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Molecular Analysis of the Bacterial Communities from Tannery Contaminated Sites in Riyadh, Saudi Arabia

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Abstract
A major problem of the tanning industry is the disposal of chromium-contaminated wastes which have a deleterious effect on the environment. Inductively Coupled Plasma Mass Spectrometer analysis of tannery dumping soils (Riyadh, Saudi Arabia) showed the presence of high concentrations of chromium (268–297 ppm) and strontium (255–435 ppm). 454 pyrosequencing was applied to characterize the bacterial communities in four contaminated sites and a reference site. A total of 5,862 bacterial clones were assembled through 16S rRNA gene clone libraries. Phylogenetic analysis at the species level demonstrated contrasting distributions, interrelationships and shared species in all sites including the reference site. The chromium-contaminated sites were dominated by bacterial phyla Proteobacteria, Bacteroidetes and Firmicutes while other genera such as Chloroflexi, Acidobacteria, Gemmatimonadetes, Cyanobacteria and Nitrospirae were not detected.

The bacterial genera Halomonas, Proteiniphilum, Alkaliphilus and Marinilabiaceae were identified only in the contaminated sites revealing their capability to tolerate and resist chromium toxicity. A vast variation in bacterial species was observed where Halomonas xinjiangensis was found at a considerable percentage in the chromium polluted soils. These findings revealed that long-term chromium stress in the tannery or dumping soil resulted in community shifts towards a dominance of chromium-resistant bacterial populations.

Keywords: Chromium-polluted 454 pyrosequencing, bacterial community, *Halomonas xinjiangensis*, 16S rRNA.

Introduction

Increased industrial activity has resulted in increasing heavy metal contamination leading to serious environmental hazards to all living organisms[1]. Heavy metal pollution is generally the result of anthropogenic activities such as mining, smelting and metal treatment to provide raw materials for industry and from different industries such as ceramics, dyeing, plastics, textile, leather tanning, colorants in oil paint, tire manufacturing industries and others; these industries are important around the world to increase economic activity in different countries and to meet consumer demands[2].

The toxicity of heavy metal pollution not only deteriorates soil fertility and crop production and quality but also pollutes the biosphere, threatening living beings through contamination of the food chain. Elevated heavy metal concentrations are potentially hazardous because of long-term and non-reversible toxicity[3]. Inappropriate disposal of industrial effluents is responsible for a substantial proportion of heavy metal contamination[4]. Among the heavy metals, chromium (Cr) always dominates the effluents of paints, pigments, wood preservatives and steel production. Cr salts are particularly important in the leather industries as the tanning process could not be done without Cr salts[5].

According to Rivela et al[6], elevated concentrations of Cr in the biosphere are mainly caused by the tannery industry which accounts for 40% of the total utilization of Cr by industry. Cr is found as either Cr(III) or Cr(VI). Cr(III) is usually used in tanneries for tanning procedures; after oxidation, it becomes Cr(VI). The toxicity of Cr(VI) is greater than that of Cr(III); Cr(VI) is more likely to cross living cell membranes and interact with nucleic acids, proteins and other biological molecules[7]. However, some reports indicate that Cr(III) can be converted to Cr(VI) through redox reactions by inorganic substances present in tannery effluents and by organic substances present in leathers[8].

Bioremediation is a novel and cost-effective approach involving the utilization of microorganisms such as fungi, algae, archaea and bacteria for degradation of hazardous and toxic environmental substances[9]. Microbes resistant to high concentrations of heavy metals can be useful for reducing contamination of heavy metals in soils[10]. Microbial degradation is the best option for reduction of Cr(VI) to Cr(III), which is the optimal strategy for remediation of Cr toxicity in the environment[11]. *Streptomyces rimosus* and *S. griseus* were both able to biologically remove Cr[12,13].

