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Original Article

Metagenomic investigation of bacterial community of arsenic-prone area in the northwest region of Bangladesh

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Metagenomic analysis provides in-depth understanding of microbe mediated Arsenic (As) metabolism. The present study aims atmetagenomic analysis of the distribution, diversity, and abundance of bacteriome in arsenic affected groundwater and surrounding soils collected fromBogra district of Bangladesh. Metagenomic DNA was extracted from two groundwater samples BCW3 andBCW4 (As content10µgL^{-1} and 500 µgL^{-1}, respectively), and two tube-well surrounding soil samples BSS1 and BCS5 (As content335 µgkg^{-1} and 492 µgkg^{-1}),where As rich water flows off. Metagenomic analysis of six hypervariable regions of 16S rRNA gene resulted in a total of 788709 processed sequence reads and 5878 operational taxonomic units (OTUs). Bacterial richness, abundance and diversity (alpha and beta) were higher in BCW4 (85 genera) than BCW3 (19 genera) whereas both soil samples exhibited almost similar richness and diversity. Predominant genera in BCW3 were *Pseudomonas, Microbacterium, Achromobacter*whereas *Acinetobacter, Thiothrix, Stenotrophomonas, Paracoccus, Dechloromonas* dominated in BCW4. Soils were co-dominated with more than hundreds of genera with a high relative abundance of *Bacillus, Rhodoplanes*and *Pseudomonas*.Metagenomic investigation explored potentialarsenotrophicbacteriome. Exploring microbial community might help to understand the biogeochemistry of As affected groundwater and surrounding soil environment.

Keywords: Arsenic, 16S rRNAmetagenome, Groundwater, Soil, Bacterial community.

Introduction

Arsenic (As) contamination in groundwater and soil has become a serious problem to human health. Endemic arsenicosisis resulting from chronic drinking of As-contaminated groundwater[1], and an accumulation of As in rice grains is resulting from As-contaminated soils[2]. In Bangladesh, the high levels of As in groundwater as well as surrounding soil environments caused serious problem among the people of the region[3]. More than 60% of the ground-water in Bangladesh contains naturally occurring As, with concentration levels often exceeding 10 µgL^{-1} which is the maximum accepted concentration of As in drinking water recommended by WHO[4,5]. Food chain contamination and loss of crop yield might result from irrigation water contaminated with high levels of As. In Bangladesh,As concentration of groundwater is periodically monitored, but the irrigation soils are far less investigated and it is likely that the rapid spread of As might enter the irrigation soil and plant population affecting the food chain[6].

Bacteria has developed different strategies to transform arsenic including arsenite oxidation, arsenate reductionand arsenite methylation[7,8]. Though novel organisms isolation and exploring their properties could be done by cultivation-dependent studies, the cultivation-independentmethods offer a more comprehensive assessment ofmicrobial diversity[9]. Several studies have used traditional molecular methods such as denatured gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP) and clone library analysis to characterize microbial communities in Bangladesh[6,10-12].But traditional molecular methods are laborious, time consuming, costly and unable to provide in-depth analysis of the composition and diversity of the bacterial community.Therefore, a comprehensive census of the microbial communities ofAs contaminated groundwater and surrounding soil in Bangladesh is still lacking.

Next generation sequencing (NGS) technologies enable fast and high-throughput analysis at a reasonable cost. Since its recent availability, it enabled analysis of larger cohorts with increased sensitivity and depth. Metagenomics based on 16S rRNA gene libraries, generated through NGS can allow the full mapping of microbial communities directly from the environment or from biological samples to be characterized without the need to isolate and culture specific bacteria[13]. Therefore, it is likely that in-depth investigation of arsenotrophic bacteria would be possible without time consuming enrichment and cultivation as well as overcoming all the biasness of conventional culture based and molecular methods. The primary objective of this study was toexplore

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bacterial diversity and abundance in the contaminated environment.To achieve this objective,16S rRNA gene based metagenomicsstudy was performed using Ion Torrent technology. Furthermore, multiple hypervariableregions of 16S rRNA gene were investigated to obtain correlative and credible microbiome data.

Materials and methods

Sample collection

Bogra district, Bangladeshwas chosen based on available report related to high arsenic pollution, published in a national newspaper. The locations selected were Shibganj and Gabtaliupazila (Geographical coordinates 25.0006° N, 89.3203°E and 24.8824° N, 89.4482° E respectively). Distance of the two sample collection sites was around 18.5 Km. While collecting water samples,enough water was purged out to get the samples from the bottom of the tub-wells.Tube well water samples identified as BCW3, BCW4 and soil sample were identified as BCS5 and BSS1.Soil samples were collected from areas where As contaminated water from tube wells flows off.

