity of river water and sediment samples

Based on the colony morphology and 16S rRNA gene sequencing a total of 129 isolates (water-86 and sediment-43) and 116 isolates (water-61 and sediment-55) were recovered in monsoon season and winter season respectively making a total of 245 isolates. The bacterial isolates belonged to 5 major phyla with the higher percentage of the bacterial isolates belonging to major phyla like the Proteobacteria, Actinobacteria and Firmicutes followed by Bacteroidetes and Deinococcus-Thermus. These phyla were represented by various classes belonging to Alpha-proteobacteria, Beta-proteobacteria, Gamma-proteobacteria, Actinobacteria, Bacilli, Flavobacteriia, Deinococci, Sphingobacteriia and Cytophagia. Representative comparison of different phyla and classes in both water and sediment samples are shown in Fig 2 and Fig 3. In the water samples Proteobacteria and Actinobacteria were dominating phyla in all the sites during both monsoon and winter season. Firmicutes were found to be prevalent during monsoon season than in winter at all the sites. Deinococcus-Thermus was a unique phylum that was detected only at site S5 which represented 5% of the total phyla at that particular site. The common classes present in water samples during both the seasons are the Alpha-proteobacteria, Beta-proteobacteria, Gamma-proteobacteria and Bacilli. Flavobacteriia and Deinococci was detected only during monsoon season whereas Sphingobacteriia and Cytophagia were two unique classes found only at site S1.

In the sediment samples, Proteobacteria, Actinobacteria and Firmicutes are the phyla commonly present in all the sites during both monsoon and winter season. Deinococcus-Thermus was present only at site S4 in monsoon season whereas in winter season Bacteroidetes was present only at site S1. At the class level, Alpha-proteobacteria, Beta-proteobacteria and Gamma-proteobacteria, Bacilli and Actinobacteria are commonly present. Flavobacteriia was detected only at site S1 in both the seasons whereas Deinococci was found only in monsoon season present only at site S4.

Bacterial diversity at the genus level

From all the samples with 244 total isolates, a total of 69 genera were identified after comparing the 16S rRNA sequence with the known sequences deposited in the public database EzBioCloud, <https://www.ezbiocloud.net/identify>. The spatio-temporal comparison of the various genera isolated from water and sediment samples are provided in the Fig 3. The isolates exhibited >97% to the previously deposited sequences. High bacterial diversity was displayed by both the water and sediment samples. The recovered bacterial genera were higher in the water samples as compared to the sediment samples.

In case of water samples, out of 138 identified genera some of the genera prevalent in the water samples in more than two sites are *Pseudomonas* (13%) followed by *Microbacterium* (5.79%), *Bacillus* (5%), *Acinetobacter* (5%), *Brevundimonas* (4.34%), *Serratia* (3.6%) *Arthrobacter* (2.8%), *Janibacter* (2.8%), and that altogether accounted for 42.33% of the total isolates identified in the water samples and the rest 57.67% isolates represented genera that were uniquely present at less than two sites. Genera like *Flavobacterium*, *Duganella*, *Pedobacter*, *Deefgea*, *Arcicella* were present only at site S1 belonging to the phylum Bacteroidetes and Proteobacteria. Genera distinct to site S2 are

Haemophilus, *Ideonella*, *Roseateles* and *Variovorax* whereas *Phycococcus*, *Nocardioides*, *Asticcacaulis* and *Vogesella* were unique only to site S3. *Myroides* and *Comamonas* were found to be present only at site S4 and *Priestia*, *Deinococcus*, *Phenylobacterium* and *Brucella* were unique to site S5 and similarly *Demacoccus*, *Agromyces* and *Gordonia* were unique genera present only at site T1. The downstream sites impacted by human intervention were represented more by the opportunistic pathogens like *Serratia*, *Acinetobacter*, *Pseudomonas*, *Microbacterium*, *Gordonia* and *Brucella*. Water is an easy means of dissemination of these pathogens and these bacteria can easily come in contact with the humans when the water is used for various purposes including recreation.