In addition, there are other strains that have been isolated with the ability to reduce or remove Cr(VI) including *Achromobacter* sp. strain Ch1, *Arthrobacter* sp., *Nesterenkonia* sp. strain MF2 and *Sphaerotilus natans*[14-17]. There are four genera namely *Modicisalibacter*, *Cobetia*, *Chromohalobacter* and *Halomonas* that are recognized as

halophiles and capable of removing Cr. Non-halophilic genera including *Zymobacter*, *Halotalea* and *Carnimonas* can be employed in bioremediation of Cr [18-20]. Guan et al[21] isolated *Halomonas xinjiangensis*, a halotolerant bacterium, from a salt-lake in China, which is capable of surviving in high salt concentrations. Cr has deleterious effects on most microorganisms including bacteria, algae and yeasts[22].

There is only limited information about the long-term effects of Cr pollution on overall microbial community structures in the soil environment. Therefore, the main goal of this study was to detect Cr-tolerant bacteria that have the potential to remove Cr from the environment, for potential use in biotechnological applications.

Material and Methods

Sample collection: Samples were collected from a remote area (24°27'01.1"N 46°52'36.8"E) located near the second industrial city to the south of Riyadh, Saudi Arabia where several old hangars housed old fashioned tannery activities. Tannery effluents are discharged to an arid site close to the hangars via sewage tankers. A total of four representative contaminated sites were selected near the dumping site and another site about 12 km away from the tannery site was selected as a reference site to represent the background profile of the area.

Approximately 50 g soil was collected from each site and transferred into sterilized bottles. The sediment was solid enough to allow gentle cutting, handling and mixing to improve homogeneity. Later, 0.5 g of each sample was used for DNA extraction and the rest was used for determination of heavy metals.

Heavy metal analysis: The soil samples were dried thoroughly and then sieved through a 2-mm sieve. An amount of sieved soil (0.1–0.15 g) was digested with a mixture of 5 mL HNO_3, 2 mL HF and 2 mL HCl. The concentrations of chromium (Cr), strontium (Sr), manganese (Mn), barium (Ba), zinc (Zn), arsenic (As), copper (Cu), cobalt (Co), nickel (Ni), lead (Pb) and cadmium (Cd) were determined by inductively coupled plasma mass spectrometer (ICP-MS; PerkinElmer SCIEX ELAN® 6100, Concord, Ontario, Canada). Recoveries of 98% to 100% were achieved using certified reference soil IAEA-SOIL-7.

DNA extraction: Genomic DNA was extracted using a Power Soil DNA Isolation Kit (MO BIO Laboratories Inc, Solana Beach, CA, USA). A Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to quantify the extracted DNA.

Microbial analysis: Bacteriological analysis was performed by Molecular Research LP (Shallowater, TX, USA) based on the method originally described by Dowd et al.[23,24] 454 pyrosequencing is a high throughput DNA sequencing method that utilizes a single strand of DNA with a length of 400–500 bp. All DNA samples were adjusted to 100 ng/µL and a 1.0 µL aliquot of each DNA sample was used as template for each 50 µL PCR reaction. Forward and reverse 16S rRNA universal Eubacterial primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used for a single-step 30 cycle PCR.

The PCR amplification was accomplished using the HotStarTaq plus Master Mix Kit (Qiagen, Valencia, CA, USA) under the following conditions: 94°C for 30 min followed by 28 cycles of 94°C for 30 sec, 53°C for 40 sec and 72°C for 1 min with a final elongation step at 72°C for 5 min. All amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA) followed by PCR. Sample sequencing was performed using the Roche 454 GS FLX+ (Roche, Nutley, New Jersey, USA) following the method described by Dowd et al.[23]

The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline by Molecular Research LP. Sequences were depleted of barcodes and primers and then short sequences of <200 bp, sequences with ambiguous base calls and sequences with homopolymer runs exceeding 6 bp were removed. Sequences were then denoised and chimeras removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences with clustering at 3% divergence (97% similarity) [23-26].

OTUs were then taxonomically classified using BLASTn against a curated Green Genes/RDP/NCBI derived database and compiled at each taxonomic level into both "counts" and "percentage" files[27]. Counts files contained the actual number of sequences while the percentage files contained the relative (proportion) percentage of sequences within each sample that mapped to the designated taxonomic classification. Statistical analysis was performed using a variety of computer packages including XLstat, NCSS 2007, "R" and NCSS 2010. Alpha and beta diversity analysis was conducted using Qiime (www.qiime.org).[23-26,28] Significance reported for any analysis was defined as $p<0.05$.

