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Molecular Study of Indigenous Bacterial Community Composition on Exposure to Soil Arsenic Concentration Gradient

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Abstract

Community structure of bacteria present in arsenic contaminated agricultural soil was studied with qPCR (quantitative PCR) and DGGE (Denaturing Gradient Gel Electrophoresis) as an indicator of extreme stresses. Copy number of six common bacterial taxa (*Acidobacteria*, *Actinobacteria*, *α-*, *β-* and *γ-Proteobacteria*, *Firmicutes*) was calculated using group specific primers of 16S rDNA. It revealed that soil contaminated with low concentration of arsenic was dominated by both *Actinobacteria* and *Proteobacteria* but a shift towards *Proteobacteria* was observed with increasing arsenic concentration, and number of *Actinobacteria* eventually decreases. PCA (Principle Component Analysis) plot of bacterial community composition indicated a distinct resemblance among high arsenic content samples, while low arsenic content samples remained separated from others. Cluster analysis of soil parameters identifies three clusters, each of them was related to the arsenic content. Further, cluster analysis of 16S rDNA based DGGE fingerprint markedly distributed the soil bacterial populations into low (< 10 ppm) and high (> 10 ppm) arsenic content subgroups. Following analysis of diversity indices shows significant variation in bacterial community structure. MDS (Multi Dimensional Scaling) plot revealed distinction in the distribution of each sample denoting variation in bacterial diversity. Phylogenetic sequence analysis of fragments excised from DGGE gel revealed the presence of *γ-Proteobacteria* group across the study sites. Collectively, our experiments indicated that gradient of arsenic contamination affected the shape of the soil bacterial population by significant structural shift.

Key words: agricultural fields, arsenic (As), bacterial community, DGGE, copy number, qPCR

Introduction

Arsenic is one of the most common toxic metalloid, present in ground water as well as in soil (Xiong *et al.*, 2010; Majumder *et al.*, 2013), and has become a significant problem to environment (Banerjee *et al.*, 2011; Ghodsi *et al.*, 2011). Although arsenic intoxication of humans through drinking water is of the primary concern (Bachate *et al.*, 2009), soil is the secondary source and transfers arsenic to the edible part of the crops (Heikens *et al.*, 2007). Agricultural fields are frequently irrigated with arsenic contaminated ground water and hence a route of arsenic contamination into the food chain was established (Ghosh *et al.*, 2014; Shrivastava *et al.*, 2014). In India and Bangladesh, around 105.000 km^2 fertile deltaic plains are widely reported to have high levels of arsenic (Mukherjee and Bhattacharya, 2001). This region is largely used for the various types of agriculture, seasonal crops and irrigated with arsenic rich ground water creating major environmental threat (Bhattacharya *et al.*, 2002; Guha Mazumder 2003). The impact of environmental contamination on microbial community composition and diversity is being increasingly considered as highly sensitive ecological parameters to provide baseline information about contamination (Dhal *et al.*, 2011). Therefore it is really important to elucidate the diverse population of microorganisms associated with arsenic mobility and transport, as they can be the sensitive indicators for contaminant stress. A follow-up study identified and reported different soil microorganisms to be resistant to lethal concentration of arsenic and linked the progressive arsenic removal to bacterial activities, chiefly their arsenic (III) oxidizing activity (Aksornchu *et al.*, 2008; Mallick *et al.*, 2014). Alternatively, the toxic and bioavailable forms of the metals often affect adversely the diversity and function of indigenous microorganisms inhabiting the environment (Sobolev and Begonia, 2008; Dhal *et al.*, 2011). In order to understand the soil microbial ecology and the biological

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processes occurring *in situ*, multiple studies should be performed to determine the bacterial diversity in the soil and sediment, with high risk of arsenic contamination or already contaminated. The real status of microbial community cannot be reflected by traditional culture methods. Most of the soil microorganisms are uncultivable under the standard laboratory conditions as they are well adapted to their environment (Vartoukian *et al*., 2010). Several studies have revealed that more than 99% of microorganisms present in their innate environment are not readily cultivable and they represent only a minor fraction, usually less than 1% of the whole diversity present in complex environmental samples, such as soil (Sharma *et al*., 2005; Felczykowska *et al*., 2015). Nowadays, prevailing molecular tools endure investigators to frame microbial community function and structure without cultivation at increasingly finer resolution (Mccaig *et al*., 1999; Fakruddin and Mannan, 2013). Development of cultivation-independent techniques can circumvent such pitfalls and permit access to the genomes of entire communities that extend our knowledge about the diversity, ecology, evolution and functioning of the microbial world in considerably more detailed and accurate manner (Ranjard *et al*., 2000; Pogacic *et al*., 2010).

The study of soil microbial communities has strongly advanced with the introduction of sophisticated molecular techniques (Fakruddin and Mannan, 2013). In this study, we have assessed the effect of arsenic contamination on soil bacterial community structure using molecular techniques. qPCR was used to quantify the abundance of the major bacterial groups in the soil environment and to determine any changes that occur in the diversity of these bacterial assemblies at varying amount of arsenic contamination of the selected sampling sites. Furthermore, the effect of arsenic contamination on total bacterial genomic diversity was estimated through 16S rDNA based PCR-DGGE analysis where predominant DGGE bands were further identified by extraction of the band from a gel and sequencing. Although investigation of bacterial communities and its phylogenetic analysis using 16S rDNA signature sequence in arsenic contaminated aquifers were made previously (Bachate *et al*., 2009; Paul *et al*., 2015; Goswami *et al*., 2015), only a few studies using molecular approach have been carried out for arsenic contaminated soil in our study area. According to Hossain *et al*., 2005, arsenic toxicity in the agricultural fields of West Bengal is the major challenge and suitable remediation schemes are still unsuccessful. The study on bacterial community composition from arsenic contaminated soil may provide better understanding about potential biomarkers of this metal contamination as well as may implement suitable and effective potentials for bioremediation.

Experimental

Materials and Methods

Study site and soil sampling. The soil samples were collected from the five different agricultural fields of Gontra village (23° 1' N, 88° 34' E) in Chakdaha, Nadia, West Bengal, India by composite soil sampling and labeled as VS, PS, TS, JS and AS respectively. From each site, three subsamples were collected from 10–15 cm depth in a 2 m^2 area and transported to the laboratory in sterile polyethylene bags at 4°C. All the soil samples were analyzed separately for the physicochemical properties and average data collected from three subsamples were used to establish the physical and chemical characteristics of the particular site. For microbiological work, subsamples were mixed and used as representative sample after clearing root debris from the soil and stored at –20°C. Bacterial community analysis was performed within a week after sampling.