In the sediment samples, total 95 genera were identified amongst which *Pseudomonas* (11.57%) was prevalent in more than two sites followed by *Microbacterium* (10.5%), *Acinetobacter* (8.4%) and *Arthrobacter* (6.3%) accounting for 36.9% of the total isolates in the sediment samples and the remaining 60.1 % accounted for the less prevalent genera. *Cryobacterium* was found only in the site S1 which is characterised by high altitude and cold temperature throughout the year. *Comamonas* was identified only from the samples of site S2 and *Deinococcus* was found to be present only at site S4 and *Hydrogenophaga*, *Paracoccus* and *Ensifer* were unique genera found at site T1.

Diversity analysis

Diversity indices are calculated to compare the community richness and evenness within the various sampling sites and seasons. Shannon-Weiner and Simpson's index gives idea about community composition. Shanno-Weiner index puts more weight on species richness whereas the Simpson's index considers species evenness (Kim et al. 2017). The Shannon richness in water samples ranged from 1.76-2.91 and 1.96-2.74 in monsoon and winter respectively. Similarly in the sediment samples, the Shannon index ranged from 1.03-2.29 and 1.38 -2.47 in monsoon and winter respectively. Shannon index values showed that the bacterial diversity was comparatively higher in the water samples as compared to the sediment samples which is also apparent from the Shannon rarefaction curve (Fig. 4). In case of water samples, bacterial diversity as indicated by the Shannon index increased as we go downstream but however no such observation was found in the case of sediment samples. The Simpson's index ranged from 0.77-1 and 0.89-1 respectively in monsoon and winter season in the water sample. In the sediment samples the values ranged from 0.6-0.95 in monsoon and 0.75-0.96 in winter season. The Margalef's richness index for water samples ranged from 2.16-4.50 in monsoon and 1.37-2.89 in winter. According to the Margalef's index calculated for the water samples high bacterial richness was observed at site S5 (downstream site) and least at site S1 (uppermost site). Similarly, in the case of the sediment samples the values ranged from 1.12-2.92 in monsoon and 1.37-3.22 in winter samples with the bacterial richness least in the upstream sites and highest in the downstream sites except for site S5 which showed less Margalef value in winter. This richness at particular sites in the river could possibly be due to its ability to grow at particular environmental conditions which are enriched by inputs from human intervention. In addition, the culturable bacterial diversity are limited to those bacteria which can grow in the provided media and given conditions therefore the relative abundance provided by the culturable bacteria does not provide a clear picture of the microbial community like the culture independent approaches (Venkatachalam et al. 2015). The summary of various diversity indices in water and sediment samples are given in Table 5. Shannon diversity index was used to study any seasonal variation and the t-test analysis showed no significant variation in diversity between seasons (monsoon vs winter) in both the water samples ($t_{df(10)} = .624$, $p = .547$) and sediments ($t_{df(10)} = -.452$, $p = .661$) respectively.

Accession numbers for the isolates

Accession numbers for all 245 isolates were obtained after submission of 16S rRNA sequences to the NCBI database <https://www.ncbi.nlm.nih.gov/>. The accession numbers of the isoaltes are as follows: MN726744-MN726749; MN727110-MN727116; MN733078-MN733085; MN733101-MN733106; MN733214-MN733218; MN733329-MN733339; MN726750- MN726753; MN727117-MN727130; MN733086-MN733122; MN733219-MN733240;

MN733340-MN733354; OK090491-OK090506; OK090511-OK090522; OK090551- OK090569; OK090524-OK090545; OK090595-OK090618; OK090572-OK090594.

Correlation analysis

Relationship between species richness and bacterial load with the various physico-chemical parameters

The species diversity and bacterial load in the water samples showed a positive correlation with each other ($r=.690$, $p<0.05$). Among all the physico-chemical parameters only temperature and Cu concentration showed a positive correlation with the species diversity (Table 6) whereas bacterial load in the water samples showed a positive association with temperature and concentrations of all the heavy metals. Species diversity did not show any significant relationship with the heavy metal concentration in the sediments. However, the bacterial load was positively correlated to Pb ($r=.664$, $p<0.05$) and Cd ($r=.813$, $p<0.001$) concentrations (Table 7).

