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Research article

Diversity of nitrogen-fixing bacteria in agricultural field soil along the Yamuna River, Delhi, India

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Abstract

Nitrogen-fixing bacteria play an important role in sustaining soil health. The diversity was studied in nitrogen-fixing bacteria using the *nif*H gene along the Yamuna River in Delhi-National Capital Region. The *nif*H gene is a part of the *nif* regulon, which codes for an Fe-protein of the nitrogenase enzyme complex, responsible for the reduction of dinitrogen (N_2) to ammonia (NH_3) during biological nitrogen fixation. Soil samples were collected from agricultural fields near the Yamuna River. Terminal restriction fragment length polymorphism analysis of soil DNA using the TReFID software package showed community comprising different nitrogen-fixing bacterial phyla, dominated by the *Proteobacteria* (26.87%) and *Actinobacteria* (2.9%). The relative abundance of these two phyla increased downstream along the river while the relative abundance of the *Cyanobacteria* decreased. The maximum overall microbial diversity index was lower downstream and during the summer season. It was concluded that the seasonal variation in temperature and moisture and the increases in the pollution of the water directly affected the microbial diversity as well as the relative abundance of the nitrogen-fixing bacterial population in agricultural fields along the Yamuna River.

Introduction

Nitrogen is one of the most abundant elements in the earth's atmosphere; however, more than 78.08% of the total nitrogen is in the form of nitrogen gas which is unavailable to living organisms (Wagner, 2011). Living organisms can use nitrogen in the form of ammonium or nitrate ions (Wagner, 2011). The process of converting atmospheric nitrogen gas into these ions is known as nitrogen fixation and it occurs either through atmospheric or biological processes, with biological nitrogen fixation being carried out by nitrogen-fixing microbes such as bacteria and algae (Wagner, 2011). Nitrogen-fixing bacteria are responsible for 90% of total biological nitrogen fixation, with the *Cyanobacteria* being the most important bacterial community (Georgiadis et al., 1992).

Biological nitrogen fixation by bacteria is carried out using a nitrogenase enzyme which is a complex of two major dinitrogenase proteins (the MoFe- and Fe-proteins) coded by the *nif*HDK gene (Georgiadis et al., 1992). Among these genes, *nif*H is the most conserved and codes for the Fe-protein of the nitrogenase enzyme complex (Georgiadis et al., 1992). It is one of the oldest and most thoroughly studied functional genes; it is easily amplified by a universal set of primers and has an extensive collection of sequences obtained from both cultured and uncultured organisms, being widely used in independent cultural studies to determine the potential nitrogen capacity of the soil (Zehr and Turner, 2001).

The Yamuna River is one of the most important rivers in India, with a number of important cities depending on its river water for their basic requirements, including irrigations and household purposes (Central Pollution Control Board, 2006). However, today the Yamuna

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River is regarded as one of the most polluted river systems in the world; Delhi, dumps more than 57% of both its treated and untreated wastewater directly into the river and consequently is responsible for almost 80% of the total pollutants (Central Pollution Control Board, 2006). A number of studies have so far focused on the quality of the river's water and the presence of high concentrations of various heavy metals and pesticides have been reported in the Yamuna River (Kumar et al., 2012; Kaur and Mehra, 2012). Approximately only 2% of the total Yamuna River water enters Delhi at Palla village as almost all the water is stopped at the Wazirabad barrage from which the Yamuna River water is virtually composed of the effluent of the 18 major drains of Delhi (Central Pollution Control Board, 2006). Therefore, the river has two distinct levels of pollution: 1) Palla to Wairabad with a lower pollution level and 2) Wazirabad to Basantpur being highly polluted. The dynamics of the nitrogen-fixing bacterial communities with the increased pollution in the River water were also analyzed.

In the present study, variation in microbial community of nitrogen fixing bacteria were access by using *nif* H gene with the help of T-RFLP technique. On the basis of different peak size and peak area, we performed in-silico approach using TReFiD software and correlate these patterns with the environmental factors and results were analyzed. Heavy metal content of field soil were also analyzed using AAS to revel their contamination level.