Physical and chemical analysis of soil samples. Soil samples were analysed for a variety of physical and chemical parameters. Total nitrogen and organic carbon content were measured by Macro-Kjeldahl method (Bremner, 1965) and Walkley-Black (Jackson, 1973) method, respectively. Soil pH was determined in suspension of soil in water (1 : 2.5) using pH meter. The same suspension was used to measure electrical conductivity (EC) using a direct reading conductivity meter. Cation exchange capacity was measured by extracting the soil with buffered $BaCl_2$ (Dewis and Freitas, 1984). Particle size distribution was determined by the International Pipette Method (Piper, 1966). Water holding capacity was analysed as described by Black (Black, 1965). Available phosphate (P_2O_5) was determined by the Bray and Kurtz method (Bray and Kurtz, 1945) and potash (K_2O) by using neutral molar ammonium acetate (Hanway and Heidel, 1952). Total arsenic content of the soil samples were determined by atomic absorption spectrophotometer (GBC Scientific Equipment Ltd., Model: GBC 932 B Plus) as mentioned elsewhere (Blas and Mateos, 1996).

Soil DNA extraction. Microbial genomic DNA was extracted from 0.5 g soil sample collected at each agricultural field using commercial kit and following the protocol described by the manufacturer (SoilMaster™ DNA Extraction Kit-Epicentre). DNA were dissolved in 50 µl TE buffer and stored at –20°C until processing. DNA quality was checked by electrophoresis in 0.8% horizontal agarose gel. Band pattern was observed using Gel Doc System (Bangalore Genei, Bangalore, India) after staining with 0.5 µg/ml ethidium bromide solution. Additionally, purity and yield of the extracted DNA were also analysed using a Nanodrop 2000 spectrophotometer (Thermo Scientific). One µl of DNA

solution was taken to determine the quantity, protein contamination ($A_{260/280}$) and co-extraction of other organic acids, mainly humic acid ($A_{260/230}$).

Soil bacterial community analysis by group specific qPCR assay. An abundance of specific phyla/class of bacteria (*Acidobacteria*, *Actinobacteria*, *Proteobacteria- α*, *β*, *γ* and *Firmicutes*) in the selected soil samples were quantified by qPCR assay using taxon-specific primers for 16S rRNA gene as reported by Philippot *et al.* (2011). These PCR primers were also tested previously for the detailed assessments of complex bacterial communities (Fierer *et al.*, 2005; Muhling *et al.*, 2008). Here, we have used SYBR Green dye for quantification as it is described as reliable method for detecting nucleic acid targets (Olioso *et al.*, 2007). The extracted genomic DNA was used as a template in the assay to detect the copy number of taxon-specific 16S rRNA gene present in the soil samples. Individual qPCR reaction was carried out for each bacterial taxa in a real time PCR system (MyiQ2, BioRad). The qPCR reactions were performed in 96 well plate as described previously (Islam and Sar, 2011) with some modification. Each 20 µl reaction mixture contained 4 µl template DNA, 0.5 µl of each forward and reverse primer (concentration: 10 pmol/µl), 10 µl 2 × master mix containing SYBR Green (BioRad) and 5 µl nuclease free water. The reaction conditions were as follows: 5 min at 95°C; 40 cycles of 20 s at 95°C and 30 s at 60°C. The quantification was based on increasing fluorescence intensity of the SYBR Green dye during amplification. The qPCR assays were performed in triplicate with unknown samples, no template control (nuclease free water), and series of plasmid standard in 10 fold dilutions.

Known concentration of plasmid standard containing single copy of 16S rRNA gene (~ 1.5 kb size) of specific bacterial taxa cloned into pGEM-T vector was a base to construct each standard curve. The threshold cycle values (Ct values) were subsequently used to calculate the target copy numbers of 16S rRNA genes in each of plasmid standards using the standard equation (Lee *et al.*, 2008). A linear regression line was obtained by plotting the logarithm of copy numbers of plasmid standards (X-axis) against the corresponding threshold cycle values (Y-axis). The quality of the standard curve was derived from the slope and the correlation coefficient (r). Targeted 16S rRNA gene copy numbers of various bacterial groups of unknown samples were calculated from the respective standard curve. Correlation between arsenic concentration and the copy number of bacterial groups in soil was also performed.

Bio-geochemical data analysis. The relation among the samples with reference to their physicochemical characteristics was determined using between-group linkage (UPGMA) method of hierarchical clustering analysis with SPSS software (21.0 version). A 2D Principal Component Analysis (PCA) was also performed to correlate the samples with respect to their bacterial community compositions. In order to correlate bacterial community composition with the soil chemical properties, correlation coefficient analysis were applied.

Denaturing gradient gel electrophoresis (DGGE) profiling of bacterial communities. DGGE specific primers, 63F (5'-CAGGCCTAACACATGCAAGTC-3') with 40 bases GC clamp at 5'end and 518R (5'-ATTACCGCGGCTGCTGG-3') were selected to produce a 495 bp fragment for the PCR amplification of V2 and V3 regions of 16S rRNA gene (Fantroussi *et al.*, 1999; Breugelmans *et al.*, 2007). Prior to DGGE analysis touchdown PCR for each soil DNA extracts were performed in 20 µl final volume reaction containing 1 µl of extracted DNA, 2 µl of 10 × PCR buffer, 0.5 µl of dNTP mix (10 mM), 1 µl of each primer (10 pmol/µl), 0.1 µl Taq DNA polymerase (5 U/µl, KAPA Biosystems) and 14.4 µl nuclease-free water. Amplification was carried out in a thermal cycler (MyiQ2, BioRad) with reaction conditions as follows: 5 min at 94°C; 10 cycles of 94°C for 30 s, 60–55°C step down for 30 s, 72°C for 30 s; 20 cycles of 94°C for 20 s, 55°C for 30 s and 72°C for 30 s; final extension was at 72°C for 7 min and then 4°C storage (Schabereiter-Gurtner *et al.*, 2003; Yu and Morrison, 2004). The PCR products were checked by 1% agarose gel electrophoresis.