Pearson's analysis was conducted to check for the relationship of the various physico-chemical parameters and bacterial classes. For the water samples, the analysis showed that temperature showed significant ($p<0.05$) positive correlation to the dissolved oxygen and bacterial load whereas it showed significant negative correlation with the abundance of Sphingobacteria and Cytophagia. Similarly, DO was found to have significant positive correlation with the bacterial load and Deinococci abundance. TDS and conductivity were found to be significant variable with respect to Actinobacteria (positive correlation) and γ -Proteobacteria (negative correlation) as depicted in Fig. 5. Heavy metals like Pb and Cd positively correlated with the bacterial load, Zn showed strong positive correlation with Cu and Cd and with the abundance of Bacilli. In one study from metal polluted area, it was found that *Bacillus* sp. was the most dominant and frequently isolated species which indicates its potential to survive in metal polluted areas and could be an indicator of metal contaminated areas (Zampieri et al 2016).

In the case of sediment samples, Pb and Cd showed significant positive correlation with the bacterial load and Pb further showed positive correlation with Deinococci. Increased heavy metals have showed higher relative abundance of abundances of Deinococci in metal exposed areas. Similarly, Zn and Cu were found to be strongly positively correlated and both metals demonstrated moderate positive correlation with β -Proteobacteria (Fig. 5).

Heavy metal tolerance of the isolates

In this study, total 107 bacterial isolates were tested for their tolerance to four heavy metals Pb, Zn, Cu and Cd and the MIC values were recorded. MICs are crucial in understanding the response of the bacterial isolates to the heavy metals and could be associated with the heavy metal concentration and toxicity (Zampeiri et al 2016).

The tolerance of the isolates to the heavy metals showed varying trend depending upon the sampling sites (Table 8). The MIC of Pb ranged from 500-2000 ppm where about 82% of the isolates belonging to the downstream sites showed MIC values 2000 ppm. The MIC values of Zn ranged from 100-1000 ppm, 6.5% of total isolates displayed MIC of 100 ppm, 38% showed MIC of 250 ppm, 42% showed MIC of 500 ppm and remaining 13% showed MIC of 1000 ppm. In the case of Cu the MIC of the isolates ranged from 250-1000 ppm with 25% showing MIC value of 250, 67% displayed MIC of 500 ppm and the remaining 8% showed MIC of 1000 ppm. Considering the background values for Cd determined in the study sites which ranged from 0.02-1.2, concentration of Cd was used at lower concentration ranging from 1-100 ppm. The MIC of the isolated ranged from 2.5-100 ppm where 2% isolated showed MIC between 2.5-5 ppm, 26% of the isolates showed MIC of 10 ppm, 38% had MIC of 50 ppm and the remaining 44% showed MIC of 100 ppm. The degree of tolerance of the isolates to the heavy metals were as follows $Pb>Zn>Cu>Cd$.

Antibiotic resistance profile of the bacterial isolates

All 107 isolates were tested for antimicrobial resistance against 15 antibiotics belonging to various classes of antibiotics. The number of isolates showing resistance to various classes of antibiotics are summarised in Table 9 and percentage wise graphical representation is shown in Fig 6. which also indicates that the frequency of the antibiotics to which bacterial isolates are resistant to increases as we move from upper stretch to lower stretch which has more human impact in terms of anthropogenic input in to the river. Considering the number of antibiotics an isolate is resistant to, MAR (multiple antibiotic resistant) index was calculated for each isolate. The MAR index range for each site and the percentage of MAR bacteria at each site is presented in the Table 10 .

