Assessment of bacterial diversity in the Gangetic river system of Uttarakhand, India

Microorganisms are the most abundant organisms in natural freshwater systems and also play a key role in ecological processes. Detailed knowledge of the diversity and function of microorganisms dwelling in freshwater habitats is an essential prerequisite for the sustainable management of freshwater resources. The Ganges is considered to be the largest river in the Indian subcontinent. It faces substantial anthropogenic activities. It originates in the state of Uttarakhand as Bhagirathi from Gaumukh in the Gangotri glacier at 30°56’N and 79°04’-79°15’E. The Alaknanda river originating near Badrinath, is the main tributary of the Ganges. Alaknanda and Bhagirathi join at Devprayag, about 200 km downstream of their origin.

Though considerable insights into the pollution status of the Ganges have been gained, little or no efforts have been made on the analysis of its bacterial diversity in Uttarakhand. One of the major constrains is that the Ganges in Uttarakhand is spread over a vast geographical area and covers difficult terrain, which makes the selection of sites difficult for sample collection. Biologists have long recognized that there are undiscovered species in almost every survey or species inventory. The estimation of species richness is important for comparing communities in conservation and management of biodiversity, for assessing the effects of human disturbance on biodiversity, and for making environmental policy decisions. India is considered as a mega biodiversity centre, and therefore, this work provides a platform where bacterial diversity of the Gangetic river system has been studied in detail. The bacterial population was isolated and characterized to estimate the limits in terms of Shannon index. As diversity indices provide more information about community composition along with relative abundances of different species, they were calculated for assessment of diversity.

The total stretch covered in this study was 440 km, comprising Alaknanda (200 km), Bhagirathi (170 km) and Lower Ganga (70 km). The study area was divided into three different stretches, i.e. upper, middle and lower. While Bhagirathi and Alaknanda both comprised upper and middle stretches, one in each tributary, the lower stretch was predominantly represented by the Ganga, i.e. downstream to Devprayag (Sangam) till Haridwar (Figure 1). The samples were carefully collected in triplicate from 32 different sites in sterile containers and transported on ice to the laboratory. Samples were collected during three seasons, i.e. winter, summer and monsoon from all selected sites.

The bacterial population used in the present work was isolated during a previous study. The water samples were subjected to microbiological analysis and bacteria were isolated on nutrient agar (total viable count) and other selective and differential media, including EC broth (HiMedia), and glucose azide broth (HiMedia). The pH of the medium and incubation temperature were adjusted according to the pH and temperature of respective sites of sample collection, unless mentioned otherwise. Isolates having different cell morphology and colony characteristics were selected and stored on nutrient agar slants at 4°C or -20°C as and when required. Medium with varying pH was also inoculated and processed as discussed above to obtain maximum culturable diversity from water samples.

Figure 1. Location map of the study area as the Gangetic river system of Uttarakhand.
Water samples were also inoculated in lactose broth tubes and incubated at 37°C for 48 h. Loop-full culture from tubes showing positive results was inoculated in eosin methylene blue (EMB) agar and Endo agar, and incubated at 37°C for 24 h. Lactose fermenting and non-fermenting colonies were isolated for further characterization. EMB (HiMedia) and KF streptococcal agar (HiMedia) were inoculated with loopful culture from tubes showing positive results and incubated at 45.5°C for 24 h for the isolation of faecal coliform and faecal streptococci respectively.

The isolates were identified using standard morphological, cultural, biochemical and physiological characteristics as described in Bergey’s Manual of Systematic Bacteriology. Selected isolates were subjected to 16S rDNA sequence analysis for establishment of their genotypic position. Briefly, pure cultures were grown in nutrient broth till log phase and genomic DNA was isolated according to Sambrook and Russel. Universal eubacterial primers 5′-(agagtttacgcttacct-3′) and 492R 5′-(ggttacctgtactgact-3′) were used for amplification of the 16S rRNA gene. A 50 μl reaction mixture included 100 ng of bacterial DNA as template, 1 mM of each primer, 1.5 U Taq DNA polymerase and 200 mM dNTPs. The reaction conditions were: initial denaturation for 3 min at 95°C, 35 cycles of denaturation for 1 min at 95°C, annealing at 56°C for 1 min, extension of 1 min at 72°C and a final extension of 10 min at 72°C. The 16S rDNA sequence was determined using ABI PRISM 377 DNA sequencer. Sequence analysis of these isolates was also performed using BLAST (blastn) search tool (http://www.ncbi.nlm.nih.gov). The 16S rDNA nucleotide sequences were submitted to GenBank (EU882823–EU882828, GU366194–GU366222 and GQ906350). Selected isolates were submitted in Microbial Type Culture Collection (IMTECH, Chandigarh), including Escherichia coli SA11C (MTCC 9541), Streptococcus thermophilus SA1S (MTCC 9540), Staphylococcus aureus RB13U1 (MTCC 9542), Shigella flexneri SB8U3 (MTCC 9543) and Klebsiella pneumoniae WB3T1 (MTCC 9544).