Materials and Methods

Site description and soil sampling

Soil samples were collected from 10 different locations (28°48'48.2"N to 077°12'38.4"E and 28°31'39.5"N to 077°20'01.4"E) from agricultural fields along the banks of the Yamuna River, thrice each year (March, July, November) for three consecutive years (2010, 2011, 2012). On the basis of the drainage entering the Yamuna River, these 10 sampling points were divided into three different locations: 1) upstream (Palla village, Christian Ashram, Jagatpur village, Sonia vihar), 2) middle stream (Wazirabad bridge, Shastri park, I.P. Power station) and 3) lower stream (Okhla, Noida bridge, Basantpur). Two of the largest drains (Nazafgarh drain and Shahdara drain) dump their waste in the Yamuna River at Wazirabad and Noida Bridge, respectively. Soil samples were collected randomly from 1–15 cm depth using a rectangular sampler (5 cm × 5 cm × 10 cm). Ten subsamples were collected from each field and mixed well into one composite sample for each site. The collected soil samples were uniformly ground and passed through a 2mm meshed sieve and stored at 4˚C before further analysis. Subsamples for microbial community analysis were stored at -20°C until further analysis.

Soil physico-chemical analysis

Sieved soil samples were air-dried and subject to physico-chemical analysis using the following methods. The soil texture of all soil samples was analyzed using a soil texture triangle. The moisture content of field soil was determined using the method described by Ohlinger (1996). Soil pH was measured using two different buffers (pH 4.0 and pH 7.0) and a pH meter (Corning, "India"). The total heavy metal content was analyzed using atomic absorption spectroscopy (Sensa AAS Dual; GBC, Australia) using an acid digestion procedure (United States Environmental Protection Agency, 1996). The C:N ratio was analyzed using the Elementar Analysensysteme Vario EL V3.00 at the University Science Instrument Center, Delhi University (New Delhi, India).

Terminal restriction fragment length polymorphism analysis of nifH gene

Total DNA was extracted from 1 g soil samples using an Ultra Clean™ Soil DNA Isolation Kit, (Mo Bio Laboratories; CA, USA) according to the manufacturer's instructions. Isolated DNA was visualized on 0.8% agarose gel and quantified at 260/280nm on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific; DE, USA). Polymerase chain reaction (PCR) amplification of the *nif*H gene was applied using 50 ng of isolated soil DNA with 1 µM FAM™ labeled PolF (5'-TGCGAYCCSAARGCBGACTC-3') and Pol R (5'-ATSGCCATCATYTCRCCGGA-3') (Poly et al., 2001) in a thermocycler (Bio-Rad; PA, USA). Final PCR reaction mixtures of 50 µL contained 200 mM of each dNTP, 2 mg/mL bovine serum albumin, 2 U of Taq polymerase and 1× Taq buffer. The final volume was made up using nuclease free water. The PCR conditions for *nif*H gene amplification were: 5 min at 94°C with 30 cycles of 1min at 94°C, 1min at 54.7°C and 2min at 72°C, with a 5min extension at 72°C for the last cycle. Amplified products were visualized on 0.8% agarose gel in the Gel Documentation system (Gel DocTM XR+; Bio-Rad; PA, USA). Amplified bands were excised and eluted using a PCR Cleanup Kit (Wizard® SV Gel and PCR Cleanup System; Promega; WI, USA). A sample of 100 ng of each eluted product was digested with 2.5 U of three different restriction enzymes, namely *Alu*I (AG^CT), *Msp*I (C^CGG) and *Hae*III (GG^CC) at 37°C for 12 hours. The digested products were sequenced by the SCI Genome Laboratory (Cochin, India). The terminal restriction fragment length polymorphism (T-RFLP) profiles were visualized using the GeneMapper® software (version 4.0; Applied Biosystems; MA, USA) which detected the fluorescently labeled terminal restriction fragments (T-RFs).

Statistical analysis

The level of significance among the physico-chemical properties of the agricultural field soil samples was assessed using two-way analysis of variance (ANOVA) and the Sigma plot version 13 software package (SYSTAT "USA"Reference or maker details as "State abbreviation, USA") while significant differences in the heavy metal concentrations of field soil samples at the three different stream locations were analyzed using three-way ANOVA.