Results

The concentrations of heavy metals in the four tannery dumping sites and the reference sample were shown in table 1. The contaminated soil contained elevated levels of Cr (five-fold increase) and Sr (two-fold increase) when compared with the uncontaminated site. At the same time, there was a small increase in the concentrations of some metals (Fe and Zn) in the contaminated sites that could be attributed to the salts used for tanning the leather. Conversely, other metals (Mn, Co, Ni, Cu and Pb) were found at normal concentrations lower than those in the reference soil (IAEA-7).

Based on 454 pyrosequencing, the bacterial communities associated with contaminated soils were identified (table 2). The results showed the existence of some bacterial genera in the contaminated sites such as *Halomonas* spp., *Proteiniphilum* spp., *Alkaliphilus* spp and *Marinilabiaceae* spp. that were not found in the reference sample. *Halomonas* and *Proteiniphilum* spp. were most dominant in the Cr-contaminated sites.

Some bacterial genera such as *Patulibacter* spp., *Solirubrobacter* spp. and *Rubrobacter* spp. were identified in the reference sample but were not present in the contaminated sites (table 2). The severe change in the composition of the microbial communities inhabiting the contaminated sites could be attributed to the effect of the high Cr levels. At the species level, bacterial community structure in response to the elevated Cr level at the contaminated sites was evaluated and calculated by principal coordinate analysis (PCoA-two-dimension; figure 1) to demonstrate the variability between the four contaminated sites and the reference site. Venn diagrams (figure 2) demonstrated the shared species in bacterial communities among reference and contaminated sites.

Discussion

The results indicated the diversity of indigenous bacterial communities in contaminated sites where some resistant clones survived under elevated Cr levels and high salinity. Soil from sites contaminated by relevant tannery processes, maintained pH and electrical conductivity at around 7.9 and 0.67 dS/m respectively [15]. The results were in agreement with those of Abdul Mottalib et al[29] who recorded extremely high Cr and Fe concentrations in soil from a tannery area of Dhaka city, Bangladesh. Similarly, Rahaman et al[30] showed that soil exposed to a huge amount of untreated tannery effluent and sludge was highly contaminated with Cr. Moreover, there were also an agreement with a study conducted in oil wells in China indicating a shift in microbial community structure but adaptation of only a few microbial species to the extreme conditions of the petroleum reservoir even after the injection of fresh surface water[31].

Table 1
Heavy metals concentration at the dumping sites as well as reference (mg/kg).

Analyte	Site 1	Site 2	Site 3	Site 4	Ref.	IAEA Soil 7
Cr	290	268	285	297	57	61
Mn	121	139	133	138	149	730
Fe	6,090	6,630	6553	6,940	5,840	25,000
Co	4	4	3	5	7	16
Ni	9	11	6	13	14	26
Cu	8	8	7	5	10	13
Zn	34	44	38	38	24	113
As	14	9	15	8	13	21
Sr	435	341	255	282	141	4.2
Ba	96	130	108	146	112	132
Pb	6	7	4	6	8	54
U	1	1	1	1	1	2

Table 2
Total Cr and associated percentage of bacterial species identified in contaminated and Ref. sites.

Analyte	Sampling Site				
	Site 1	Site 2	Site 3	Site 4	Ref.
Cr (mg/kg)	**290**	**268**	**285**	**297**	**56**
Percentage of Bacterial Species					
Halomonas spp.	13	17	15	17	0
Proteiniphilum spp.	2	7	11	6	0
Alkaliphilus spp.	2	4	3	3	0
Marinilabiaceae spp.	2	3	2	2	0
Patulibacter spp.	0	0	0	0	4
Solirubrobacter spp.	0	0	0	0	4
Rubrobacter spp.	0	0	0	0	17

The findings of our study were similar to those of Mabrouk et al[32] who isolated a Cr-resistant *Halomonas* sp. strain from a tannery effluent in Egypt. This strain demonstrated high capability to grow in the presence of elevated Cr-concentrations up to 3,500 ppm and 20% NaCl under alkaline conditions (pH 10). *Halomonas* sp. was used for the remediation of high concentrations of Cr(VI) from chromite ore processing leachates[33]; the bacterial cells were able to rapidly remove high concentrations of aqueous Cr(VI) at pH 11. *Proteiniphilum* spp. were reported as obligatory anaerobic strains that were identified in an anaerobic sludge blanket reactor treating brewery wastewater and also as a member in a consortium dedicated for conversion of coal to methane *in situ* and *ex situ*[34,35].

