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Rhizospheric soil of *Typha angustifolia* L. from heavy metal contaminated and free sites: Comparative profiling reveals selective abundance of γ-proteobacteria and β-proteobacteria

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Typha angustifolia L. commonly called Lesser bulrush or Narrowleaf cattail, is one of the most predominant flora of the heavy metal enriched wetland of Jaduguda Uranium mine tailings, India. The luxurious growth of this plant is due to its ability to tolerate heavy metals, such as iron and manganese.This plant also grows abundantly in natural wetlands of Kolkata. Rhizospheric eubacterial community of *Typha* growing in Jaduguda and Kolkata were compared by cloning of 16SrDNA sequences followed by ARDRA (Amplified rDNA Restriction Analysis) and sequencing of unique clusters. Sequencing results were subjected to computational analysis. In the natural wetland of Kolkata, the predominant group of eubacteria was found to be β-proteobacteria, which isabsent in heavy metal enriched wetland of Jaduguda where γ-proteobacteria was found to be predominant. The data was statistically validated using a simple χ^2 test which established the diversity of the populations in context to the bioavailability of heavy metals.

Keywords: 16S rDNA, ARDRA, Heavy metals, Lesser bulrush, Narrow leaf cattail, Wetlands

Soil microbial population is regulated by the climatic, chemical and physicochemical characteristics of the soil and water availability in a particular area. Rhizospheric microbes are a special class of soil bacteria that inhabit the soil around a plant root and maintain a symbiotic relationship with the host. The composition of rhizospheric microflora is highly dependent on root exudates from the host plant as well as the soil physicochemical properties[1].

Typha angustifolia is a perennial herbaceous plant growing in both heavy metal enriched and heavy metal lacking wetland. Common names are lesser bulrush, narrowleaf cattail or lesser reedmace. No observable variation in the morpho/physiological characteristics of *Typha* such as total biomass, root and shoot length was recorded in either of the sampling sites. Since Fe is the major contaminant of the mine tailings of Jaduguda (10^5 mg kg^{-1}), *Typha* has opted suitable mechanism to maintain Fe homeostasis and combat with the Fe toxicity. Previous works with *Typha* have shown the existence of a selective barrier controlling root to shoot transport of Fe^{+2}. Heavy metals play a key role in controlling the soil microbial composition. In this work, we tried to establish a difference in the pattern of diversity in microbial population of two different rhizospheric soils of *Typha* growing in heavy metal enriched wetlands of Jaduguda (22°23'N 86°13'E) India and in the heavy metal lacking wetlands of Kolkata (22°34'N 88°22'E) India.

Materials and Methods

Soil analysis

Rhizospheric soil of *Typha angustifolia* growing in Kolkata wetland and Jaduguda tailings were collected aseptically. For each type of wetlands, multiple plants were primarily identified to serve as representative

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samples for the entire sampling sites and rhizospheric soils associated with the superficial layer of the root was collected from a depth of 15cm and pooled aseptically in sterile containers. Chemical analysis of Kolkata wetland soil was done for its total and available nitrogen, available phosphorous, organic carbon content, Fe and Mn concentration from SGS India private Ltd., Kolkata, India. The physiological parameters of the Kolkata soil that include pH, moisture content and conductivity were measured in the laboratory by Water Analyzer from ELGA. Following analyses the physicochemical properties of Kolkata wetlands were compared with the previously published data of Jaduguda wetlands by Dhal & Sar[3] and Chakraborty *et al*.[4].