The number of species divided by the square root of the number of individuals results in species richness. The diversity of the culturable bacterial communities in each region was estimated using the Shannon diversity index:

\[ H' = -\sum_{i=1}^{s} p_i \ln p_i, \]

where \( H' \) is the species diversity index, \( s \) the number of species, and \( p_i \) the proportion of individuals of each species belonging to the \( i \)th species of the total number of individuals.

Evenness of species was also evaluated using the formula:

\[ \text{Evenness} = H / H_{\text{max}}. \]

A total of 43 different bacterial species were obtained, which were distributed in 24 genera. Of these, 31 species were isolated from Alakanda, 25 from Bhagirathi and 28 from Lower Ganga in Uttarakhand. Species richness index for the entire Ganga river system of Uttarakhand was found to be 1.5. Overall, E. coli presented the highest diversity index (0.883), followed by Enterobacter (0.718), Streptococcus faecalis (0.681) and Pseudomonas sp. (0.583) in the complete study area. E. coli was also the most prevalent organism in all three stretches with diversity index of 0.268, 0.293 and 0.322 respectively. Interestingly, higher diversity index was obtained for Lower Ganga compared to Alakanda and Bhagirathi, in spite of the fact that higher species abundance was obtained for the Alakanda river. Overall greater diversity (\( H' = 2.773 \)) was observed in the Alakanda river than in the Bhagirathi river (\( H' = 2.528 \)) and Lower Ganga (\( H' = 1.256 \)), which might be attributed to the large number of sampling areas and sites in the Alakanda region. Hafnia alvi, Deinococcus radophillus, Fratulosa auranti and Edwardsiella sp. were isolated in low frequencies from different stretches of the Ganga river system of Uttarakhand. These genera have not been previously isolated from the region.

In the Alakanda river system, diversity index of E. coli was followed by S. faecalis (0.25), Enterobacter sp. (0.225), Pseudomonas sp. (0.197), Bacillus sp. (0.184) and Proteus sp. (0.161; Figure 2a). It was invariably observed that the pathogenic genera constituted very high diversity index. Similarly, E. coli was again the most diverse representative in the Bhagirathi river with an index of 0.293 (Figure 2b). Interestingly, this value was higher than that obtained for the Alakanda river in spite of the fact that higher number of sampling sites was present in the Alakanda (16) than the Bhagirathi (9). The values for diversity index ranged from 0.293 for E. coli, followed by Enterobacter sp. (0.236), Bacillus sp. (0.218), S. faecalis (0.176) and Proteus sp. (0.16). The lowest value was 0.022 obtained for Serratia sp., Sarcina ventriculi, Salmonella typhimurium, Proteus vulgaris, Kurthia sp., Fratulosa auranti, Bacillus circulans and Azotobacter sp. Further, E. coli was the most diverse organism in the Lower Ganga too (Figure 2c). Interestingly, the highest value for diversity index, i.e. 0.322 in the case of E. coli was recorded in the Lower Ganga. Enterobacter sp. presented a diversity index of 0.257, followed by S. faecalis (0.255), S. aureus (0.24), Citrobacter sp. (0.165), Proteus sp. (0.137), and Pseudomonas sp. and Bacillus sp. with 0.12 each. Edwardsiella sp. and D. radophillus were isolated from the Lower Ganga; it was not previously reported in the Ganga river. Overall, the number of isolates obtained from the Lower Ganga was 205, which is less than that obtained from Alakanda (375) and Bhagirathi (219), but the highest values for diversity index for the respective genera were obtained in the case of the Lower Ganga. This suggests the contribution of anthropogenic activities as they are more prevalent in the Lower Ganga.

The highest values for evenness were recorded for E. coli – 2.69, 2.436 and 2.289 in the Lower Ganga, Alakanda and Bhagirathi respectively. The values of diversity index were found to be in proportion to the evenness, suggesting that the predominant genera were evenly distributed in the complete study area, whereas occasionally isolated genera were confined to specific sites. Density and distribution patterns of some prevalent genera were also determined. The three most frequent genera were E. coli, Enterobacter sp. and S. faecalis followed by Bacillus sp., Pseudomonas sp., Proteus sp. and Staphylococcus sp. Highest values were obtained in the lower stretch of the Ganga and that too particularly during rainy season. Higher values were obtained in the middle stretch of the Alakanda for E. coli, Enterobacter sp., S. faecalis, Pseudomonas sp. and Proteus sp. compared to the upper stretch of the Alakanda and both stretches of the Bhagirathi river. However, in the case of Bacillus sp., the highest values were obtained in the upper stretch of the
SCIENTIFIC CORRESPONDENCE

Bhagirathi, which varied from the general pattern observed.