Hydrological and geochemical parameters of the samples

Low range field arsenic test kit (Hatch, Loveland, Colorado, USA) was used to measure the total arsenic concentration in tube well water. The arsenic test strips provide results within a range of 0 - 500 μgL^{-1}. This reliable method recovers 100% of inorganic arsenic in samples and allows detection of arsenic levels in the field to assess compliance with the US Environmental Protection Agency (EPA) Arsenic Rule (EPA 816-F-01-004 January 2001). For soil arsenic measurement, the samples were digested following heating block digestion procedure[14] and the total As concentration were estimated by Atomic absorption Spectrophotometer (Perkin Elmer, Analyst 400) accompanied with hydride generation system. Chemical parameters of the tube well waters such as pH, conductivity, DO (dissolved oxygen), TDS (Total dissolved solid), total alkalinity and acidity, nitrate, nitrite, phosphorous as orthophosphate and sulphate concentration was determined according to the standard methods for examination of water and wastewater[15]. Soil pH was measured by Electrometric method with the help of a pH meter using combination Glass electrode. Chloride, fluoride, nitrate, phosphate, sulphate, bromide contents were estimated by methods described by APHA[16].

Metagenomic DNA extraction of soil and water samples

Total DNA from soil samples was extracted using PowerSoil® DNA Isolation Kit (Cat. 12888-100). DNA was extracted from the groundwater samples according toprotocols [17].

PCR amplification of 16S rRNAhypervariable regions and sequencing

Sixhypervariable regions (V2, V3, V4, V6-7, V8, and V9) of bacterial 16S rRNA gene were amplified using Ion 16S™ Metagenomics Kit (Thermo Fisher Scientific). The amplicons were processed to make the DNA library using Ion Plus Fragment Library Kit(Ion Torrent; Life Technologies, USA). Template preparation was done using the Ion OneTouch™ 2 System(Ion Torrent; Life Technologies, USA) and the IonPGM™ Hi-Q™ OT2 Kit(Ion Torrent; Life Technologies, USA) and sequenced on Ion PGM™ Systems(Ion Torrent; Life Technologies, USA).

Data processing

The fastq files were quality filtered using fastqfilter scriptfrom USEARCH suite (fastq_filter command).It discarded sequences shorter than 100bp or if it contained a Q score less than 20. Then the read IDs were editedand sample name was added as prefix of each sequence using fastx_relabel script. The four fasta files were merged for downstream analysis. Reads were de-replicated and sequences present less than 3 copy number were discarded using the UPARSE pipeline. OTU clusteringand chimera checking were performed using cluster_otus command (UPARSE). Reads were mapped back to the merged fasta file using USEARCH otutab command with a minimum identity of at least 97%. Taxonomy assignment was performed using QIIME (assign_taxonomy.py).

Forfamily level analysis, OTU counts were determined by summing over multiple OTUs assignedto the same family and for genus level analysis the same was performed by summing over multipleOTUs assigned to the same genus level. Any OTU not classified up to family and/orgenus level was labeled "Unassigned". The sequencing reads were deposited at the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) database under the study accession number PRJEB28492 (http://www.ebi.ac.uk/ena/data/view/PRJEB28492) and PRJEB28491 (http://www.ebi.ac.uk/ena/data/view/PRJEB28491) for groundwater and soil samples respectively.

Results

Groundwater chemistry

Between the two shallow groundwater samples, BCW3 was collected from higher depth than BCW4.But BCW3 had higher dissolved oxygen content and lower Asconcentration. Alkalinity was higher in BCW4 than BCW3.BCW3 had higher DO, total suspended solid, nitrite, sulphate and iron. Whereas BCW4 had higher alkalinity, total dissolved solid, pH, chloride ion and manganese (Table S1a and S1b).

Soil chemistry

Two soil samples BSS1 and BCS5 were collected from the surroundings of tube well where the Ascontaminated tube well water flows off. The As contents of these two tube-well water were 0.1 mg/L and 0.3 mg/L respectively. BSS1 soil sample contained0.33521 mg/kg of As and BCS5 contained 0.49233mg/kg of As. BCS5 had higher chloride and sulphate ion concentration. The nitrite ion in BCS5 was below detection level whereas BSS1 contained 5.557 mg/L of nitrite. Among the metal ions only chromium was detected in both of the soil samples, iron and manganese were not detected. The pH of BSS1 (8.69) was higher than BCS5 (pH 7.16) (Table S2).