Amplification, Cloning and Identification of 16S rDNA

Total genomic DNA was isolated from 500mg of rhizospheric soil using HiPURA soil DNA extraction kit from HIMEDIA, India according to manufacturer's protocol. From this isolated genomic DNA 16SrDNA fragment were amplified using universal 16S primers 27f (5'AGAGTTTGATCCTGGCTCAG3') and 1492r (5'TACGGTTACCTTGTTACGACTT3')[5], using the following cycle: Initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s, extension at 72°C for 1½ min and then a final extension at 72°C for 7 min. The amplified 1.5 kb fragment was cloned into TOPO TA vector using Invitrogen TOPO TA cloning kit according to manufacturer's protocol. Positive clones were confirmed by colony PCR using vector specific primers and plasmid was isolated from the positive clones using Qiagen spin miniprep kit following the manufacturer's protocol. The 16S rDNA fragments was amplified from each isolated plasmid using vector specific M13f (5'GTAAAACGACGGCCAG3') and M13r (5'CAGGAAACAGCTATGAC3') primers. The amplified fragments were then subjected to ARDRA with Hha1 at 37°C for 1 h.

Amplicons with different restriction patterns were selected for sequencing using vector specific primer along with two universal 16SrDNA specific internal primers 515f (5'TGCCAGCAGCCGCGGTAA3') and 907r (5'AAACTCAAAGGAATTGACGG3')[6] in 3130x Genetic analyzer from Applied Biosystem. The contigs were generated using the Contigexpress™ (Invitrogen) software and were subjected to BLAST analysis against NCBI database. Sequences were submitted directly to Genbank for archival and public release.

Statistical analysis

To statistically validate the level of significance of the difference among the classes in these two population χ^2 test was performed at 5% level of significance at 12 degrees of freedom. Phylogenetic and evolutionary relationship among the identified species of a particular site was analysed by preparing the ancestral tree and time tree along with molecular clock analysis[7], Tajima's neutrality test[8] using MEGA6 software[9].

Results and Discussion

Comparison of physico-chemical properties of two soils

Unlike normal soil, the tailing material consists of metal laden sludge and is low in organic content. The organic carbon content of the Kolkata wetland was found to be at the range of 0.82% (w/w), which is much higher than the reported 0.18% (w/w) organic carbon content in Jaduguda tailings[3]. Decomposed plants and released exudates being the source of the organic matter. Jaduguda tailing was found to be highly contaminated with heavy metals like Cr, Co, Ni, Pb, Cd. However, in the *Typha* rhizospheric region, Fe and Mn were found to be the predominant metal contaminants that were present at the concentration of about 10^5mg.kg^{-1} and 3×10^3mg.kg^{-1}, respectively[4]. In contrast, the concentration of Fe and Mn in the rhizospheric soil of *Typha* growing in Kolkata wetland was estimated and found to be 2.1×10^4 mg.kg^{-1} and 2.82×10^2 mg.kg^{-1}, respectively, which is much less than that is found in Jaduguda tailings (Table 1). Nitrogen content of Jaduguda tailings was 150 mg.kg^{-1} and that of Kolkata wetland was 200 mg.kg^{-1}. A significant difference was observed in the available phosphorous content in Jaduguda wetland compared to the Kolkata wetland. Jaduguda tailings contained relatively high phosphorous content (400 mg.kg^{-1})[3] with compare to 20 mg.kg^{-1} in Kolkata soil (Table 1).

The heavy metal enriched fresh mine tailing of Jaduguda wetland has been reported to possess a circumneutral pH of 6.9 with 89.5% moisture and 4340 µScm^{-1} conductivity[3]. In contrast, Kolkata wetland was found to have a neutral pH of 7.2 with 89% moisture and 4246 µScm^{-1} conductivity (Table 1) indicating high clay content in both kinds of soils.

Table 1—Comparative analysis of chemical properties of Kolkata and Jaduguda soil

Chemical properties

Soil Type	Fe	Mn	Total Nitrogen	Phosphorous	Organic Carbon
Kolkata Soil	2.1×10^4 ppm	282.89ppm	200ppm	20ppm	0.82%
Jaduguda Soil (Active tailing pond)	10^5ppm*	3000ppm*	150ppm[#]	400ppm[#]	0.18%[#]

Physical properties

	pH	Moisture content	Conductivity (μScm^{-1})
Kolkata Soil	7.2	89	4246
Jaduguda Soil (Active tailing pond)	6.9	89.5	4340

[#] From Dhal and Sar[3]; * From Chakraborty *et. al*.[4]