The Shannon-Wiener diversity index (*H'*) was calculated from the T-RFLP profiles obtained with the AluI, MspI and HaeIII restriction enzymes using Eqation 1:

$$H' = -\Sigma(Ni/N) \times \ln (Ni/N) \quad (1)$$

where N_i is the number of individuals of species *i* or the % peak area of species *i* and N is the total number of individuals of all species or the total sum of the peak areas of individual samples.

Hierarchical clustering on the basis of the unweighted paired group method with arithmetic mean (UPGMA) and principal component analysis (PCA) were performed using the multivariant statistical package MVSP v3.1 (Kovach Computing Services "U.K." to quantify the variation in the relative abundance and richness of the microbial community of nitrogen-fixing bacteria at the three different stream locations along the Yamuna River. These statistical analyses helped to determine the relationship between microbial operational taxonomic units (OTUs) distribution and community composition as a function of the habitat structure (Kent et al., 2007).

Results

Physico-chemical properties of agricultural field soil

The physico-chemical properties of field soil irrigated from the Yamuna River were examined using the relevant methods. All the soil samples belonged to the silty clay loam type with the average moisture content ranging from 14.58% in July to 24.6% in November. The soil pH remained slightly alkaline in all the soil samples, ranging from 7.4 to 7.6. The average C:N ratios of the soil samples significantly decreased moving downstream. The difference was highest in the soil samples collected during March (41.62% upstream and 2.76% lower stream) followed by July (22.70% and 3.47%, respectively) and November (19.05% and 2.93%, respectively).

The total heavy metal content of all soil samples was higher than their permissible levels as recommended by the World Health Organization (WHO, 2011) for cobalt (Co), zinc (Zn), iron (Fe), aluminum (Al) and lithium (Li), as shown in Table 1. Some elements, sodium (Na), potassium (K), magnesium (Mg) and calcium (Ca) had significant ($p < 0.001$) increases in their concentrations compared to the other metals present in the lower stream section followed by upstream and middle stream. Though the concentrations of the other metals increased lower stream, they did not exceed their permissible safe limits.

Analysis of nifH gene sequencing

The TReFID v1.2.1 software was used for *nif*H gene data analysis. The TReFID program and the *nif*H gene databases were downloaded from the website: *www.trefid.net*. The web based software was used to analyze the T-RFs digested with multiple restriction enzymes. The TReFID databases are based on gene bank entries. The input data file for a TReFID analysis is stored in the project extension and the output results are a list of organisms that match the T-RFLP data. The T-RFLP profile data of the *nif*H gene consisted of the peak size (length) in the base pair, peak height, peak area and a data point for each T-RF in each sample. The relative abundance of individual T-RFs was calculated as the percentage of the total peak height and T-RFs with a relative abundance >1% were considered for further analysis.

Nitrogen fixing bacteria retrieved from field soil samples

The T-RFLP sequences of the *nif*H gene were analyzed using the TReFID software. Seven different nitrogen-fixing bacterial communities were identified in the agricultural field soil samples. In total, 9,163 OTUs were obtained from the 28,384 different sequences of the *nif*H gene retrieved during the complete study period. Of these, 2,463 belonged to the *Proteobacteria* (1,070 for *α-Proteobacteria*, 246 for *β-Proteobacteria*, 1,023 for *γ-Proteobacteria*, 124 for *Delta-Proteobacteria*), 273 OTUs to the *Actinobacteria* (*Frankia*), 79 OTUs to the *Cyanobacteria* (*Anabaena*, *Nostoc*, *Trichodesmium*,

Table 1 Heavy metal content (mean±SD, parts per million) in agricultural field soil at three different locations along the Yamuna River