DGGE analysis of PCR amplicons was carried out essentially as described previously with slight modifications (Smalla *et al.*, 2001). The DGGE gel contained 0.5 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8) and 8% (w/v) acrylamide gel with a linear denaturant gradient from 40% to 60% made of urea and formamide (100% denaturant contains 7 M urea and 40% (v/v) formamide) using a DCode System (Bio-Rad, Munich, Germany). Equal amounts of amplified PCR products were loaded onto 1 mm thick vertical gels and electrophoresis was carried at a constant temperature of 60°C, with 150 V for 8 h. After completion of electrophoresis, gel was stained in an ethidium bromide solution (0.5 µg/ml) for 10 min followed by distaining in distilled water for 20 min. The gel band images were visualized using Chemi Doc System (Bio-Rad, Munich, Germany).

Sequencing, phylogenetic analysis and nucleotide accession number. The DNA fragments to be sequenced were excised from DGGE gel, placed in sterilized vials with 20 µl nuclease free water and kept overnight at 4°C. The eluted DNA was used as template for re-amplification using the primers 63F (without GC clamp) and 518R. PCR products were purified and cloned into pTZ57R/T vector (InsTAclone PCR Cloning Kit, Thermo Scientific) and the resulting plasmids were used as templates for sequencing reactions. At least one positive clone from each selected DGGE bands

was sequenced. The sequences were aligned using the CLUSTAL W program (Thompson *et al*., 1994) to the 16S rDNA sequences from the database of the National Center for Biotechnology Information using a BLAST search (Altschul *et al*., 1997) and deposited in NCBI Genbank database under accession numbers KJ136644, KJ136645, KJ403747, KJ403748, KJ403749, KJ403750 and KJ403751. The sequences from DGGE profiling were compared with known sequences listed in the GenBank nucleotide sequence database. The BLAST search of NCBI database was used to find the evolutionary relationship of the sequences. A phylogenetic tree was constructed using MEGA 5.1 by neighbor-joining method (Saitou and Nei, 1987).

Analysis of DGGE banding patterns. DGGE fingerprints were interpreted by cluster analysis, MDS and diversity indices estimation. DGGE image was first digitized and analyzed using Quantity One software (version 4.65, BioRad, USA) in order to compare the fingerprint patterns. Assuming each band in a lane as a single unique phylotype (operational taxonomic unit/OTU), the band number, band intensity and relative position of each band were determined to estimate the richness value (Huang *et al*., 2013). Identification of DGGE bands was based on the magnified image, absorption peak and similarity in each lane. Band analysis was performed using the rolling disk method and setting background subtraction at 15 (Alele *et al*., 2014). Bands with intensity < 0.05 were excluded from the analysis. Unweighted pair-group methods with arithmetic mean (UPGMA) trees were generated using Quantity One software with Sorensen's similarity index. Based on the presence or absence of individual bands in each lane, a binary matrix was constructed. The binary data representing the banding patterns were used to generate a pair wise Dice distance matrix. A dendogram was generated using UPGMA cluster analysis of Quantity One software. For constructing a MDS diagram, the distance matrix was used, where each DGGE fingerprint was placed as one point in respect with a two dimensional map with artificial X and Y axis. The diagram represented similar samples plotted together. The MDS analysis was performed using SPSS software (21.0 version). In order to correlate the DGGE fingerprinting analysis with multiple aspects, the Shannon-Weaver index of diversity (*H*) (Hedrick *et al*., 2000) and equitability index (*E*) (Smit *et al*., 2001) were calculated.

Results and Discussion

Soil characteristics of the study site. The long term and continuous use of arsenic contaminated ground water for irrigation purpose in agricultural fields is a threat to various life forms in the study site. The

Table I
Physical and chemical characteristics of the soil samples (Mean and SE are shown for each soil samples)

Serial Number	Soil type	Moisture content (g)	Particle size distribution			Moisture holding capacity %	Cation exchange capacity [cmol (p^+) kg^{-1}]	Amount of total arsenic in soil (ppm)	Soil pH	Carbon and organic matter content %	Electrical conductivity (d Sm^{-1}); (1:2.5)	Total nitrogen (kg ha^{-1})	Available P_2O_5 (kg ha^{-1})	Available K_2O (kg ha^{-1})
			Sand %	Silt %	Clay %									
VS	Clay loam	0.82±0.01	30.8±0.47	36.0±0.17	33.2±0.2	44.40±0.22	8.45±0.04	6.65±0.04	6.93±0.07	0.36±0.007	0.22±0.004	692±2.73	27.2±0.22	182±2.03
PS	Clay loam	0.63±0.02	26.8±0.5	40.0±0.09	33.2±0.25	46.07±0.57	10.2±0.18	8.25±0.03	6.42±0.12	0.3±0.008	0.14±0.002	494±2.65	30.5±0.15	150±5.52
TS	Clay loam	0.50±0.008	23.1±0.12	41.2±0.22	35.7±0.19	74.51±0.18	13.4±0.15	21.01±0.020	6.96±0.02	0.42±0.003	0.32±0.01	568±2.6	35.7±0.35	193±0.9
JS	Sandy clay loam	0.65±0.02	52.8±0.25	20.0±0.2	27.2±0.22	46.34±0.14	10.2±0.15	23.03±0.02	6.9±0.03	0.47±0.02	0.45±0.01	448±0.9	35.2±0.19	176±0.9
AS	Sandy loam	0.53±0.02	53.5±0.5	34.2±0.15	12.3±0.18	43.60±0.19	5.7±0.09	56.67±0.1	6.55±0.04	0.27±0.006	0.31±0.008	324±0.9	26.2±0.2	124±5.37

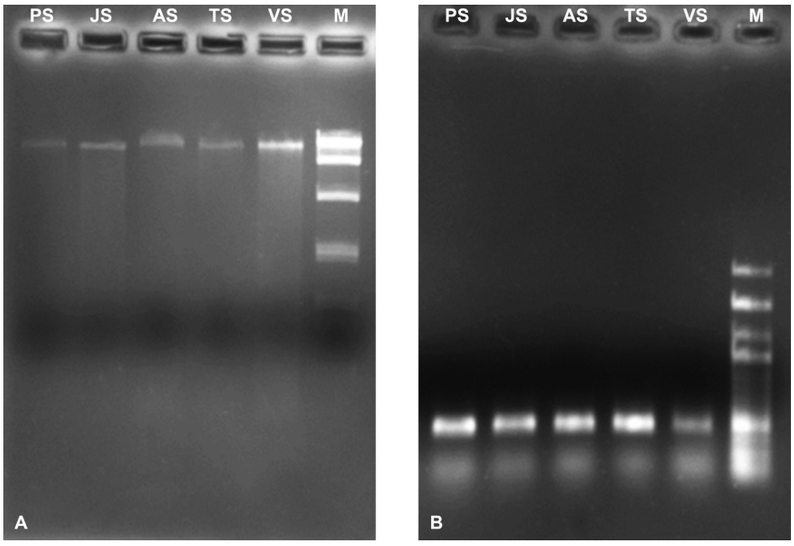

Fig. 1. (A) Agarose gel electrophoresis image of bacterial genomic DNA isolated from arsenic contaminated soil samples. M indicates molecular weight marker (lamda phage DNA/*Hin*dIII digest) and (B) Agarose gel image of PCR amplicons. M indicates 100 bp DNA Ladder.