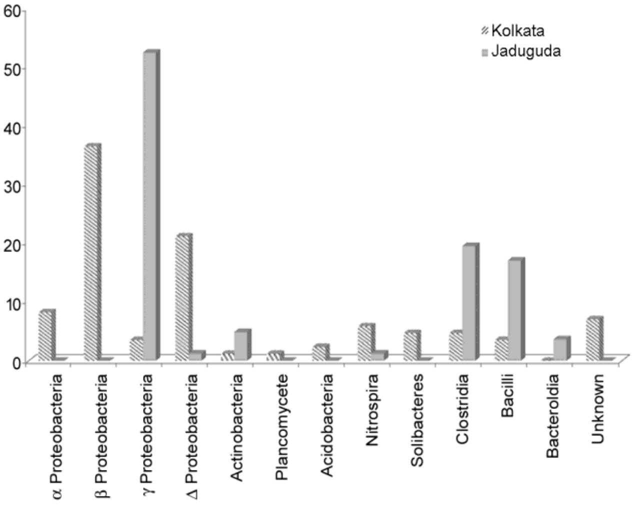

Fig. 1 — Relative distribution and class wise bacterial diversity present in soil. [Comparative analysis of the relative abundance of each class between heavy metal enriched Jaduguda wetland non-enriched Kolkata wetland. The dashed bars represent the relative percentage of a particular class in Kolkata soil, whereas the plain grey bars indicates the same for Jaduguda soil. A significant difference in the distribution of each class, specially the β and γ between these two different wetlands has been observed]

Population density study

Total 386 clones from Kolkata wetland and 318 clones from jaduguda wetland were studied. ARDRA[10] revealed the existence of 21 different restriction patterns out of 386 clones isolated from Kolkata wetland and fifteen different restriction patterns out of 318 clones from Jaduguda wetland. (Data not shown). All these different clones were sequenced and identified using NCBI BLAST. Thirteen different classes were obtained by clustering together the twenty one and fifteen clones from Kolkata and Jaduguda wetland respectively (Fig. 1). The β-proteobacteria constituting about 36.47% of the total population was most predominant class found in Kolkata wetlands. Members of this family include uncultured *Azoarcus* species KO4 (KX170867), uncultured *Denitratisoma* species KO8 (KX170871), uncultured *Sclegelella* species KO6 (KX170869), uncultured *Chitinimonas* species KO21(KX170884), and uncultured β-proteobacterium species KO13 (KX170878) (Fig. 2A). Members of Δ-proteobacteria that include uncultured *Geobacter* species KO1 (KX170864) and KO14 (KX170877), uncultured Δ-proteobacteria species KO3 (KX170866) and KO17 (KX170880) constituted 21.18% (Fig. 2A) of the total population was the next abundant class found in Kolkata wetlands whereas γ-proteobacteria inclusive of uncultured *Povalibacter* species KO2(KX170865) and uncultured *Acinetobacter* species KO16 (KX170879) constituted only 3.53% of the total eubacterial population. The most prevalent bacterium was uncultured *Schlegelella* species KO6 (15.29%) (Fig. 2A), which belongs to the Rubrivivax subgroup. Other two important genera those were present in the Kolkata wetland were *Geobacter* (4.71%) (Fig. 2A) which have two different species, Uncultured *Geobacter* sp. KO1 and KO14 and *Nitrospira* (5.88%) (Fig. 2A) as uncultured *Nitrospira* sp. KO11 (KX170874). Beside these, existence of, uncultured *Methylosinus* species KO9 (KX170872) (8.24%), uncultured *Acidobacteria* species KO10 (KX170873), uncultured *Solibacter* species KO12 (KX170875)