Heavy metals	Upstream	Middle stream	Lower stream	Safe limit	Least mean square value
Pb	0.30 ± 0.001e	0.40 ± 0.003e	0.50 ± 0.002e	0.200	0.0400
As	0.02 ± 0.001e	0.02 ± 0.001e	0.02 ± 0.001e	0.020	0.0200
Cr	0.18 ± 0.090e	0.18 ± 0.080e	0.40 ± 0.030e	0.400	0.1100
Co	0.03 ± 0.008e	0.03 ± 0.006e	0.05 ± 0.003e	0.001	0.0300
Ni	0.009 ± 0.001e	0.01 ± 0.001e	0.02 ± 0.004e	0.070	0.0100
Cu	0.20 ± 0.070e	0.10 ± 0.060e	0.20 ± 0.050e	0.100	0.0200
Zn	1.37 ± 0.400e	1.53 ± 0.300e	1.94 ± 0.400e	0.300	1.2000
Cd	0.0006 ± 0.040e	0.0006 ± 0.070e	0.0009 ± 0.030e	0.003	0.0009
Fe	24.76 ± 3.000d	25.76 ± 6.000d	26.21 ± 5.000d	NA	25.5800
Al	1.30 ± 0.150e	1.30 ± 0.160e	1.411 ± 0.130e	0.005	1.2400
Sn	0.02 ± 0.009e	0.03 ± 0.005e	0.05 ± 0.008e	NA	0.0300
V	0.01 ± 0.004e	0.06 ± 0.007e	0.09 ± 0.008e	NA	0.0100
Mg	34.16 ± 4.000b	31.65 ± 6.000b	42.25 ± 9.000b	325	36.0200
Li	3.73 ± 0.100e	3.76 ± 0.400e	3.77 ± 0.300e	1.600	3.7600
Na	337.41 ± 10.00a	423.88 ± 19.00a	441.88 ± 20.00a	NA	392.0600
K	14.93 ± 5.000de	14.63 ± 6.000de	19.71 ± 4.000de	NA	13.7600
Ca	27.34 ± 7.000bcd	27.49 ± 6.00bcd	32.89 ± 8.000bcd	NA	29.2400

NA = not applicable. Mean values (± SD) are mean of all soil samples collected three times each year for 3 yr. Lower cases (a, b, c, d and e) denotes significant difference among heavy metal concentration at $p < 0.001$ according to Tukey's test. Values with same letter are not significantly different from each other.

Cyanotheca), 64 to the *Firmicutes* (*Clostridium beijerinckii*), 171 to the *Spirochetes*, 165 to the *Euryarchaeota*, 38 to the *Nitrospirae* and the remaining 5,910 sequences were unclassified. The percentage relative abundance values of these bacterial communities were calculated; the *Proteobacteria* with 26.87%, had a significantly ($p < 0.001$) higher relative abundance followed by the *Actinobacteria* (2.9%), *Euryarchaeota* (1.8%), *Spirochetes* (1.8%), *Cyanobacteria* (0.8%), *Firmicutes* (0.6%) and *Nitrospirae* (0.4%) (Fig 1).

Seasonally, summer had greater bacterial populations (3,536 OTUs) followed by winter (3,437 OTUs) and the rainy season (2,190 OTUs). Similarly, the relative abundance was higher during summer (38.59%) followed by winter (37.50%) and the rainy season (23.90%). Along the different stream locations, the lower stream (3,295 OTUs) had a higher number of nitrogen-fixing bacteria followed by the middle stream (2,993 OTUs) and upstream (2,875 OTUs). The relative abundance levels of the *Proteobacteria, Firmicutes, Spirochetes, Nitrospirae* and *Actinobacteria* increased along the Yamuna River from upstream to the lower stream while the relative abundance of the *Cyanobacteria* and *Euryarchaeota* decreased (Fig 1). Similar results were obtained for the rarefaction curves constructed on the basis of the number of OTUs present in the different stream locations (Fig. 2A) as well as for the different seasons (Fig. 2B).

The mean *H'* values (± SE) of nitrogen-fixing bacteria were higher in the lower stream (4.90 ± 0.4) followed by middle stream (4.03 ± 0.3) and upstream (3.80 ± 0.1). Among the different seasons, summer (5.04 ± 0.4) had a higher diversity index followed by winter (4.10 ± 0.1) and the rainy season (3.50 ± 0.4).