Composition of the bacterial communities

From groundwater samples, total 280,716 reads were obtained after processing ion torrent sequencing data containing 2,184,775 sequences. The numbers of processed sequences were 62,362 and 218,354 from BCW3 and BCW4 respectively. 854 operational taxonomic units (OTU) were detected in these two groundwater samples (Table 1).

Table 1. *Diversity indices based on the 16S rRNA gene sequences from the four samples contaminated with different levels of arsenic. Shannon index- higher numbers represent higher levels of diversity.*

Sample ID	No. of sequences	Richness (No of OTUs)	Shannon index
BCW3	62362	186	5.140
BCW4	218354	808	6.916
BSS1	218673	4025	10.253
BCS5	289320	4471	10.574

From soil samples total 507,993 reads (218,673 reads from BSS1 and 289,320 reads from BCS5) were obtained by quality filtering and processing 2,400,017 raw sequences (Table 1). 5,024 operational taxonomic units were detected in these two soil samples.

Principle coordinate analysis (PCoA) of the 16S rRNA gene sequences was performed by QIIME todetermine the overall variation among bacterial communities (Figure1).The results showedthat bacterial community structures differed based on the amount of Aspresent.The PCoAplot explained 100% of the observed variation, with the first axis explaining 58.01% of thevariations, second axisexplained 35.24% of the variation and the third axis 6.71%.

Changes in the bacterial community composition

Figure2 summarizes the relative bacterial community abundance at the phylum level for each Groundwater and Soil samples. Proteobacteria was the dominant phylum for both of the water samples accounting for 86.46% in BCW3 and 87.8% in BCW4. Actinobacteria was the second dominant phylum in both samples representing 12.25% and 6.8% respectively. Firmicutes was detected in BCW3 and BCW4 comprising 0.03% and 1.79% respectively.15other phyla were detected in BCW4. Among them Bacteroidetes comprised 1.45% and others were less than 1%.

Total 35 phyla were detected in the two soil samples (BSS1 and BCS5). Proteobacteria was the dominant phylum for both of the soil samples accounting for 48.58% in BSS1 and 35.68% in BCS5 (Figure 2). Acidobacteria was the second dominant phylum in both samples representing 15.26% and 18.16% respectively. The subsequent dominant groups for BSS1 were Chlorobi, Planctomycetes, Actinobacteria, Nitrospirae, Gemmatimonadetes, Chloroflexi, Firmicutes, Bacteroidetes comprising 5.43, 5.23, 4.91, 3.4, 3.3, 2.98, 2.75% respectively. For BCS5 sample, subdominant groups were Actinobacteria, Gemmatimonadetes, Firmicutes, Nitrospirae, Chloroflexi, Chlorobi, Planctomycetes, Verrucomicrobia, WS3 (Latescibacteria) comprising 12.61, 6.72, 5.52, 4.41, 4.03, 3.61, 2.96, 1.26, 1.1% respectively (Figure 2).

In BCW3, Gammaproteobacteriacomprised 90.59% of the Proteobacteria, followed by Betaproteobacteria (5.34%) and Alphaproteobacteria (4.07%).In BCW4, Alphaproteobacteria comprised 42.78% of the Proteobacteria, followed by Gammaproteobacteria (38.25%) and Betaproteobacteria (17.93%). Other two classes Deltaproteobacteria and Zetaproteobacteria were also detected in BCW4 at less than 1% (Figure 3).

Alphaproteobacteria of BCW3 was dominated by Rhizobiales (99.72%), but BCW4 was dominated by Sphingomonadales (71.96%) followed by Rhodobacteriales (12.1%), Rhizobiales (10.63%) and two other classes (Figure 4A).