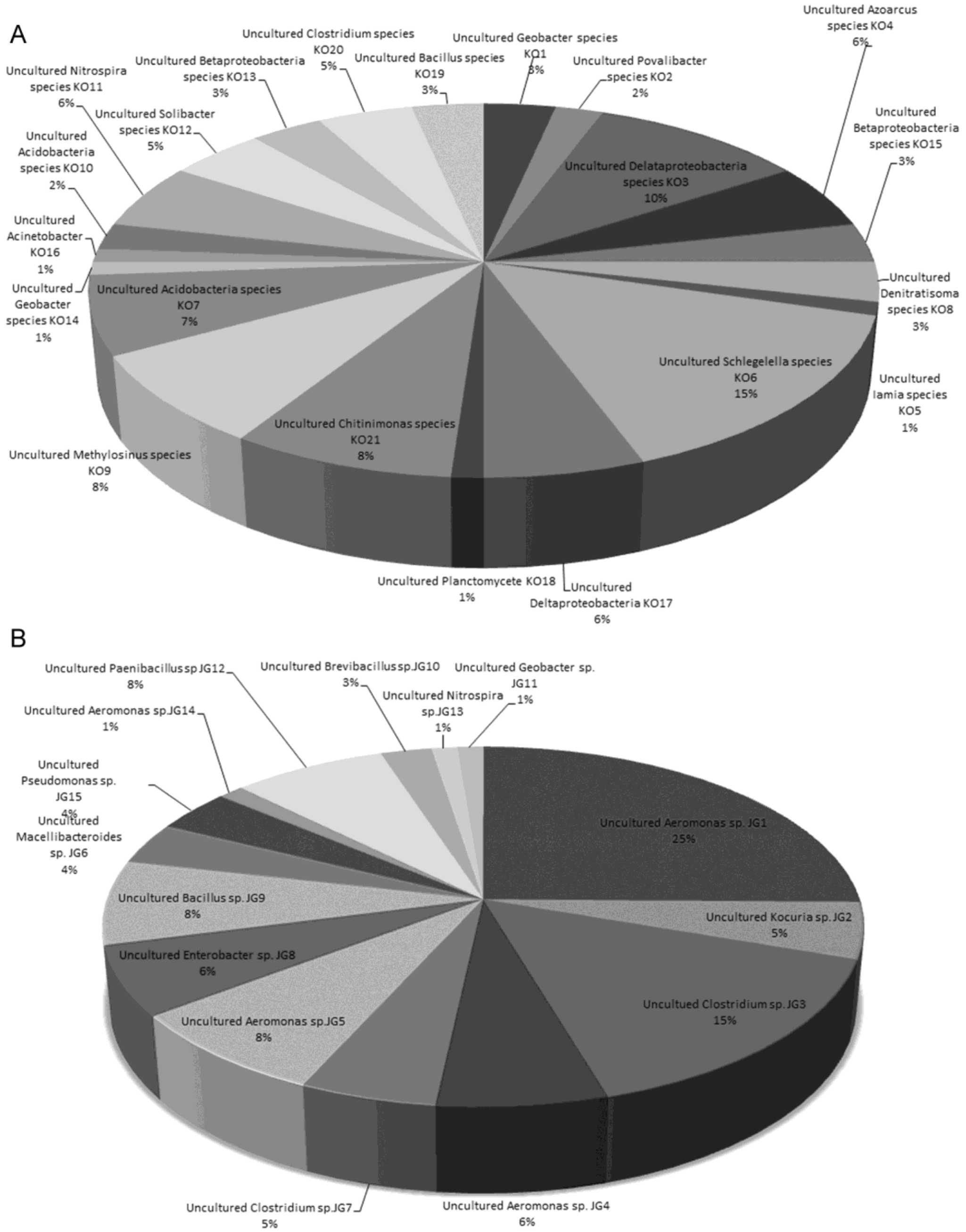

Fig. 2 —Relative abundance (in %) of different species determined after analysis of the 16S rDNA. [16S rDNA sequence of clones having different ARDRA patterns have been analysed using Blast tool and the number of clones that have been appeared to be of same species were expressed in relative percentage (%) of the corresponding species. (A) Kolkata soil; and (B) Jaduguda soil]

(4.70%), uncultured *Planctomycetes* species KO18 (KX170881) (1.18%), uncultured *Bacillus* species KO19 (KX170882) (3.53%) and uncultured *Clostridium* species KO20(KX170883) (4.70%) were also found (Fig. 2A).