The link identified among the studied soil samples were further investigated using a hierarchically clustered UPGMA based on the Bray-Curtis index. The outcomes were presented in a dendrogram which showed the percentage of divergence among the different bacterial groups on the basis of their percentage relative abundance. Two different clades (monophyletic and paraphyletic) were evident in which bacterial phyla with similar values of percentage relative abundance are grouped together (Fig. 3). The *Proteobacteria* (0.877%), *Actinobacteria* (0.167%) and *Cyanobacteria* (0.254%) were clustered independently in the dendrogram as they did not show any similarity in their abundance with other bacterial groups. The *Firmicutes* had a similar value of percentage dissimilarity (0.075%) with the *Nitrospirae*, while the *Spirochetes* and the *Euryarchaeota* (0.106%) were clustered together (Table 4). The *Proteobacteria* had a high percentage of dissimilarity (0.877%) in their richness with respect to the other phyla and consequently were clustered autonomously in the dendrogram.

Principle component analysis was applied to the microbial communities of nitrogen-fixing bacteria at the three different streams. The microbial communities present in the agriculture field soil samples were distributed unevenly. The *Proteobacteria, Actinobacteria* and *Cyanobacteria* were distant from the other bacterial groups (*Spirochetes*, *Firmicutes*, *Nitrospirae* and *Euryarchaeota*). The first two principal components (PC1 and PC2) explained 88.53% and 11.46% of the total variation, respectively, with the *Proteobacteria* and *Cyanobacteria* as the leading bacterial communities, respectively (Fig. 4A). With the exception of the *Cyanobacteria*, all the other members of the nitrogen-fixing bacterial community had analogous behavior and consequently were clustered together on the right side of PC1 and explained 88.53% of the total variation while the *Cyanobacteria* on the left side accounted for only 11.46% of the total variation, signifying entirely different behavior. Overall, both principle components represented 99.99% of the total discrepancy of the different microbial communities.

Among the three different streams, the upstream and middle stream locations accounted for 93.12% of the total variation and were grouped on the right side of PC1 while the lower stream explained 6.8% and was located on the right side of PC2 (Fig. 4B). All three stream locations were positioned at the three different edges of the convex hulls signifying that these stream sampling locations had nitrogen-fixing bacterial communities that differed markedly in their relative abundances.

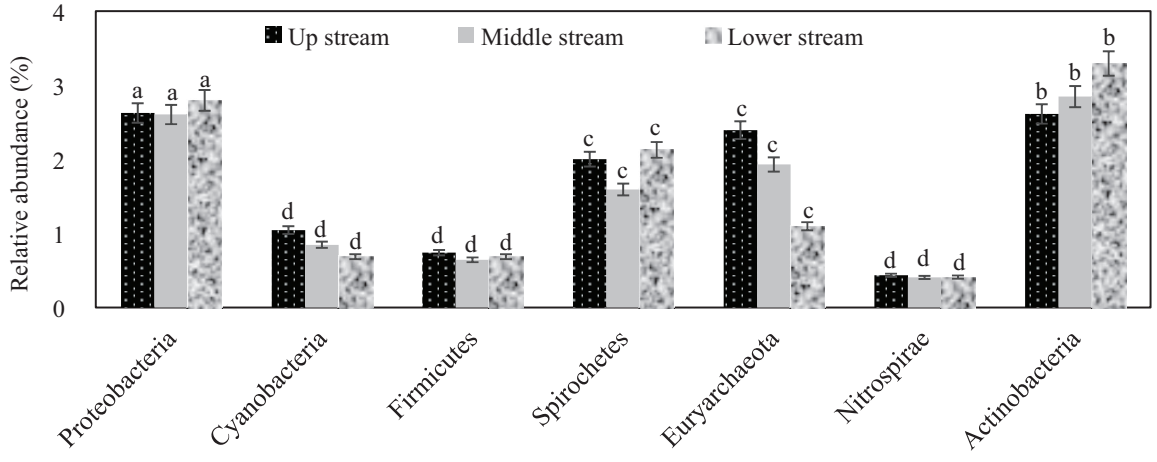

Fig. 1 Relative abundance of dominant phyla of nitrogen-fixing bacteria from agricultural field soil on the basis of terminal restriction fragment length polymorphism profiles. Values are means of all soil samples during the study period.