In both the soil samples abundance of Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Deltaproteobacteria was in the range of 20 to 30% of

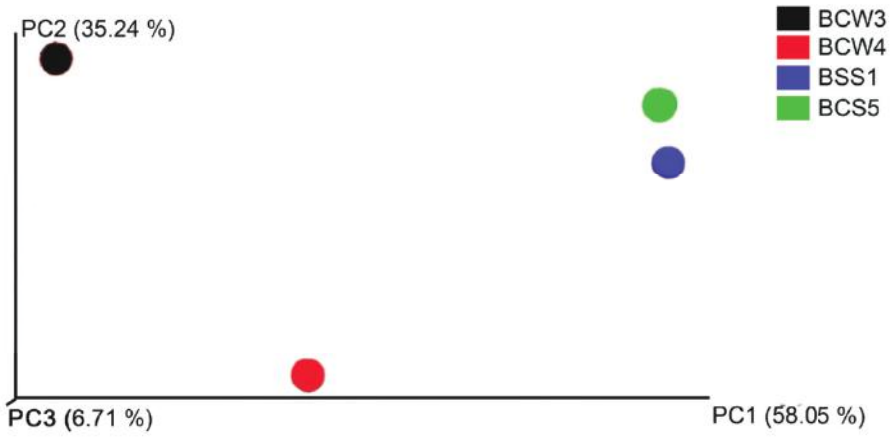

Fig. 1. *Principle coordinate analysis (PCoA) plot showing differences in bacterial community structure.*

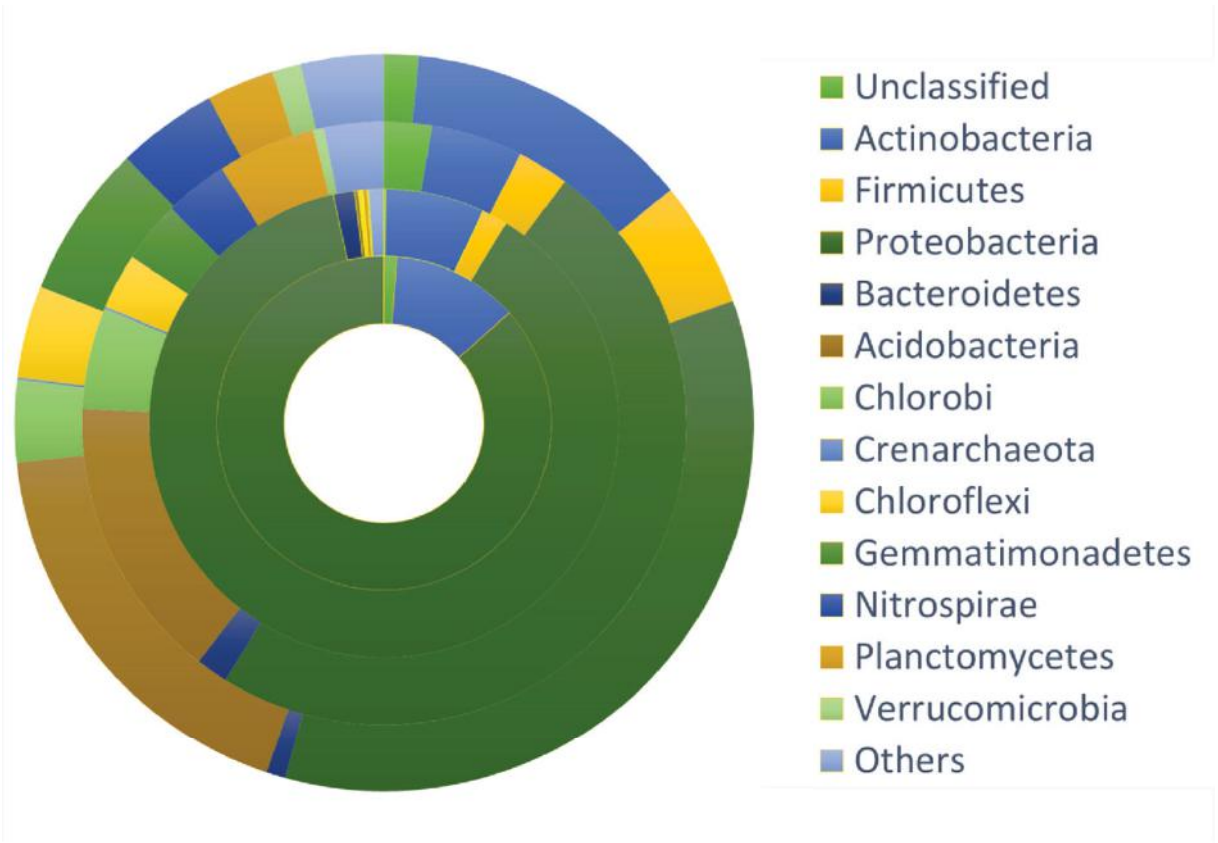

Fig. 2. *Composition of microbial communities in the two groundwater and two soil samples at the phylum level. Circles from inside out corresponds to the BCW3, BCW4, BSS1 and BCS5 respectively.*