The MAR index at site S1 ranged from 0.13-0.2, at S2 it ranged from 0-0.46, followed by S3 that ranged from 0.2-0.53, at S4 it ranged from 0.13-0.53, TS5 showed 0.2-0.46 and finally at T1 it ranged from 0.13-0.53. An increase in the number of isolates showing multiple antibiotic resistance was observed at the downstream sites (Table 10). Highest percentage of isolates 92% and 95% was observed at sites S4 and S5 respectively. S4 is a site characterised by human settlement and several pharmaceutical companies along the banks of the river and S5 is a site downstream to S4 The percentage of MAR bacteria increased downstream along the length of the river (Table 10).

Amongst the various isolates tested for resistance to antibiotics, *Pseudomonas* and *Acinetobacter* showed the highest MAR values that ranged from 0.2-0.6 and 0.2-0.53 respectively followed by *Bacillus* for which MAR index ranged from 0-0.46 and *Paenarthrobacter* showed 0.4 MAR index. All the *Serratia* and *Microbacterium* isolates showed MAR index of 0.33 and for *Stenotrophomonas* the values ranged from 0.26-0.33 whereas *Brevundimonas* and *Arthrobacter* showed MAR index range from 0.13-0.33 and *Exiguobacterium* and *Brevibacterium* showed 0.26 MAR index and least of values of 0.2 was shown by *Cryobacterium* and *Janibacter* whereas *Kocuria* and *Flavobacterium* showed only 0.13 MAR index value. The presence of heavy metals in the environment causes selective pressure leading to increase in bacterial resistance to heavy metals and antibiotics. Metals like Pb, Zn, Cu and Cd have huge applications in industries and also enter the environment via various vehicular emissions, batteries, mining activities and gasoline spillage (Mahato et al 2017) these heavy metals are known to increase the concentrations of selected antibiotic resistance genes (Li et al 2019). The spread of antibiotic resistance can occur between related and unrelated bacteria including pathogenic and non-pathogenic ones. Mobile genetic elements such as plasmids and integrons encoding the antibiotic resistance genes are spread in the microorganisms through horizontal gene transfer as a result of which resistance genes are disseminated in the environment and water is an easy means of dissemination (Khan et al 2013).

Conclusion

This study showed that the levels of heavy metals like Pb and Zn at certain human impacted areas to be elevated as compared to the other sites. The bacterial load showed significant variations among the sites with higher load at the downstream sites possibly explained by the anthropogenic organic matter input from adjoining industrial and domestic areas. Bacterial diversity was higher in the water samples however our study found that there was no effect of season on the diversity. Heavy metals alter the bacterial composition and function and here we isolated bacterial strains showing tolerance to both heavy metals and antibiotics and the MAR bacteria increased in the downstream sites.

Declarations

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Conflict of interest/ Competing interests: The authors declare no competing interest.

Author contributions: SRJ conceived the study. UC conducted the experiments. SRJ and UC wrote the manuscript and analysed the data.

Availability of data material: The study did not involve any data deposition

Code availability: NA

Ethics approval: The study did not require any ethical clearances to conduct the study.

Consent to participate: Authors declare their consent to participate in the present work.

Consent for publication: Both the authors have consented to publication of the present research findings.

Permission: Necessary permission to carry out the study as per the existing regulations was obtained from Department of Forest, environment and Wildlife Management, Office of the Chief Conservator of Forest (T&HQ) cum CWLW, Govt of Sikkim, India.

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Tables

Table 1. Site description and location of the sampling sites

Site ID	Elevation (m)	Coordinates	Site description
S1	5183	N 28°2.10.2' E 088°42.32.6'	Uppermost stretch of the river with minimum human interference
S2	1790	N27°36.355' E088°39.062'	Moderate human settlement with tourism industry and hydro-project in the vicinity
T1	516	N27°15.544' E088°33.118'	Tributary of river Teesta passing through human settlement and industrial areas
S3	426	N27°13.896' E088°29.517'	Site with intense human settlement and mining activities
S4	333	N27°10.864' E088°30.027'	Industrial belt along with human settlement
S5	295	N27°04.954' E088°26.654'	River banks under forest cover with human settlement and mining activities