Fifteen different genera were obtained from the 16S rDNA clones from heavy metal enriched active tailings of Jaduguda wetland (Fig. 2B). Unlike the Kolkata wetland, here the most prevalent class was γ-proteobacteria that constituted 52.44% of the total population. (Fig. 1)The most prevalent bacterium of this class was uncultured *Aeromonas* (30.48%) (Fig. 2B) which have four different species JG1 (KX180895), JG4 (KX180898), JG5 (KX180899) and

JG14 (KX180908). Other members of γ-proteobacteria class were uncultured *Enterobacter* species JG8 (KX180902) that constituted 6.09% and uncultured Pseudomonas species JG15 (KX180909) that constituted 3.66% of the total eubacterial population (Fig. 2B). Uncultured *Clostridia* that constituted 19.51 % of the total eubacterial population was the next abundant class (Fig. 1). It has two different species JG3 (KX180897) and JG7 (KX180901). Third important bacteria found in Jaduguda wetland were uncultured *Bacillus* species JG9 (KX180903), uncultured *Brevibacillus* species JG10 (KX180904), uncultured *Paenibacillus* species JG12 (KX180906) that together constitute 17.07%, the third abundant population of Firmicutes(Bacilli) (Fig. 1). Rest of the eubacterial population of this wetland was made up of uncultured *Kocuria* JG2 (KX180896) (4.88%), uncultured *Macellibacteroides* JG6 (KX180900) (3.66%), uncultured *Nitrospira* species JG13 (KX180907) (1.22%), and the members of Δ-proteobacteria like uncultured *Geobacter* species JG11 (KX180905) (1.22%) (Fig. 2B). Though few percentage of the selected clone (7.06% of the total population) remain undefined in Kolkata wetland, but, interestingly along with α-proteobacteria, β-proteobacteria, the most predominant class of Kolkata wetland was also absent altogether in the rhizospheric soil of Jaduguda (Fig. 1) indicating a large difference between the rhizospheric population.

Statistical analysis

The χ^2 value at 5% significance level with 12 degrees of freedom also support the rejection of the Null-hypothesis and statistically confirms the wide degree of variation in the distribution of classes between the rhizospheric eubacterial population of these two sites (Supplementary data). The diversity and distribution of bacterial population was found to be statistically significant which may be due to the differences in the physicochemical composition of the soil.

Phylogenetic and Evolutionary Insights

Comparative analysis of phylogenetic tree of both the members of Jaduguda and Kolkata wetland (Fig. 3 A & B) showed there was a significant difference in the nucleotide substitution during the course of evolution.

Two distinct ancestral states were obtained by posterior probability calculations[11] of the two sites. The OTUs of Jaduguda wetland were found to have evolved with the substitution in the A nucleotide while that of the Kolkata wetland resulted from the substitution of G nucleotide. The rate of divergence among the concerned OTUs in the Jaduguda wetland ranges from 0% (among different species of uncultured *Aeromonas*) to 17% (between uncultured *Macellibacteroides* sp. JG6 and uncultured *Nitrospira* sp. JG13). The minimum rate of divergence found in the members of Kolkata wetland was 2% (between uncultured *Schlegelella* sp. KO6 and uncultured *Denitratisoma* sp. KO8 as well as between uncultured *Geobacter* sp. KO1 and uncultured *Geobacter* sp. KO14) which suggest that the members of this part were diversified with the substitution of at least two bases, even if they exist in same genera (Fig. 4).