physical and chemical properties of the soil samples measured in this study are listed in Table I. Soil texture ranged from sandy loam to clay loam with pH close to neutral (6.42–6.96). We found that there are no significant differences in the chosen parameters of the five different agricultural soils including soil type. It confirms that the present irrigation system of the agricultural lands did not affect the general properties of soils. On the contrary, the arsenic contamination varies significantly among these sampling sites. The total arsenic contents ranged from 6.65 to 56.67 ppm creating a gradient of arsenic contamination. Thus, the observed trend of physicochemical characteristics of the selected sites is a key finding of our study. From Table I, it is clear that the selected soil parameters will not influence the microbial community as there is no such variation except soil arsenic content, which may affect the microbial community structure. In our study, it was observed that the maximum arsenic content in agricultural fields of Gontra village in Chakdaha is 56.67 ppm, which is much above the maximum acceptable limit *i.e.* 20 mg per kg as limited by the European Commission (Rahman *et al*., 2007; Bhattacharya *et al*., 2009). Based on our current study it could be concluded that due to continuous and long term irrigation by arsenic contaminated ground water, arsenic content of the studied area is above alarm limit.

Soil DNA extraction. The cultivation independent evaluation of microbial diversity has been mainly based on the extraction of total DNA from environmental samples (Yuan *et al*., 2012). Here, we successfully obtained detectable amount of DNA from soil using commercial kit as shown on agarose gel (Fig. 1A). Agarose gel electrophoresis revealed the yield of high molecular weight DNA approximately 10 kb in the total soil DNA extracts. DNA concentration of the soil samples ranged between 9.0 ng/µl to 11.1 ng/µl as measured by nanodrop (Table II). The $A_{260/280}$ (> 1.7) and $A_{260/230}$ (> 1.9) of extracted DNA indicate minimum protein and other organic contaminations respectively.

Soil bacterial community analysis by group specific qPCR. The qPCR has been used for rapid and reliable quantification of 16S rDNA copy number. Investigation reports suggested that the *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Firmicutes* etc. are the most common phyla of soil bacterial communities (Fierer *et al*., 2005; Muhling *et al*., 2008; Philippot *et al*., 2011). The qPCR data demonstrated that the separate

Table II
Spectrophotometric measurement of extracted DNA from soil obtained by Nanodrop 2000 spcetrophotometer (Thermo scientific)

Sample Name	DNA Concentration (ng/µl)[a]	$A_{260/280}$ [b]	$A_{260/230}$ [c]
VS	9.7	1.74	1.99
PS	9.0	1.70	2.01
TS	11.1	1.77	1.97
JS	9.8	1.73	1.99
AS	9.7	1.77	1.98

[a] Calculated based upon A_{260} against a standard response curve.
[b] $A_{260/280}$, Ratio of A_{260} to A_{280} (a high ratio [> 1.7] is indicative of pure DNA, whereas a low ratio is indicative of protein contamination).
[c] $A_{260/230}$, Ratio of A_{260} to A_{230} (a high ratio [> 2] is indicative of pure DNA, whereas a low ratio is indicative of phenolic and humic acid contamination).