Table 2. Physico-chemical parameters of the water

Parameters		S1	S2	T1	S3	S4	S5	BIS
Temperature	Mn	5±0	17±0	18±0	18±0	18.25±0.53	18.5±0.71	-
	Wn	2±0	9±1.41	14.5±0.71	14.5±0.71	15±1.41	15±01.41	
pH	Mn	7.4 ± 0.23	7.59±0.07	7.71±0.42	7.29±0.4	7.75±0.07	7.53±0.33	6.5-8.5
	Wn	7.26±0.09	7.59±0.04	7.43±0.35	7.79±0.01	7.47±0.14	7.39±0.15	
TDS (ppm)	Mn	26.65±0.69	25.2±0.76	25.73±5.13	28.32±1.3	26.62±0.42	25.47±0.51	500
	Wn	34.69±0.26	35.9±0.67	54.04±0.91	49.82±2.49	44.74±0.78	45.18±0.35	
Conductivity (µS/cm)	Mn	49.92±1.73	47.20±1.59	47.11±10.89	52.64±2.43	49.52±0.93	47.45±0.91	300
	Wn	62.64±0.78	66.14±0.78	99.23±0.9	91.72±2.27	82.32±0.28	85.08±0.48	
DO (ppm)	Mn	5.32±0.17	6.96±0.79	6.55±0.78	7.4±0.06	7.36±0.09	8.45±0.3	5
	Wn	5.05±0.07	7±0.28	6.38±0.2	6.76±0.06	6.3±0.42	6.38±0.09	
BOD (ppm)	Mn	0.2±0	0.32±0.11	1.05±0.35	3.9±0.48	0.87±1.32	1.85±1.15	5
	Wn	0.29±0.18	1.62±0.2	2.76±0.45	1.96±0.06	2±0.57	1.18±0.23	

Mn-Monsoon; Wn-Winter

Table 3. Heavy metal concentration in the sites during monsoon and winter season

Heavy metal concentration in water samples (ppm)					
Sampling sites	Seasons	Lead	Zinc	Copper	Cadmium
S1	Mn	0.0048±0.004	0.0022±0.004	0.0009±0.000	0.0002±0.000
	Wn	0.0029±0.003	0.002±0.003	0.0005±0.003	0.0001±0.000
S2	Mn	0.0063±0.003	0.0139±0.008	0.0014±0.003	0.0003±0.000
	Wn	0.0086±0.003	0.0097±0.007	0.0015±0.004	0.0002±0.000
T1	Mn	0.0161±0.008	0.1007±0.016	0.0237±0.018	0.0009±0.000
	Wn	0.014±0.006	0.0846±0.009	0.0245±0.014	0.0005±0.000
S3	Mn	0.0082±0.004	0.0264±0.005	0.0041±0.004	0.0004±0.000
	Wn	0.0033±0.004	0.0063±0.002	0.0017±0.002	0.0002±0.000
S4	Mn	0.0330±0.021	0.0414±0.002	0.0056±0.002	0.0007±0.000
	Wn	0.0046±0.016	0.007±0.005	0.0024±0.000	0.0003±0.000
S5	Mn	0.0149±0.014	0.0490±0.0169	0.0102±0.003	0.0003±0.000
	Wn	0.0112±0.009	0.0737±0.021	0.0172±0.015	0.0006±0.000
BIS		0.01	15	1.5	0.003
Heavy metal concentration in sediment samples (ppm)					
S1	Mn	3.6±0.002	37.4±0.018	5.4±0.002	0.04±0.000
	Wn	5.6±0.004	1.8±0.008	1.5±0.003	0.2±0.000
S2	Mn	8.8±0.007	53.4±0.014	8.4±0.002	0.06±0.000