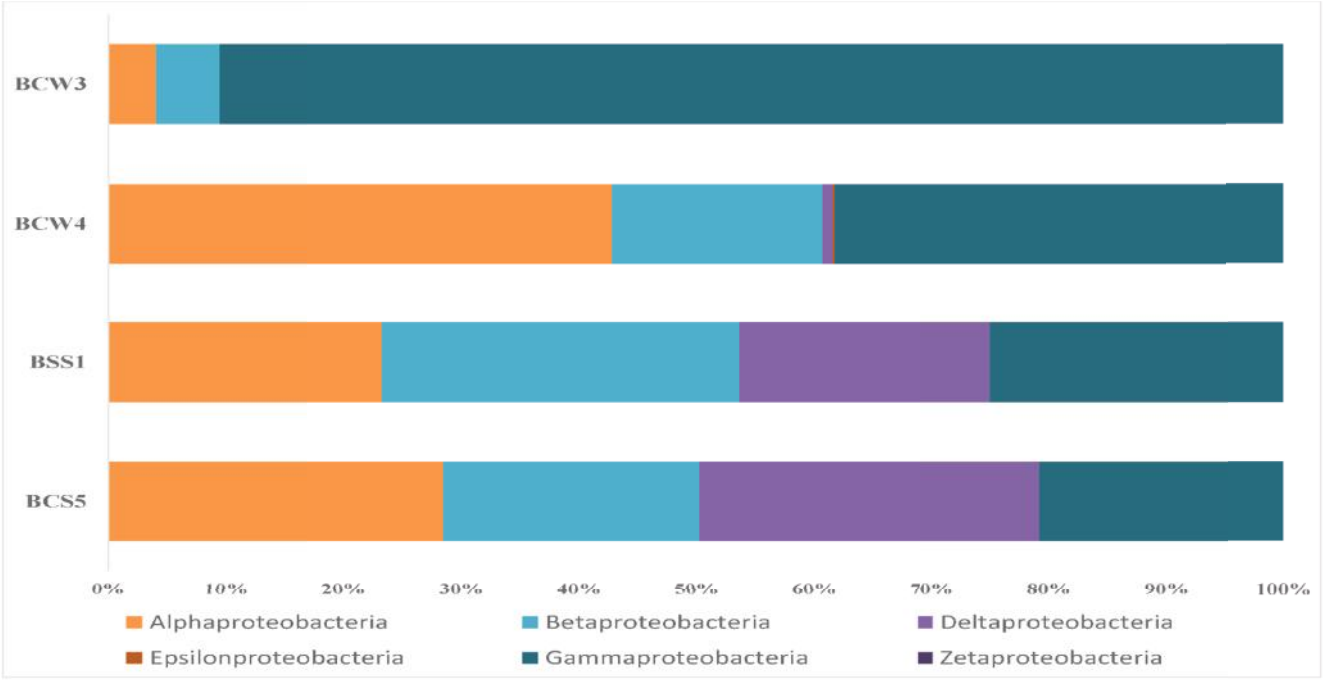

Fig. 3. Relative abundance of the Proteobacteria community composition in the water and soil samples.

Proteobacteria. Percentage of Alphaproteobacteria and Deltaproteobacteria increased in BCS5 compared to BSS1 while percentage of Betaproteobacteria and Gammaproteobacteria decreased.

Within Gammaproteobacteria, three taxa were identified in BCW3, Pseudomonadales (69.82%), Enterobacteriales (15.85%), Xanthomonadales (14.33%) whereas 9 taxa were identified in BCW4-Pseudomonadales (48.89%), Thiotrichales (31.45%), Xanthomonadales (11.38%) and other with8.27% dominance (Figure 4B).

Only Burkholderiales was detected in Betaproteobacteria of BCW3. Though Burkholderiales (71.72%) was dominant of Betaproteobacteria in BCW4 but 6 other orders (28.28%) were detected.

Total 133 orders were detected (BSS1- 110, BCS5- 118) among them 95 were shared by both of the two soil samples. 15 were found only in BSS1 accounting for less than 0.5% of the total sequences. 23 were present only in BCS5 accounting for about 0.75% of the total sequences.