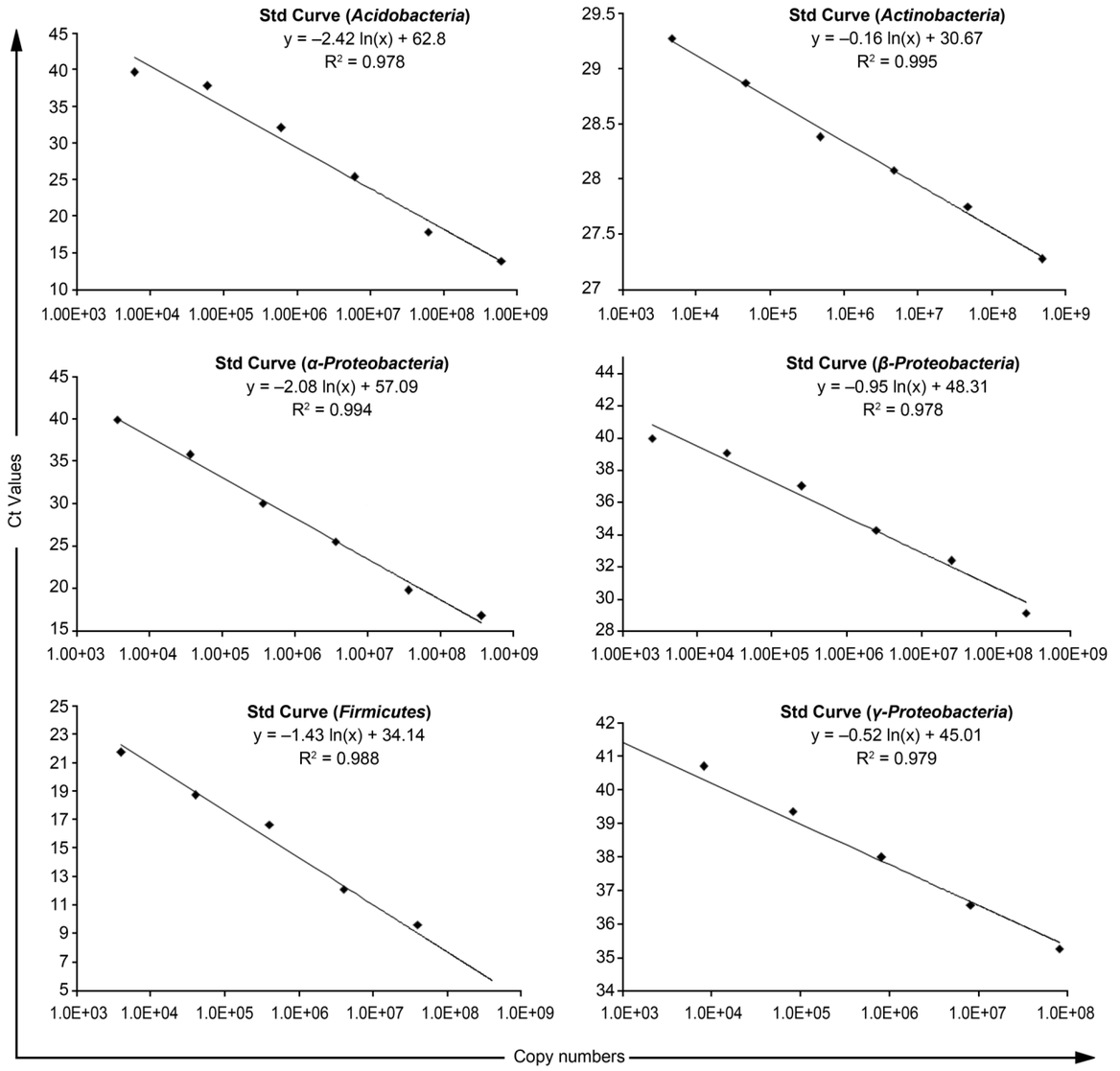

Fig. 2. qPCR standard curves for 16S rRNA gene of different bacterial taxa.

peaks and different threshold values (Ct values) were related to the standard samples. Non-specific amplification was not detected. Copy numbers of the standard samples were calculated and a linear standard curve was generated for each set of qPCR analysis (Fig. 2). Amplification efficiency was calculated from the slops to be ≥ 0.97. Finally, the copy number of the 16S rRNA gene of six targeted bacterial groups in the extracted soil DNA was detected by putting the Ct values in the equation derived from the standard curve. We used the qPCR data to estimate the relative abundance of above mentioned bacterial groups in the five distinct agricultural fields. It was observed that *Actinobacteria* and *Proteobacteria* represented the highest copy number and thus constituted the major bacterial taxa of the selected agricultural fields, whereas *Acidobacteria* and *Firmicutes* with lower copy numbers were estimated as subdominants (Table III). Comparative analysis of the bacterial community composition among the contaminated sites revealed a shift in a relative abundance of dominant group towards increasing arsenic contamination. At lower concentration of arsenic at contaminated sites (< 10 ppm) *Actinobacteria* was the most abundant phylum whereas in the case of higher arsenic contamination (> 10 ppm) the difference in their abundance at phylum level was noted (Fig. 3). Thus this study underlies the fact that the copy number of *Actinobacteria* gradually decreases with increasing concentration of arsenic, on the contrary to the growth of the *Proteobacteria* copy number. Our study also revealed that arsenic contamination influenced the resident microbial communities and dominant group might represent the major arsenic

Table III
The copy number of the bacterial taxa specific 16S rDNA present in the soil samples.

Soil samples	Acidobacteria (μl^{-1})	Actinobacteria (μl^{-1})	Firmicutes (μl^{-1})	α-Proteobacteria (μl^{-1})	β-Proteobacteria (μl^{-1})	γ-Proteobacteria (μl^{-1})
VS	2.41×10^{5}	2.33×10^{9}	9.43×10^{3}	1.48×10^{5}	2.74×10^{8}	8.81×10^{7}
PS	1.16×10^{5}	4.27×10^{8}	4.59×10^{3}	1.79×10^{5}	3.34×10^{8}	8.24×10^{8}
TS	1.75×10^{5}	4.54×10^{8}	4.25×10^{3}	1.41×10^{5}	2.76×10^{9}	3.18×10^{9}
JS	1.74×10^{5}	1.88×10^{7}	2.29×10^{3}	1.89×10^{4}	2.39×10^{9}	1.04×10^{9}
AS	5.95×10^{4}	7.28×10^{5}	2.34×10^{3}	1.11×10^{4}	7.19×10^{9}	5.83×10^{9}

Table IV
Simple linear correlation coefficients relating the abundance of bacterial groups in the different agricultural fields to the varying level of arsenic contamination and other soil parameters (See Table I for soil properties).

Bacterial Groups	Total Arsenic	Total Nitrogen	Carbon & organic matter	Soil pH	Available P_2O_5	Available K_2O
Actinobacteria	–0.94**	0.92**	0.38	0.27	0.27	0.72
Acidobacteria	0.81*	0.88*	0.73	0.63	0.44	0.90**
Firmicutes	–0.72	0.91**	–0.01	0.12	–0.28	0.43
α-Proteobacteria	–0.84*	0.80*	0.12	0.03	0.14	0.53
β-Proteobacteria	0.62	–0.82*	0.02	–0.13	0.41	–0.31
γ-Proteobacteria	0.76	–0.79	–0.14	–0.11	0.41	–0.38

*Significant values ($P < 0.05$) and ** highly significant values ($P < 0.001$) as determined by correlation coefficient analysis.

resistant indicator groups for effective remediation strategies. Thus, the different studies identified *Actinobacteria* as a major heavy metal resistant microorganism in soil (Schmidt *et al*., 2005; Baz *et al*., 2015), however some other reported them as minor group (Sheik *et al*., 2012). In present scenario, it was important to understand the effect of arsenic contamination on the resident soil microbial communities to make effective remediation strategies. Therefore, phylum level shift may give a significant indication suggesting *Proteobacteria* as a major arsenic tolerant organism. Previous studies indicated that *Proteobacteria* are capable for metal transformation (Gillan *et al*., 2005; Cai *et al*., 2009).

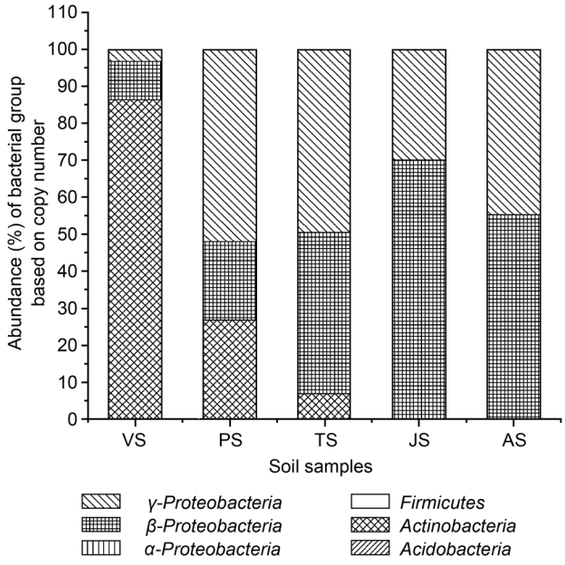

Fig. 3. Relative abundance of major taxonomic groups across the sampling sites representing bacterial community shifting towards *Proteobacteria* with increasing soil arsenic contamination.