	Wn	7.7±0.003	3±0.015	2±0.004	0.6±0.000
T1	Mn	11.7±0.012	55±0.010	9.4±0.008	0.17±0.001
	Wn	19.56±0.042	3.7±0.003	3.2±0.002	0.6±0.000
S3	Mn	7.2±0.005	54.4±0.011	10.8±0.005	0.10±0.000
	Wn	12.5±0.003	2.6±0.006	3.4±0.012	0.5±0.000
S4	Mn	27.4±0.017	83±0.012	12.7±0.015	0.65±0.004
	Wn	14.8±0.006	6±0.017	9.2±0.006	1.2±0.001
S5	Mn	21.0±0.042	85.6±0.026	11.2±0.007	0.21±0.001
	Wn	19.56±0.042	3.7±0.003	3.2±0.002	0.6±0.000
IRS		11	16	28	-

Mn-Monsoon; Wn-Winter

Table 4. Total viable bacterial count

Season	Average CFU/ml in the samples					
	CFU X 10 ⁴					
	S1	S2	T1	S3	S4	S5
WATER SAMPLE						
Monsoon	3.1 ± 0.35	15.56 ± 2.6	28.8 ± 3.4	19 ± 1.35	25.2 ± 6.36	24.1 ± 2.5
Winter	2.8 ± 0.9	7.6 ± 0.9	11 ± 1.78	7.7 ± 0.9	9.7 ± 2.12	8.5 ± 0.9
SEDIMENT SAMPLE						
Monsoon	4.6 ± 0.9	96 ± 28.6	115 ± 27.5	123 ± 20.4	165 ± 9.5	111 ± 25.5
Winter	4.4 ± 1.3	141 ± 30.3	156 ± 11	163 ± 23.0	175 ± 22.0	155 ± 8.0

The values represent the average of three replicates with standard deviation

Table 5. Various indices calculated for water and sediment samples

Diversity indices	Water sample											
	Monsoon						Winter					
	S1	S2	T1	S3	S4	S5	S1	S2	T1	S3	S4	S5
Shannon	1.76	1.91	2.63	2.67	2.71	2.91	1.96	2.01	2.13	2.68	2.19	2.74
Simpson	1	0.77	0.97	0.94	0.95	0.97	0.89	1	0.9	0.98	0.93	0.97
Margalef	2.16	2.65	3.62	3.88	3.81	4.50	2.28	2.50	2.61	3.75	2.61	3.90
Evenness	1.46	0.85	1.39	1.2	1.25	1.32	1.18	1.50	1.21	1.46	1.27	1.40
Diversity indices	Sediment sample											
	Monsoon						Winter					
	S1	S2	T1	S3	S4	S5	S1	S2	T1	S3	S4	S5
Shannon	1.03	2.11	2.29	2.05	1.89	1.89	1.89	1.84	2.47	2.21	2.04	1.38
Simpson	0.6	0.95	0.93	0.93	0.93	0.93	0.93	0.91	0.96	0.96	0.87	0.75
Margalef	1.12	2.57	2.92	2.40	2.23	2.23	2.23	2.06	3.22	2.89	2.50	1.37
Evenness	0.94	1.37	1.24	1.29	1.33	1.33	1.33	1.25	1.32	1.40	1.09	0.99

Table 6. Spearman correlation between the Shannon species diversity for water (SDW) and Bacterial load for water (BLW) with different physio-chemical parameters and heavy metal concentration in the water samples.

		SDW	BLW	Temp	pH	TDS	Pb	Zn	Cu	Cd
SDW	Correlation Coefficient	1.000	.690*	.675*	.294	.063	.503	.517	.643*	.504
	Sig. (2-tailed)	.	.013	.016	.354	.846	.095	.085	.024	.095
BLW	Correlation Coefficient	.690*	1.00	.806**	.357	-.175	.795**	.771**	.830**	.814**
	Sig. (2-tailed)	.013	.	.002	.254	.586	.002	.003	.001	.001

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 7. Spearman correlation between the Shannon species diversity for sediment (SDS) and Bacterial load for sediment (BLS) with various physico-chemical parameters and heavy metal concentrations