At the family level, a total of 84 families (TableS3) were obtained- 18 in BCW3 and 80 in BCW4. 14 families including Pseudomonadaceae, Microbacteriaceae, Enterobacteriaceae, Xanthomonadaceae, Alcaligenaceae, Methylobacteriaceae, Burkholderiaceae, Comamonadaceae, Methylocystaceae, Hyphomicrobiaceae, Oxalobacteraceae, Rhizobiaceae, Phyllobacteriaceae, Paenibacillaceae, Aurantimonadaceae, Bradyrhizobiaceae, Brucellaceae were shared by both of the water samples. There were 4 families (Paenibacillaceae, Methylocystaceae, Xanthobacteraceae, Burkholderiaceae)that appeared in only BCW3 sampleand accounted for 3.97% of the sequences. There were 66 families including Erythrobacteraceae, Moraxellaceae, Thiotrichaceae, Rhodobacteraceae, Rhodocyclaceae, Intrasporangiaceae, Dietziaceae, Bacillaceae, Sphingomonadaceae, Alteromonadaceae and other with less than 1% abundance each appeared in only BCW4 sample and accounted for 64.55% of the sequences.

At the family level, a total of 141 families were obtained- 117 in BSS1 and 125 in BCS5. 101 families including Syntrophobacteraceae, Sinobacteraceae, Hyphomicrobiaceae, 0319-6A21, Rhodospirillaceae, Bacillaceae, NB1-I, A4b, RB40, Pseudomonadaceae, Hyphomonadaceae were shared by both of the soil samples. There were 16 families (Euzebyaceae, Nitriliruptoraceae, Armatimonadaceae, Sphingobacteriaceae, Enterococcaceae, Lactobacillaceae, Streptococcaceae, Peptostreptococcaceae, Tissierellaceae, Rhodobacteraceae, Rickettsiaceae, Erythrobacteraceae, OM27, Marinicellaceae, Spirochaetaceae, CV106) that appeared in only BSS1 samples and accounted for 0.57% of the sequences. There were 24 families including Solibacteraceae, Geobacteraceae and many others appeared in only BCS5 sample and accounted for 0.775% of the sequences.

Families with more than 1% abundance in the four samples (a total of 29 families) were compared (Figure 5A).

93 genera were detected in the two water samples- 19 in BCW3 and 85 in BCW4. Among the 93 genera, 8 were present only in BCW3 including *Paenibacillus, Pleomorphomonas, Labrys, Burkholderia, Comamonas, Delftia, Herbaspirillum, Erwinia* accounting for 1.83% of total abundance, 74 were only in BCW4 including *Acinetobacter, Thiothrix, Paracoccus, Dietzia, Dechloromonas* and others with less than 1% abundance each.

99 genera were detected in the two soil samples- 73 in BSS1 and 78 in BCS5. Among the 99 genera, 21 were present only in BSS1 including *Pseudonocardia, Nonomuraea, Euzebya, Fimbriimonas, Sporocytophaga, Sphingobacterium, Haliscomenobacter, Brevibacillus, Sporosarcina, Enterococcus, Lactobacillus, Lactococcus, Rhodobacter, Novosphingobium,*