Bio-geochemical data analysis. Correlation coefficient analysis revealed a significant relationship between the bacterial population structure and soil arsenic contamination. *Actinobacteria*, *Acidobacteria*, *α-Proteobacteria* and *Firmicutes* displayed significant negative correlation but *β-Proteobacteria* and *γ-Proteobacteria* were positively correlated with soil arsenic contamination. Other soil parameters were also correlated with the bacterial abundance, showing negligible correlation, except total nitrogen content (Table IV). Relations between the samples were assessed separately in respect to their physicochemical properties and bacterial community composition (Fig. 4). The PCA of bacterial community composition among the studied samples were performed (Fig. 4A), where the two principal components (principal component 1 and principal component 2) represented 91.3% of variation among the five soil samples. Principal component plot indicated a distinct resemblance among all the samples collected from high arsenic soil content while PS and VS with low arsenic content remained separated from other clusters. Between-group linkage cluster method

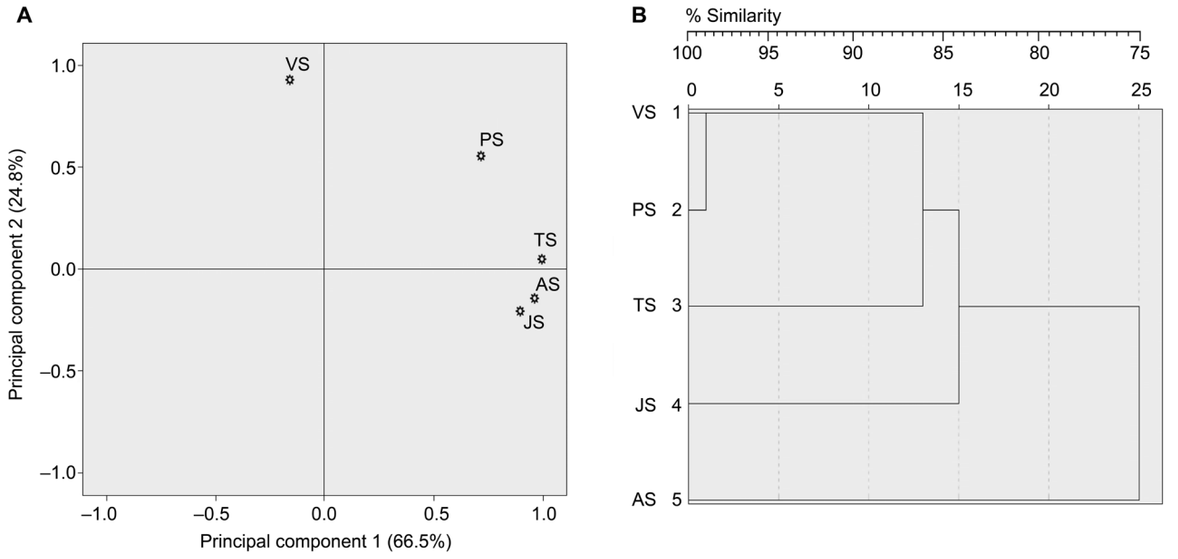

Fig. 4. Relationship among biogeochemical factors of five different soil samples. (A) PCA plot based on bacterial community composition and (B) hierarchical cluster analysis of geochemical data.

(UPGMA) was applied and the measurement was performed on Euclidean distance for the analysis on soil characteristics. The cluster analysis distingushed three clusters, each of which are related to the arsenic concentration of studied samples (Fig. 4B). VS and PS indicated 99% similarity while AS with highest arsenic content remain separated from the other samples showing positive relation with 75% similarities with JS, TS cluster (81.5%). In summary, it was shown that arsenic contamination greatly affects the microbial population.

DGGE profiling of arsenic contaminated soil bacterial communities, diversity estimations and phylogenetic analysis. Bacterial community structure of the defined arsenic contaminated agricultural soils was investigated by PCR-DGGE method based on band intensity and position. The touchdown PCR was performed to avoid nonspecific primer binding and further amplification (Maiwore *et al*., 2012). Agarose gel electrophoresis of PCR amplified products (Fig. 1B) suggested that the extracted DNA was as a good substrate for the amplification of bacterial 16S rDNA fragments. The DGGE image depicted the number and intensity of migrating bands of the DNA profiles from all samples (Fig. 5A) and provided a fingerprint of the microbial community found in soil samples indicating high diversity of 16S rRNA genotypes. The bands from DGGE profile corresponded to the 495 bp 16S rDNA fragments with different nucleotide sequences represented variation in dominant microbial populations in the community. The DGGE profiling showed a large number of bands in case of PS and AS but low number of bands appeared in analysis of VS site. A total of 50 different band positions were detected in the DGGE gel image of the five different soil samples. The number of bands per lane varied from 34 to 40. Thirty seven DGGE bands were very similar in the analyzed samples indicating the presence of a large number of equally abundant ribotypes. The other bands were unique to particular sampling sites suggesting that the samples had different dominant bacterial population.