A null hypothesis was formulated to test whether the rate of evolution among the representative taxa of the sampling sites display equal evolutionary rate. For this the Molecular Clock calculations were made using MEGA 6.0 and the maximum likelihood (ML) values obtained for the given topology, with and

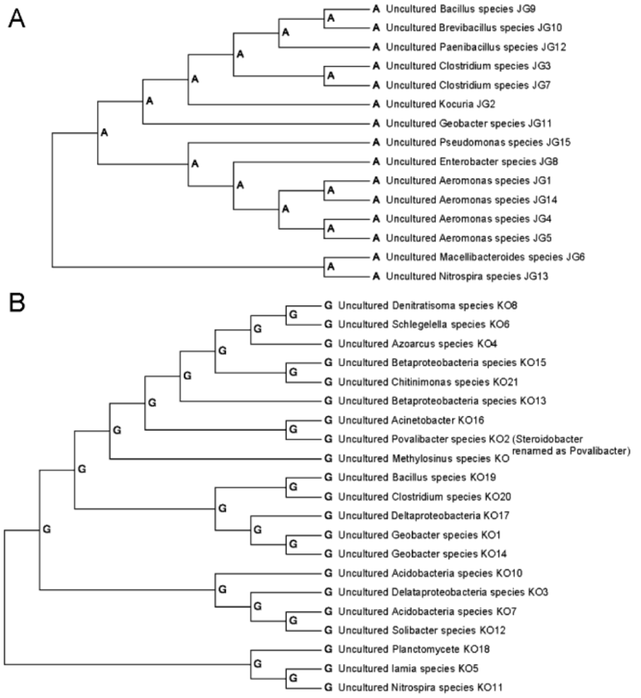

Fig. 3 —Phylogenetic trees of two different population showing the relation among the members of the corresponding population using MEGA6 software. (A) Jaduguda Community; and (B) Kolkata Community

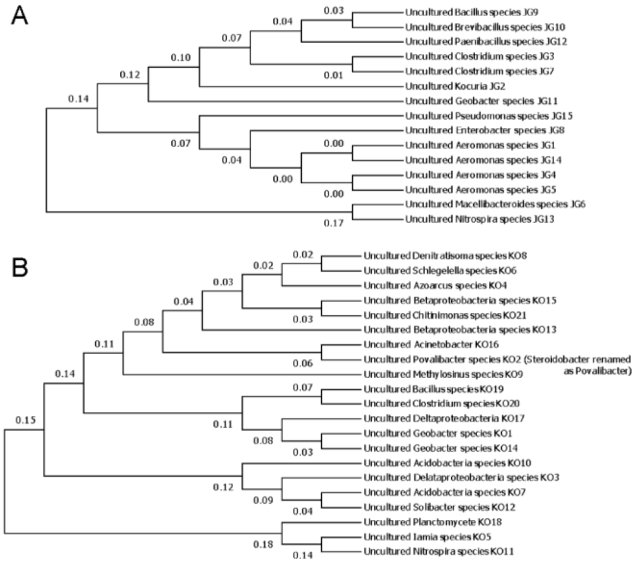

Fig. 4 —Time divergence trees obtained by alignment of 16S rDNA sequences of the members of the community using MEGA6 software. Numerical values indicate the rate of divergence from the ancestral node. (A) Jaduguda community; and (B) Kolkata Community

without the molecular clock constraints; rejected the Null hypothesis of equal evolutionary rate throughout the tree at 5% significance level for both the members of Jaduguda and Kolkata wetland with p values of 8.14036546625179e^{-8} and 0.00264083057926493, respectively. The analysis of neutrality was further extended to Tajima’s neutrality test that differentiates between DNA sequences evolving randomly and one evolving under a non-random process, the p values obtained were below 1 in Tajima’s neutrality test for both the samples of Jaduguda and Kolkata. The values of Tajima’s D in the case of Jaduguda and Kolkata were noted to be 1.428387 and 1.574083.

The phylogenetic analysis and base substitution pattern of both the samples along with their consistencies in the ancestral states of the two populations further indicate towards their neutrality in evolution and association. Tajima's D value of both the samples indicates that the neutral evolution of the microbial population at the two sites are under balancing selection with the presence of excess intermediate variants an indication of admixture in the population structure. If we correlate the findings of the molecular clock analyses and Tajima’s neutrality calculations then it is safe to comment that the individual populations at the two locations follow neutral evolution under conditions of balancing selection influenced by the associated environmental and anthropogenic factors consistent to the specific areas.