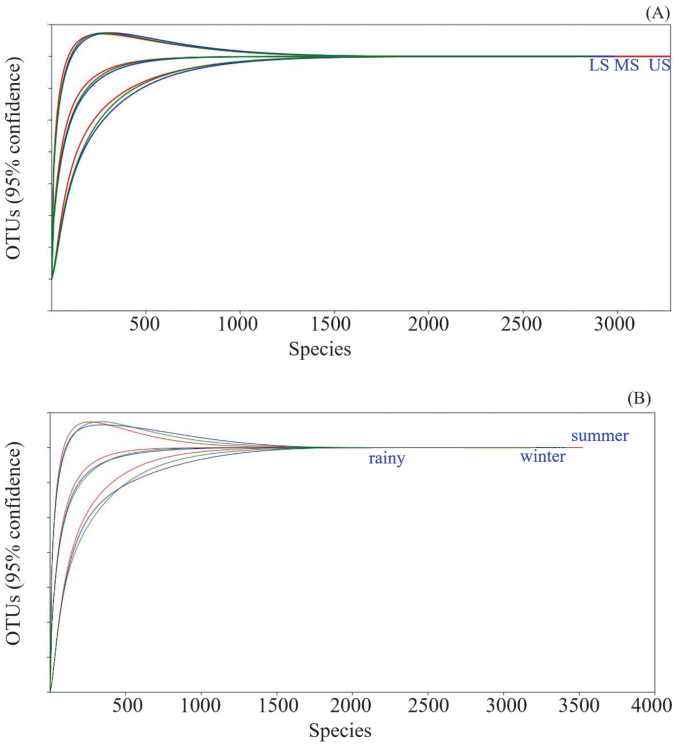

Fig. 2 Rarefaction curve showing variation among: (A) three different locations along the Yamuna River, where US = upstream, MS = middle stream and LS = lower stream; (B) three different seasons on the basis of operational taxonomic units present in the Yamuna River

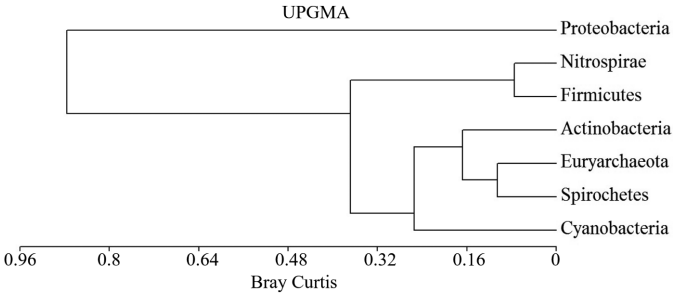

Fig. 3 Unweighted paired group method with arithmetic mean (UPGMA) dendrogram showing coefficient of dis-similarity between different phyla of nitrogen-fixing bacteria present in agricultural field soil along the Yamuna River using the Bray-Curtis index on the basis of terminal restriction fragment length polymorphism profiles.