However, the findings of Desta et al[36] also supported the results of this study regarding the relationship and sharing of the bacterial populations among aerobic, anaerobic and artificial wetland sites. *H. xinjiangensis* was isolated from the sediment samples from a lake in Xinjiang Province, north-west China[21] where it might utilise Fe as its primary source of energy followed by Mn.[37,38] *H. xinjiangensis* was dominant at all four contaminated sites. Atypically, the genus *Rubrobacter* has few species including *R. bracarensis*, *R. calidifluminis*, *R. naiadicus*, *R. radiotolerans*, *R. taiwanensis* and *R. xylanophilus*; more than 17% of the bacterial community in the reference sample was represented by species from genus *Rubrobacter*.

Some *Rubrobacter* sp. are known as radio-resistant species like *R. radiotolerans*[39] or thermophilic species like *R. xylanophilus*[40]; *R. taiwanensis* is well known for both characteristics[41]. The order of abundance of selected bacterial phyla was *Proteobacteria* > *Bacteroidetes* > *Firmicutes* > *Actinobacteria* > *Synergistetes* > *Chloroflexi* > *Acidobacteria* > *Gemmatimonadetes* > *Cyanobacteria* > *Nitrospirae* identified in tannery contaminated and reference area soils.

The most abundant phylum, *Proteobacteria*, demonstrated their resistance and ability to survive in heavy metal contaminated soils; the proportion of *Proteobacteria* in contaminated sites was higher than that in the reference site indicating their resistance and potential to degrade heavy metal pollutants like Cr. The abundance of bacterial phyla in the tannery-contaminated sites and reference site was demonstrated using 16S rRNA 454 pyrosequencing (figure 3). *Proteobacteria*, *Bacteroidetes* and *Firmicutes* represented around 90% of the sequences generated in the contaminated sites. *Proteobacteria* was the dominant phyla in sites 1 (54%), 2 (38%), 3 (36%) and 4 (32%) while a lower percentage was observed in the reference site (11%).

A similar trend was recorded for *Bacteroidetes* which represented 19%, 18%, 25%, 33% and 3% in sites 1, 2, 3, 4 and the reference site respectively. Phylum *Firmicutes* was also dominant in the contaminated sites representing 5%, 25%, 20% and 11% in sites 1, 2, 3 and 4 respectively whereas very low abundance of *Firmicutes* was recorded in the reference site. The major phylum of the bacterial community in the reference site, *Actinobacteria* (45%) was present at 3%, 1%, 3% and 6% in sites 1, 2, 3 and 4 respectively. Phylum *Synergistetes* was detected only in contaminated sites at low percentages Phyla *Chloroflexi*, *Acidobacteria*, *Gemmatimonadetes*, *Cyanobacteria* and *Nitrospirae* that were recorded in the reference site at low level but were not detected in the contaminated sites. These results are in agreement with those of Sheik et al[42] who showed a clear shift in the composition of the microbial communities in leather tanning industry polluted soil in Pakistan.