After the analysis of the digitized image with the Quantity One software it was observed that some bands dominated in samples from particular sites as showed by high band intensity whereas bands in the other samples were always faint. DGGE banding patterns showed that similarity indices varied significantly, suggesting genetic diversity with distinction among the studied samples. The Quantity One software generated dendrogram revealed similarity of PCR-DGGE fingerprints from the gel on the basis of visual comparison. Cluster analysis showed that the samples fell into two groups and correlated with the arsenic concentration of the collection sites (Fig. 5B). The first cluster included samples from VS and PS sites of arsenic contamination below 10 ppm while the second cluster consisted of samples from TS, JS and AS sites of > 10 ppm arsenic contamination. MDS analysis was performed to investigate the changes in the bacterial community structure due to arsenic contamination and illustrated the similarity of all possible pairs of each gel track. The two dimensional MDS plot pointed out that the soil samples containing bacterial community were not grouped together, suggesting significant variations in the community structure (Fig. 6A). Beside these studies, we also compared the bacterial communities of the different samples by calculating the Shannon diversity

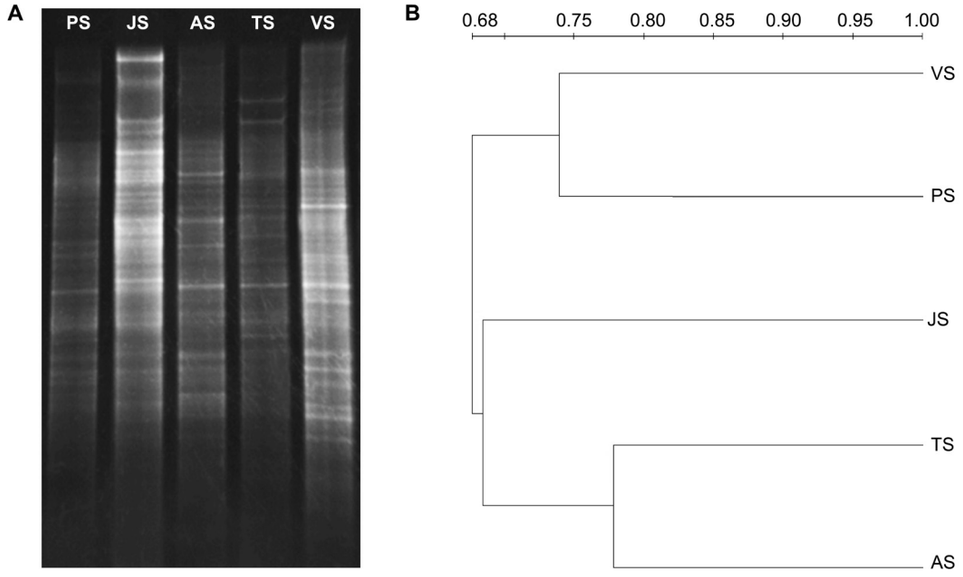

Fig. 5. (A) DGGE fingerprints of PCR-amplified 16S rRNA gene fragments of bacterial communities from five different soil samples and (B) Quantity One generated UPGMA dendogram of DGGE profile showing two clusters: the first cluster (VS and PS) having arsenic contamination < 10 ppm while the second cluster (TS, JS and AS) having arsenic contamination > 10 ppm.

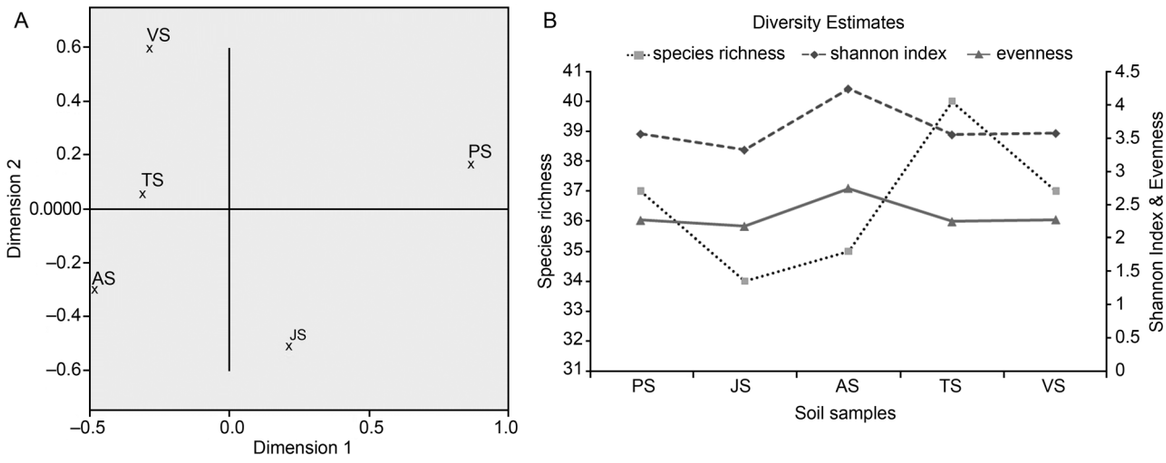

Fig. 6. (A) Two-dimensional MDS plot obtained from DGGE profile representing significant variation in the bacterial community structure of different soil samples and (B) Comparative representation of diversity estimates calculated from DGGE profile.

index (*H*), based on the DGGE banding patterns of the soil samples. The two diversity indices; species richness (*S*) and species evenness (*E*) were determined for each sample. The ability to quantify diversity in this way was then shown to be an important tool for biologists trying to understand community structure (Moura *et al*., 2009). The Shannon diversity index (*H*) has often been discussed in the analysis of DGGE fingerprints (Moura *et al*., 2009; Gafan *et al*., 2005). *H* values differed significantly ranging from 3.32 to 4.24, indicating the difference in bacterial community structures across the samples. When analyzed by Shannon index the highest value ($H = 4.24$) was found in the case of the highest arsenic contaminated soil indicating high diversity in the bacterial community. Simultaneously the bacterial diversity was low in less arsenic contaminated soils according to Shannon index. The observed data of calculated *H* index and its correlation with arsenic content of the soils showed significant positive value (0.81) at $P < 0.05$ level. Moreover, the indices for evenness of the bacterial community were also significantly different among the soils and showed high values ranging from 2.17 to 2.74. Soil bacterial diversity, as estimated by phylotype richness and diversity varied across the arsenic contaminated agricultural fields. Comparison of all diversity estimates is shown in Fig. 6B indicating

Table V
Identification of DGGE bands by 16S rDNA sequencing.

Excised Band No.	Isolation Source	No. of nucleotide compared	Identification	Closest type strain and accession code		Identity	Our GenBank Accession number
G14	Soil	454	Uncultured *Aeromonas* sp.	Aeromonas sp.	KP716703.1	99%	KJ136644
G15	Soil	455	Uncultured *Aeromonas* sp.	*Aeromonas* sp.	KP716703.1	99%	KJ136645
G16	Soil	443	Uncultured *Acinetobacter* sp.	*Acinetobacter* sp.	KT003253.1	97%	KJ403747
G2	Soil	451	Uncultured *Klebsiella* sp.	*Klebsiella oxytoca*	HQ683969.1	99%	KJ403748
G3	Soil	464	Uncultured *Acinetobacter* sp.	*Acinetobacter calcoaceticus*	KM585587.1	99%	KJ403749
G1	Soil	454	Uncultured *Klebsiella* sp.	*Klebsiella oxytoca*	KC462193.1	99%	KJ403750
G4	Soil	456	Uncultured *Aeromonas* sp.	*Aeromonas media*	HF937047.1	99%	KJ403751

distinct bacterial diversity among the samples. By analyzing the DGGE banding patterns using the Shannon index of diversity in combination with the evenness and species richness of the soils, we were able to monitor a whole range of community responses from all the contaminated soil samples.