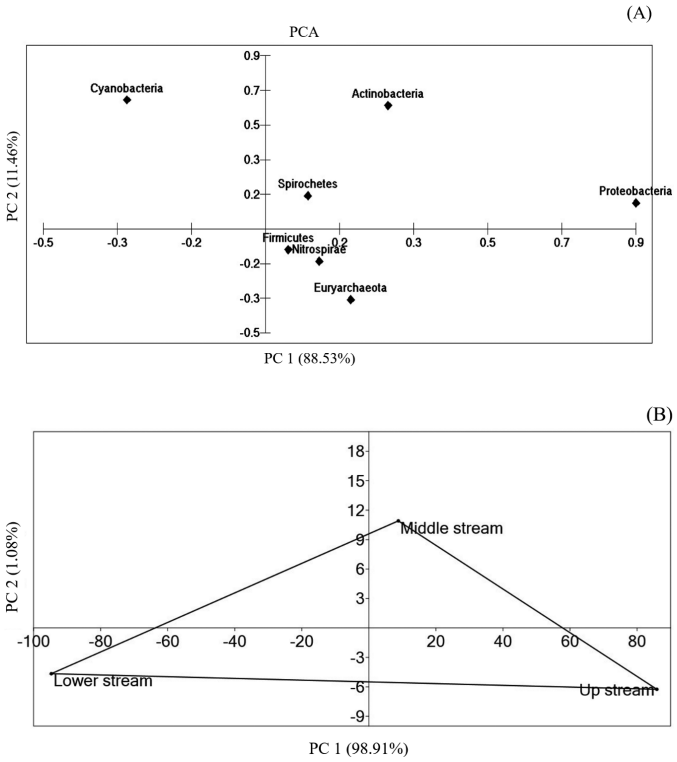

Fig. 4 Percentage of variance calculated using principal component analysis for principal components 1 and 2 (PC1 and PC2 respectively) among: (A) different phyla of nitrogen-fixing bacteria retrieved from agricultural field soil along the Yamuna River on the basis of terminal restriction fragment length polymorphism profiles; (B) three different locations showing the variability with respect to the relative abundance of nitrogen-fixing bacterial group present at each location

Discussion

Nitrogen-fixing bacteria play an important role in the nitrogen cycle and crop production as they fix the atmospheric nitrogen into a plant-accessible form and retain nitrogen, thus increasing the soil fertility (Wagner, 2011). A number of environmental conditions such as moisture and temperature affect the function and abundance of nitrogen-fixing bacteria (Tai et al., 2012). Therefore, keeping in mind the importance of nitrogen-fixing bacteria in retaining soil health, the current study focused on the distribution and composition of nitrogen-fixing bacterial communities present in agricultural field soil along the Yamuna River.

The physico-chemical analysis of soil samples showed that the C/N ratio significantly decreased in the lower stream compared to upstream in all seasons. The decrease in C/N ratio indicated the increase in the relative abundance of N in soil compared to C. Since, most of the agricultural fields in and around the Yamuna River are irrigated using water from the River, the large amount of nitrogenous waste being dumped lower stream via various municipal drains might have been responsible for the increase in the soil nitrogen content. The two largest (the Shahdara and Nazafgarh drains) directly dump their untreated waste into the Yamuna River at Noida bridge (lower

stream). This could have also accounted for the increase in heavy metal concentration in te the lower stream. Even though the metal contents of Co, Zn, Fe, Al and Li were marginally above their safe limits, the regular use of the River water for agronomical practices may have enhanced the concentration of the other heavy metals.

The T-RFLP technique was used to explore the structure and composition of the nitrogen-fixing bacterial communities in agriculture field soil along the Yamuna River. The microbial diversity as well as number of OTUs of the nitrogen-fixing bacterial community was maximum in the nitrogen-rich lower stream compared to the upper and middle stream samples. The presence of abundant nutrients in the form of various nitrogenous waste might have favored the growth of these bacterial communities. Temporal studies showed that summer (July) had higher numbers of *nif*H OTUs followed by winter and the rainy season. The higher temperature and low moisture content during the summer season favors the growth of nitrogen-fixing bacteria in agricultural field soil (Mohammadi et al., 2012; Bucci et al., 2014).

The majority of nitrogen-fixing bacteria in the present study belonged to the *Proteobacteria*, *Actinobacteria* and *Cyanobacteria*. These organisms are grouped into the Diazotrophs and use nitrogenase enzyme complexes to mediate nitrogen fixation (Rapley, 2006). Raymond et al. (2004) classified nitrogenase-encoding genes into five distinct evolutionary groups: I, Mo-Fe nitrogenases, mostly members of the *Proteobacteria* and *Cyanobacteria*; II, anaerobic Mo-Fe nitrogenases from mostly anaerobic organisms such as *Clostridia*, Acetogenic bacteria and some methanogens; III, alternative nitrogenases, including *anf* and *vnf* (but exclusing *vnf*H); IV. uncharacterized *nif* homologs detected only in methanogens and anoxygenic photosynthetic bacteria; and V. bacteriochlorophyll and chlorophyll biosynthesis genes in phototrophs (Raymond et al., 2004). Out of these, the Mo-Fe group of nitrogenases is the most proficient system at binding and reducing N_2 during nitrogen fixation (Joerger and Bishop, 1988). Most of the bacteria which fix nitrogen using this protein complex belong to the *Proteobacteria, Actinobacteria* and *Cyanobacteria* groups (Smil, 2000; Rapley, 2006).