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

		SDS	BLS	Pb	Zn	Cu	Cd
SDS	Correlation Coefficient	1.000	.239	.120	.021	.070	-.081
	Sig. (2-tailed)	.	.454	.711	.948	.828	.802
BLS	Correlation Coefficient	.239	1.000	.664*	-.042	.217	.813**
	Sig. (2-tailed)	.454	.	.018	.897	.499	.001

Table 8. Tolerance of the isolates to heavy metals

Sites	No of isolates	Heavy metal concentration in ppm with number of isolates having corresponding MIC values					
		50	100	250	500	1000	2000
Lead							
S1	5				2	3	0
S2	18					2	16
T1	21				1	0	20
S3	14					2	12
S4	26					7	19
S5	23					2	21
Zinc							
S1	5			5			
S2	18		2	10	4	2	
T1	21		1	11	9		
S3	14			8	4	2	
S4	26		4	1	15	6	
S5	23			6	13	4	
Copper							
S1	5			5			
S2	18			4	14		
T1	21			12	9		
S3	14				12	2	
S4	26			3	17	6	
S5	23			3	20		
Cadmium							
		1	2.5	5	10	50	100
S1	5				5		
S2	18			1		9	8
T1	21		1	1	8	7	4
S3	14				2	7	5
S4	26				1	16	10
S5	23				2	2	19

Table 9. No of isolates showing resistance to the antibiotics

Antibiotic class	Antibiotics	Number of isolates resistant to antibiotics at each site					
		S1	S2	S3	S4	S5	T1
		n=5	n =18	n = 14	n = 26	n = 23	n = 21
B-lactams	MRP	0	0	0	0	0	0
	I	0	1	1	13	3	0
Quinolone	NA	4	5	3	1	9	10
	CIP	0	0	0	0	4	0
Aminoglycosides	NET	0	0	0	6	2	0
	N	0	0	0	0	2	3
	NV	0	9	9	18	11	3
Macrolide	E	0	7	9	11	7	9
Sulfonamide	COT	0	4	9	13	7	8
Cephalosporin	CA	4	16	14	24	19	19
Polypeptide	PB	0	2	3	6	10	7
Polyketide	RIF	2	9	10	21	17	6
Glycylcycline	TGC	0	0	0	0	0	3
Antifolate	TR	1	7	9	12	12	1
Lincosamide	L	1	17	11	14	11	14

MRP-Meropenem, I- Imepenem, NA- Nalidixic acid, CIP- Ciprofloxacin, NET- Netillin, N-Neomycin, NV- Novobiocin, E- Erythromycin, COT- Co-trimoxazole, CA- Ceftazidime, PB- Polymyxin B, RIF- Rifampicin, TGC- Tigecycline, TR- Trimethoprim, L- Lincomycin

Table 10. Antibiotic resistance profile of the isolates

Sites	No. of isolates	Number of isolates showing resistance to corresponding number of antibiotics										Percentage of MAR bacteria at each site
		1	2	3	4	5	6	7	8	9	MAR index range	
S1	5	5	3	2							0.13-0.2	Nil
S2	18	17	17	14	11	6	5	4	1	1	0-0.46	61
T1	21	21	20	15	13	6	3	3	3	-	0.13-0.53	62
S3	14	14	14	13	9	9	9	9	1	-	0.2-0.53	64
S4	26	26	26	24	24	17	8	8	6	-	0.13-0.53	92
S5	23	23	23	23	22	18	3	2	-	-	0.2-0.46	95

Figures

Figure 1

Map of Study area

Figure 2

Graphical representation of bacterial diversity at phylum (A) and class (B) level for the water and sediment samples

Figure 3

Figure 4

Rarefaction curves for water and sediment samples

Figure 5

Pearson's correlation coefficient rank between and among the physico-chemical properties of water (A) and sediment (B) and bacterial taxa abundances. The values in the box represent the level of significance ($p < 0.05$).

Figure 6

Graphical representation of the percentage of isolates resistant to various antibiotics at each sites