This study reveals that most of the bacterial isolates from the wetland of Kolkata was found to be the members of β-proteobacteria that may be associated with the Nitrogen cycle by means of denitrification (*Steroidobacter* sp.) and nitrate reduction (*Denitratisoma* sp.)[12]. Another member of β-proteobacteria found in Kolkata wetland was *Azoarcus* sp., whichis a well established endophytic diazotroph. Literature reveals that members of β-proteobacteria group like *Azoarcus* sp., *Denitratisoma* sp. are able to supplement the nitrogen requirement in plants by means of nitrogen fixation and ammonia oxidation[13]. The most prevalent genus of Kolkata wetland *Schlegelella* sp. KO6 is also a β-proteobacterium found to be associated with PHB degradation[14]. The only member of the γ-proteobacteria group in Kolkata wetland is *Acinetobacter* sp, which is known to be associated with bioremediation of common xenobiotic compounds.

The predominant existence of γ-proteobacteria and *Clostridia* in the rhizosphere of *Typha* growing in heavy metal enriched Jaduguda wetland may be important for homeostasis as they have been reported to be associated with bioremediation[15,16] required to enrich the fertility of the soil. The study of the bacterial diversity of Jaduguda wetland reveals the presence of fifteen different genera, which is less than that of the Kolkata soil which contains twenty one different varieties. Proteobacteria are considered to be the most suitable candidates for survival under oligotrophic environment that probably plays an important role in metal reduction and resistance. Previous report says that these groups of bacteria might participate in the immobilization of uranium[15]. *Clostridia* is well documented for its ability to reduce uranium through hydrogen metabolism and fermentation[17]. Microbial population found from the heavy metal enriched fresh mine tailing of Jaduguda wetland was flooded with the heavy metal detoxifiers like *Pseudomonas* sp., *Paenibacillus* sp., and *Bacillus* sp. These microbes may play important roles in minimizing the toxic effect of heavy metals especially iron, by playing their roles as iron oxidizer and facilitating the formation of iron plaque on the root which act as a sieve that selectively regulate the entry and partitioning of iron into the plant[2].

Although the physical parameters of both these soils are almost equivalent, the heavy metal enriched wetland of Jaduguda uranium mine tailings possessed about 20 fold excess iron and 10 fold excess manganese with respect to Kolkata wetland. In addition to iron and manganese, the Jaduguda wetland also possessed many other heavy metals like cadmium, lead, zinc[2] which contribute towards the heavy metal induced toxicity of the soil. The difference in heavy metal loads in these two wetlands may contribute to the significant variation between the rhizospheric eubacterial populations. Heavy metal stress and low organic carbon and nitrogen content of Jaduguda wetland employs additional selection pressure facilitating the growth and proliferation of fittestmicrobes[18].

Conclusion

Replacement of γ-proteobacteria with β-proteobacteria and decreased Bacilli population in Kolkata wetland suggest the probable involvement of γ-proteobacteria in attributing metal tolerance and growth promotion in *Typha* under heavy metal enriched environment. As also reported earlier, enrichment of iron oxidizing bacteria in the rhizosphere of *Typha* in Jaduguda suggests iron oxidation as one of the mechanisms by which iron availability towards plant is controlled in this wetland. This diversity of rhizospheric eubacterial population may enable the plant to sequester as well as breakdown and assimilate the potential toxic compounds that impair normal metabolic functioning of the plant. The differences in the rhizospheric communities observed in this study may thus be attributable to the soil physicochemical properties. The independent rates of divergence among the microbial community members of the sampling sites further emphasizes the importance of soil characteristics as a major contributor to microbial population dynamics. More detailed effect of rhizospheric bacteria in combating soil stress in *Typha* would be better understood by amplicon sequencing on next generation sequencing platform.

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Conflict of interest

The authors declare that they have no conflict of interest.

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