Among all major bands, seven bands with distinct migration distance in the DGGE gel were successfully sequenced. The bands were selected on the basis of their intensity as well as availability in the study sites. The 16S rDNA sequences of all isolates were subjected to nucleotide BLAST and the bacteria were classified according to their similarity to sequences in the GenBank database. The sequences were derived from unculturable organisms and were representing *Aeromonas* sp. (strain KJ136644, strain KJ403751 and strain KJ136645), *Acinetobacter* sp. (strains KJ403749 and KJ403747), *Klebsiella* sp. (strain KJ403748 and strain KJ403750) (Table V). Phylogenetic analysis revealed that all the isolates belonged to *γ-Proteobacteria*. The organisms belonging to the genera of *Aeromonas*, *Acinetobacter* and *Klebsiella* were previously reported as arsenic resistant and arsenic accumulating organisms and found to grow effectively at more than 100 ppm arsenic (Anyanwu and Ugwu, 2010). The genus *Acinetobacter* is broadly represented as arsenic resistant and isolated from various arsenic contaminated sites exhibiting arsenite oxidase activity (Achour *et al*., 2007). *Klebsiella* was reported as highly resistant to arsenic and able to survive even at high arsenic concentration by converting arsenite into less toxic form arsenate (Singh, 2011). Other reports also showed the presence of the genera *Aeromonas* among arsenic resistant bacteria (Anyanwu and Ugwu, 2010). Our findings re-established these representative species as the valuable indicators of the arsenic contaminated soils and confirm that these arsenic resistant or accumulating bacteria are widespread in the polluted environment. A phylogenetic tree was constructed (Fig. 7) using the nucleotide sequences of major DGGE bands and related sequences obtained from the DNA database. A bootstrap analysis was performed and values greater than 50% were indicated. Phylogenetic analysis of these sequences suggested that they were deeply branching

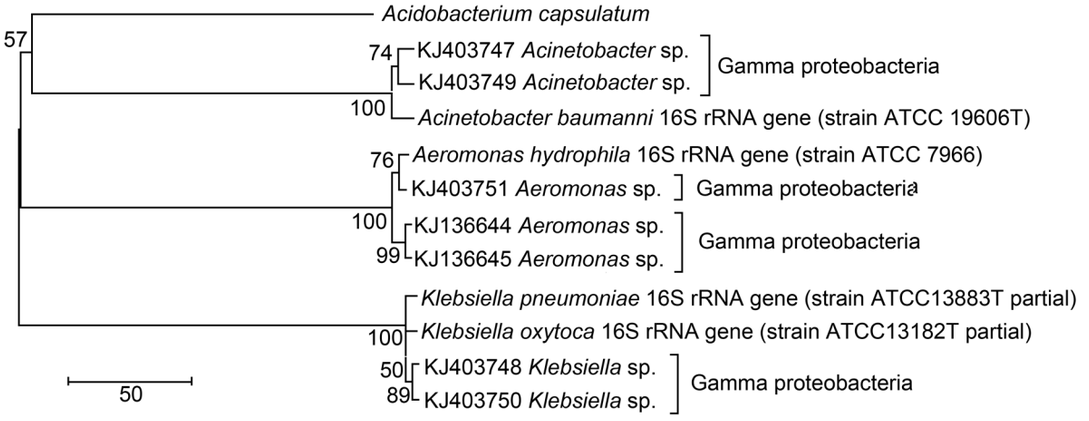

Fig. 7. Phylogentic tree based on 16S rRNA gene sequences of bands excised from DGGE gel and their closet type strains using neighbor joining method. Bootstrap values of > 50% are mentioned at the nodes.

members of the *γ* subclass of the *Proteobacteria* and grouped within a tight phylogenetic cluster. Moreover a significant correlation among bacterial copy number detection and DGGE analysis was observed. Here the combined approach of DGGE and PCR became a promising area of research denoting fluctuations at bacterial phylum level due to arsenic contamination. The consistency between the inferences made by both the molecular approaches again establishes the effect of arsenic contamination on community profiling. All the data in combination provide an indication of the nature of the bacterial communities under arsenic stress and supply important information to microbial ecology. It is therefore reflecting bacterial diversity of soils exposed to arsenic contamination.

Conclusion

In this study we utilized the culture independent techniques to gain a better understanding of the impact of arsenic on soil bacterial community. Overall study indicated significant structural variations between bacterial taxonomic groups throughout the arsenic affected sampling areas. It provides the knowledge of the distribution of bacterial community structure in such contaminated environment. The qPCR data suggested that *Proteobacteria* were the dominant group of the contaminated soils irrespective of the soil type and soil character. Therefore it can be assumed that most of the arsenite oxidizing or arsenate reducing bacteria present in soil may belong to these groups. The bacterial composition of soil contaminated with highly concentrated arsenic significantly differs from that of low arsenic concentration. *Actinobacteria* were mostly present in low arsenic containing regions but bacterial community structure of the selected soils shifted from *Actinobacteria* to *Proteobacteria* with increasing arsenic level. DGGE profiling of soil samples further confirmed the presence of bacteria (*Aeromonas* sp., *Acinetobacter* sp., *Klebsiella* sp.) belonging to *γ-Proteobacteria*. Furthermore, the diversity analysis indicated a diverse bacterial population in the soils as well as high positive correlation with arsenic content and bacterial diversity of the studied area. The observed trend of *Actinobacteria* to be highly sensitive to arsenic concentration indicates the possibility that it may be the stress determinant to monitor arsenic contamination. While dominance of *γ-Proteobacteria* at high arsenic contaminated areas makes it possible to apply this observation to enhance the bioremediation of arsenic contaminated sites. The findings reported here improve the knowledge about the abundance and patterns of soil bacterial community in arsenic contaminated agricultural fields that will enrich the future studies of soil ecology.

Conflict of Interest

The authors declare no conflict of interest.

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