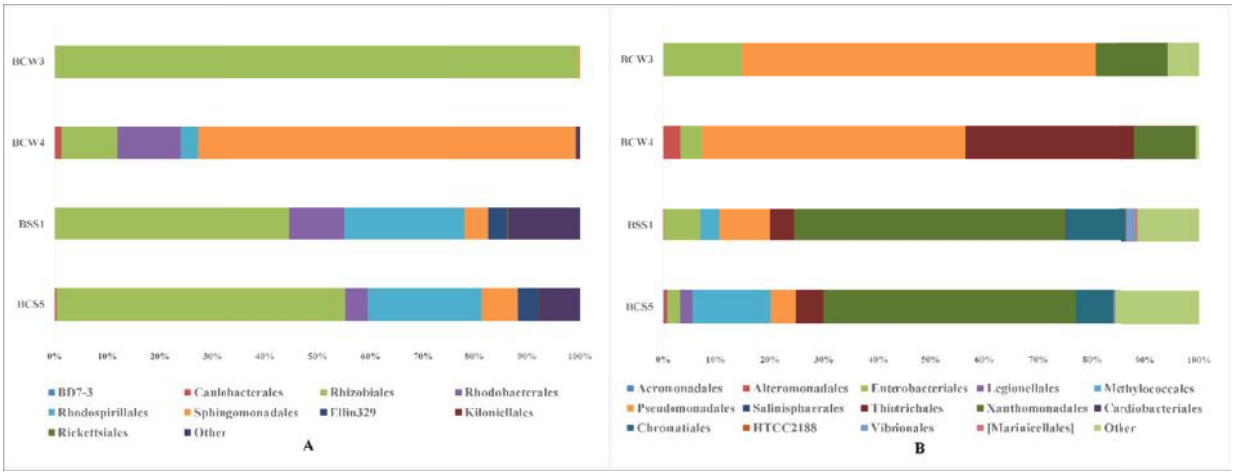

Fig. 4. *Relative abundance of the Alphaproteobacteria (A) and Gammaproteobacteria (B) community composition in the two groundwater samples.*

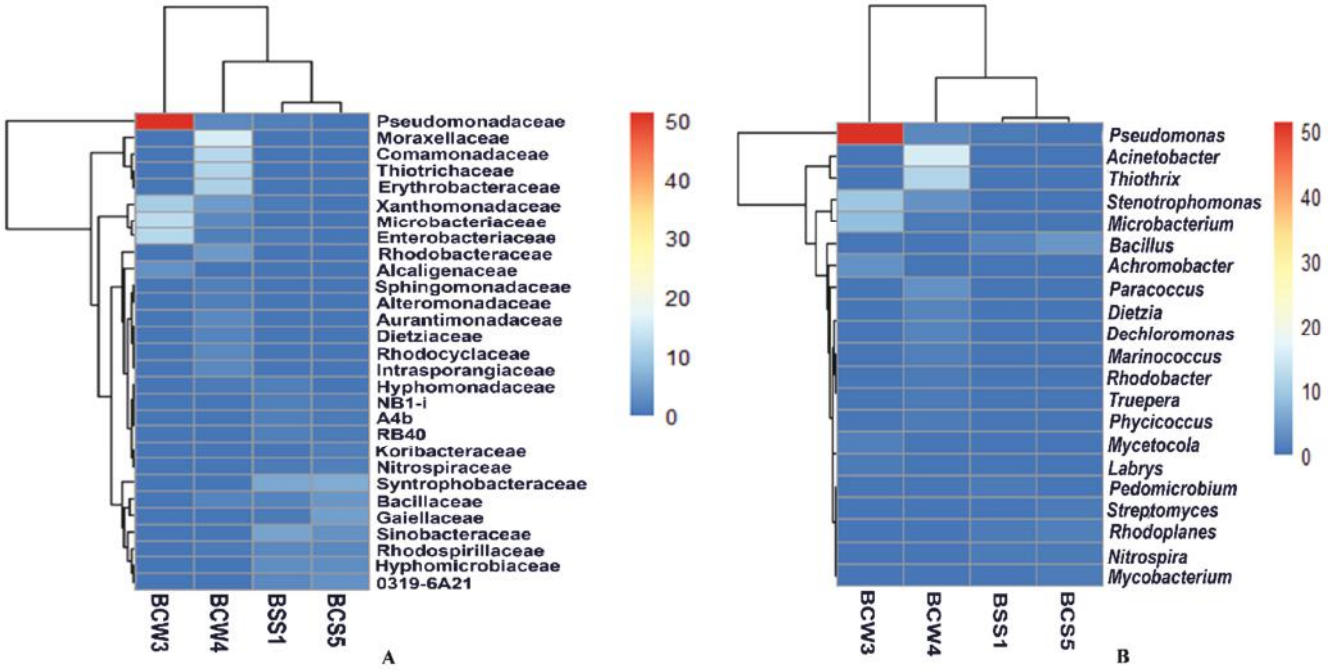

Fig. 5. *Heatmap of the most abundant families (A) and genera (B). Families with more than 1% abundance and genera with more than 0.5% abundance in each samples were selected and their abundances were compared to those in other samples. The color intensity in each cell shows the percentage of a family and genus in a sample.*

Achromobacter, Nannocystis, CandidatusEntotheonella, Morganella, Serratia, Acinetobacter, Opitutus accounting for 0.42% of total abundance, 26 were only in BCS5 including *CandidatusNitrososphaera, CandidatusSolibacter, Virgisporangium, Amycolatopsis, Kitasatospora, Sphaerisporangium, Conexibacter, Flavisolibacter, Alicyclobacillus, Cohnella, Symbiobacterium, 4-29, JG37-AG-70, Methylosinus, Sphingomonas, Roseateles, Thiobacillus, Chromobacterium, Vogesella, Hydrogenophilus, Geobacter, Anaeromyxobacter, Phaselicystis, Arcobacter, Allochromatium, Methylomicrobium* with less than 0.99% abundance.