The *Proteobacteria* have the ability to fix atmospheric nitrogen into an easily available form for plant growth as well as being ecologically important in transforming various accumulated toxic compounds in the field soil into non-toxic forms (Gupta et al., 2014). These are a phenotypically diverse bacterial group which decompose nutrients and wastewater and catalyze nitrification. Among the *Proteobacteria*, upstream had a higher abundance of *Azospirillum, Azotobacter* and *Bradyrahizobium* while *Azomonas, Xanthomonas, Alcaligens, Burkholderia, Vibrio, Pantoeaagglomerans, Serratia, Methylosinus, Azomonas, Rhodopseudomonas, Methanobrevibacter* and *Azoarcus* were higher in the lower stream.

Actinobacteria (such as the genus *Frankia*) have also been reported as playing a role in nitrogen fixation (Gtari et al., 2012). Physiologically, it is also a very diverse group of bacteria and extensively disseminated in the environment comprising soil, sediments and sea water. They producenumerous extracellular enzymes and metabolic products (including antibiotics) to degrade harmful chemicals and xenobiotic compounds which might account for their increased relative abundance lower stream compared to the upper and middle stream (Benimeli et al., 2011). *Frankia* is also used to improve the fertility and nitrogen content of degraded land under aerobic and anaerobic conditions (Diagne et al., 2011). The ability of this strain to survive even in harsh conditions may explain the increase in the relative abundance of the *Actinobacteria* in field soil lower stream.

In contrast, the abundance of the *Cyanobacteria* decreased in the field soil from lower stream followed by middle stream and upstream. The *Cyanobacteria* are a well-known photosynthetic group of bacteria. Nitrogen-fixing genera (*Cyanothece*, *Nostoc*, *Anabena* and *Trichodesmium*) were more abundant up stream compared to lower stream. The presence of xenobiotic compounds and heavy metals restricts the growth and functional role of *Cyanobacteria* in the environment (Singh et al., 2013) and might explain why the relative abundance of *Cyanobacteria* declined with an increase in River water pollution.

Principle component analysis and the UPGMA cluster analysis of current data confirmed the existence of an inconsistent structure and composition of nitrogen-fixing bacteria along the Yamuna River at the three different streams. In both types of statistical analysis, the *Proteobacteria*, *Actinobacteria* and *Cyanobacteria* displayed higher percentage levels of inconsistency and were thus grouped individually. All three stream locations, up, middle and lower stream showed variation in the abundance of nitrogen-fixing bacteria, thus located at three different edges of a convex hull. Even though it is often difficult to state the key factor controlling the unevenness in the relative abundance of nitrogen-fixing bacteria, the ebb and flow in soil properties and water quality of the Yamuna River may play a major role in this functional capriciousness.

Pollutant concentration upstream and in the lower stream may have caused variation in the abundance of nitrogen-fixing bacteria. Non-pathogenic, free-living nitrogen fixers flourished upstream and they are also used as bio-fertilizers while lower stream. the presence of household and drainage waste may have been helpful in the adaptation of the free-living pathogenic nitrogen-fixing bacteria which are well-known for their proficiency in transforming heavy metals and PHA's – polycyclic aromatic hydrocarbons into innocuous forms; hence, they might be also responsible for an upturn in the richness of this group (Lenart-Boroń and Boroń, 2014). Thus, this study concluded that nitrogen-fixing bacterial communities present in agriculture field soil along the Yamuna River were similar in their richness, but differed in their abundance at the three different stream sampling locations. Seasonal variation in temperature and rainfall affected the microbial population of nitrogen-fixing bacteria as the summer season had higher numbers of *nif*H OTUs compare to the rainy season and winter. Most of the bacterial communities belonged to the *Proteobacteria* which it is proposed evolved under the influence of contamination, leading to the selection of those species which are resistant toward pollutant contamination. The decrease in the relative abundance of the *Cyanobacteria* suggested their susceptibility to increased soil contamination. Thus, the present study concluded that the seasonal variations in the environment and increased water contamination influenced the microbial diversity of nitrogen-fixing bacteria in agriculture field soil from along the Yamuna River.

Conflict of Interest

The authors declare they do not have any conflict of interest.

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