The Himalayan region has been recognized as a hotspot for biodiversity, and therefore its microbial diversity also needs the attention of the scientific community. Changing global environment and pollution alter aquatic biota in the river water, sediments and aquifers connected to river basins. Among all aquatic microorganisms, freshwater microorganisms are rarely studied. As a result, available knowledge on them is limited\textsuperscript{15}. In the present study, \textit{E. coli} was observed to be the most diverse isolate and was evenly distributed in the entire stretch. In fact, it is known that the population present in high numbers in a particular habitat must be considered a living resident of that habitat, because only with growth it could achieve such dominance\textsuperscript{16}. Persistence of \textit{E. coli} over other bacterial genera highlights the anthropological pressure faced by the Ganges in Uttarakhand, supported by high value for diversity index (0.322) obtained for \textit{E. coli} in the Lower Ganga. Growth in the population, unchecked immigration, industrialization and unsustainable development of this region have affected the quality of Ganga water at these long stretches that are facing a risk of deterioration\textsuperscript{17}. In fact, during religious festivals millions of pilgrims take bath daily, affecting the natural microflora of this stretch of the Ganga.

Several other members of the family Enterobacteraceae were isolated, which are ubiquitous microorganisms found in the soil, water and vegetation, and are part of the normal intestinal microbiota of majority of animals and humans\textsuperscript{16}. \textit{Pseudomonas, Bacillus, Alcaligenes, Aeromonas, Sarcina, Deinococcus, Hafnia} and \textit{Lactobacillus} were amongst the heterotrophic genera isolated. Earlier, Halda-Alija and Johnston\textsuperscript{17} studied the diversity of culturable heterotrophic aerobic bacteria in pristine stream-bed sediments and found that Gram-negative bacteria comprised of most of the heterotrophic aerobic isolates (66.7%), which was similar to the findings in the Ganges of the Uttarakhand region. Further, Zwart \textit{et al.}\textsuperscript{18} described the habitat-specific clustering of freshwater bacteria, and coined the term ‘typical freshwater bacteria’ for these organisms. Many groups of typical freshwater bacteria have a cosmopolitan distribution\textsuperscript{19–20}, which is in accordance with the findings of the

Figure 2. Shannon diversity index of bacterial isolates from Alaknanda river (a); Bhagirathi river (b) and Lower Ganga (c).
present study. The bacterial genera predominating in the Ganges river system of Uttarakhand exhibited an even distribution pattern in the entire study area. Interestingly, most of these strains were pathogens, which are believed to have been introduced in the river water by anthropogenic activities. However, strains with low evenness value were isolated from specific sites in low numbers. For instance, Azotobacter sp. isolated only from Gangotri (Bhagirathi). Similarly, D. radophillus was isolated from Kodiyala (Lower Ganga), Fratrustia aurentia from Gangnani (Bhagirathi), Hafnia alvi from Shivpuri (Lower Ganga), Kurthia gibsonii from Hanumanchatti (Alaknanda) and S. ventriculii from Uttarkashi (Bhagirathi) only, with very low values of Shannon diversity index. This suggests that these strains were intrinsic and indigenous to that particular site of the river system.

Earl, Halda-Alia and Johnston observed that bacterial populations decreased in winter compared to summer conditions, and concluded that temperature influences the survival of bacteria during different seasons. Similar to their results, higher counts were obtained in summer season for E. coli, Enterobacter sp. and Pseudomonas sp. Also, higher counts of S. faecalis, Bacillus sp., Proteus sp. and Staphylococcus sp. were obtained during rainy season, which suggests the role of precipitation on the sources and extent of microbial pollution. Kistemann et al. observed that in the case of rainfall, the microbial loads of running water suddenly increase and reach reservoir bodies quickly, which might explain the increase in bacterial contamination from the upper to the lower stretches.

In this study the culture conditions were varied substantially to minimize the gap between actual diversity and that obtained using experimental approach. Earlier, Pontes et al. emphasized on the improvement in microbe culture techniques and combination of different methods to allow a more appropriate assessment of bacterial diversity. It is apparent that the literature available on bacterial diversity of the Ganges in Uttarakhand is scarce and mostly confined to pollution status and coliform counts. However, the prime objective of this work was to assess bacterial diversity on the basis of phenotypic and genomic properties.

The data provided here give an insight into the bacterial diversity, ecosystems stability and their role as pollution indicators in one of the most important freshwater ecosystems of the country, i.e. the Ganges in Uttarakhand. Further functional analyses are required for understanding the functional diversity and its possible exploitation for human welfare.


ACKNOWLEDGEMENTS. P.P., S.S. and A.S. thank the Uttarakhand State Council of Science and Technology for grant received (UCS&T/R&D/LS43/06-07/1173) to carry out this study and the Management of S.B.S.P.G.I., Dehradun for providing necessary research facilities. We also thank the Royal Life Sciences, Secunderabad for DNA sequencing services.

Received 11 January 2010; revised accepted 15 October 2010

ANCHAL SOOD1  
PYUSH PANDEY1*  
SANDEEP BISHI1  
SHIVESH SHARMA1  
MANJU P. GURAIN2  
O. P. GURAIN2

1Department of Microbiology, S.B.S. (P.G.) Institute of Biomedical Sciences and Research, Dehradun, 2Department of Zoology and Biotechnology, Hemvati Nandan Bahuguna Garhwal University, Srinagar-Garhwal 246 174, India
2*For correspondence. e-mail: pyushgkp@rediffmail.com