Discussion

Arsenic acts as one of the key environmental factors that contribute to the difference in the microbial community structure in both groundwater and soil environments[18]. Many studies suggest that there is a strong effect of As on microbial community. One study reported change in the soil microbial community due to long term arsenic contamination and thus provide selective advantages to resistant species[19]. Metagenomicsinvestigation was done to carry out an in-depth analysis of the bacterial diversity in As-prone groundwater and in nearby soil where Ascontaminated water drainsout.

The two soil samples used in this study got continuous supply of As through contaminated tub-well water. Richness and diversity of the microbial communities in the soil samples were higher than the groundwater samples. The dominant microbial communitywere also different between groundwater and soil samples. This difference might be due to the difference in geochemical conditions.

The species richness (Table 1) was higher in BCW4 than BCW3 andalso higher in BCS5 than BSS1 soil sample. At each taxonomic level higher diversity was observed. In both of these two samples As concentration were higher than their groundwater and soil counterparts. The high arsenic containing water sample BCW4 was characterized by low sulphate and nitrite which is consistent with previous studies[20]. Sultana *et al.* also described higher diversity of bacterial community in the high-arsenic containing groundwater of the shallow aquifer than low As groundwater of higher depth which is consistent with present investigation[21].

The predominant groups detected using the Ion Torrent sequencing approach in this study were consistent with previous results derived from traditional sequencing methods[20-22]. However, the relative abundances of the dominant population were different.

Groundwater sample BCW3 was dominated by *Pseudomonas* genus which covered more than 50% of all organisms. It was also the dominating bacteria found in low arsenic groundwater samplesin Hetao Basin ofInner Mongolia, China[20].*Pseudomonas* is an aerobic bacteria known for its metabolic diversity and can tolerate a variety of physical conditions. *Pseudomonas* was reported as both arsenite oxidizing[23], arsenate reducing[24]. Of the total community composition, *Stenotrophomonas* genus comprised 9.8% of the bacterial population. *Stenotrophomonas*

was previously reported as aarsenite oxidizing isolate from low arsenic containing soil[25]. *Microbacterium* genus formed the 3rd abundant genus comprising 8.97% of the community. *Microbacterium*was previously reported as both As(III) oxidizing[26] and As(V) reducing[27]. It was also reported demethylating both methylarsonic acid and methylarsonous acid to mixtures of arsenate and arsenite[28]. *Achromobacter (3.3%)* has been reported as arsenite oxidizing carrying multiple metalloid transporter a genomic arsenic island[29].

The dominant families/genera found in high As containing groundwater, *Acinetobater (14.17%)*, Comamonadaceae (11.12%), *Thiothrix* (10.56%), *Paracoccus* (2.97%), *Stenotrophomonas* (3.2%), *Dechloromonas* (1.59%), *Pseudomonas* (2.24%) were reported in previous studies[10,17,18,30-32]. But Erythrobacteraceae (18.22%) were not detected previously.

In contrastvery high bacterial diversitywith lower relative abundance was observed in soil samples. This might be due to the availability of diverse and abundant nutrients in soil that helps to thrive a diverse microbes. The soil bacteria also got continuous supply of high concentration of arsenic from the flowing tube-well water. Soils with long-term arsenic contamination may result in the evolution of highly diverse arsenite-resistant bacteria and such diversity was probably caused in part by horizontal gene transfer events[33].This higher diversity is due to the adaptation of resident microbial communities to metal or metalloid stress[34]. Thus they develop intrinsic mechanisms to As tolerance/ resistance. Sanyal*et al.* reported *Pseudomonas, Bacillus* from As contaminated soil through culture technique[10].

These diverse bacteria detected in high As containing groundwater and surrounding soil are involved in transformation of different As species including oxido-reduction which lead to precipitation, solubilization, and adsorption, desorption processes[35]. Thus they play a key role in As biogeochemical cycling.

The present study provides valuable insight intothebacterial communityof As affected groundwater and soil which previously has not been explored with the traditional molecular techniques. Expanding the current understanding of the bacterial composition of samples with contrasting As level, It might provide valuable insights about microbial community structure of low and high arsenic groundwater and surrounding soil of tube-well with high arsenic containing water.

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Conflict of interest

The authors declare that they have no conflict of interest.

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