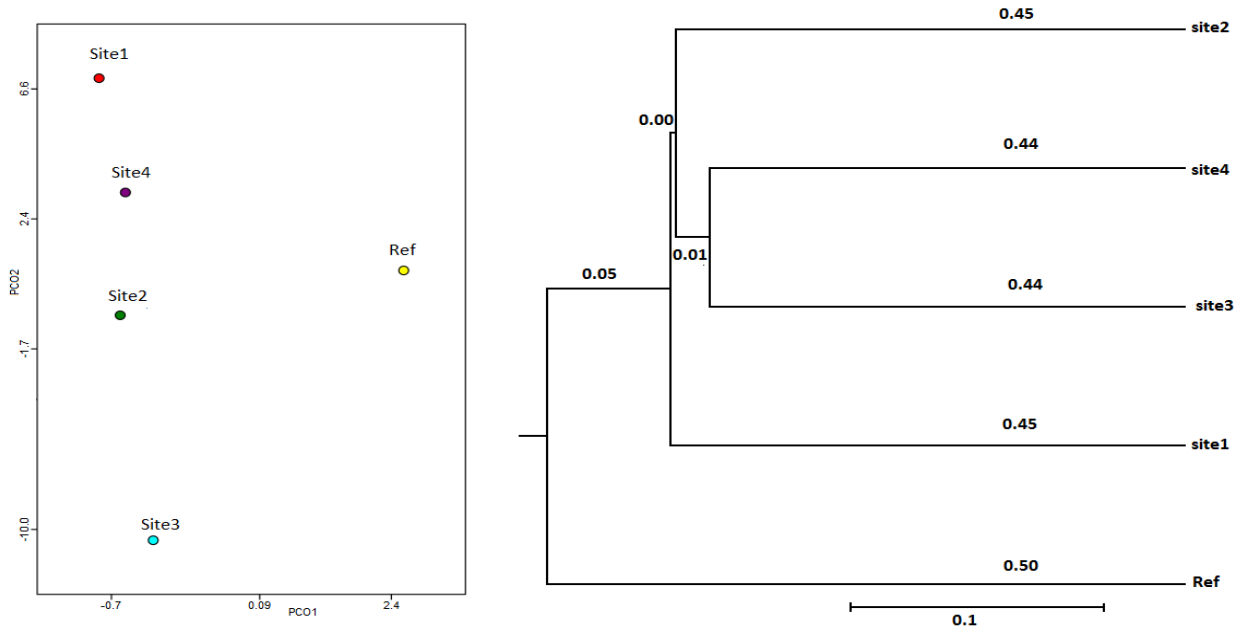

Fig. 1: Plot of the principal coordinate analysis (PCoA) and phylogenetic tree for the four contaminated sites and reference site. ●, Site 1; ●, Site 2; ●, Site 3; ●, Site 4; ●, Reference Site.

The contaminated soils were dominated by *Proteobacteria*, while *Actinobacteria* and *Acidobacteria* were found as minor components of the bacterial community. The shift in phylum level dominance suggests that *Proteobacteria*, as a group, might be the most metal tolerant organisms found at metal contaminated sites.

Previous studies showed that *Proteobacteria* are favoured after a stressor is applied to an ecosystem which is perhaps a reason for their dominance[43,44]. Regarding the existence of *Firmicutes* in the contaminated sites at high levels, Desai et al[45] showed that long-term Cr-stress shifted the bacterial community towards a dominance of *Firmicutes* in the soil environment. *Firmicutes* dominated the microbial activities when tannery wastewater was treated in constructed wetlands[46].

The dominance of *Firmicutes* within soil bacterial communities exposed to high levels of Cr might be because of their functional capacity to resist and reduce Cr toxicity. The presence of the phylum *Bacteroidetes* in the tannery contaminated sites is similar to the results of Branco et al[47] who recorded *Bacteroidetes* as one of the dominant phyla in a river system subjected to long-term Cr contamination. *Bacteroidetes* showed the potential to degrade complex carbon compounds[48]. Moreover, they were resistant to dye wastewater treatments and played a promising role in the degradation of selected re-tanning chemicals in tannery wastewater[49]. Meanwhile, the disappearance of some bacterial phyla in the presence of Cr contamination suggests the inability of these bacteria to cope with long-term Cr-stress.

Conclusion

The current study demonstrated the response of indigenous soil bacterial communities exposed to long-term Cr pollution. In the tannery dumping sites, a shift in the bacterial communities to *Proteobacteria*, *Bacteroidetes* and *Firmicutes* could serve as a bio-indicator of Cr pollution. *H. xinjiangenesis* was the most abundant Cr-tolerant species identified among the communities. The results might reflect a baseline for microbial-based metal remediation and aid in the isolation of Cr-resistant bacteria from contaminated soils, facilitating their evaluation and potential application for the bioremediation of chromate-contaminated environments.

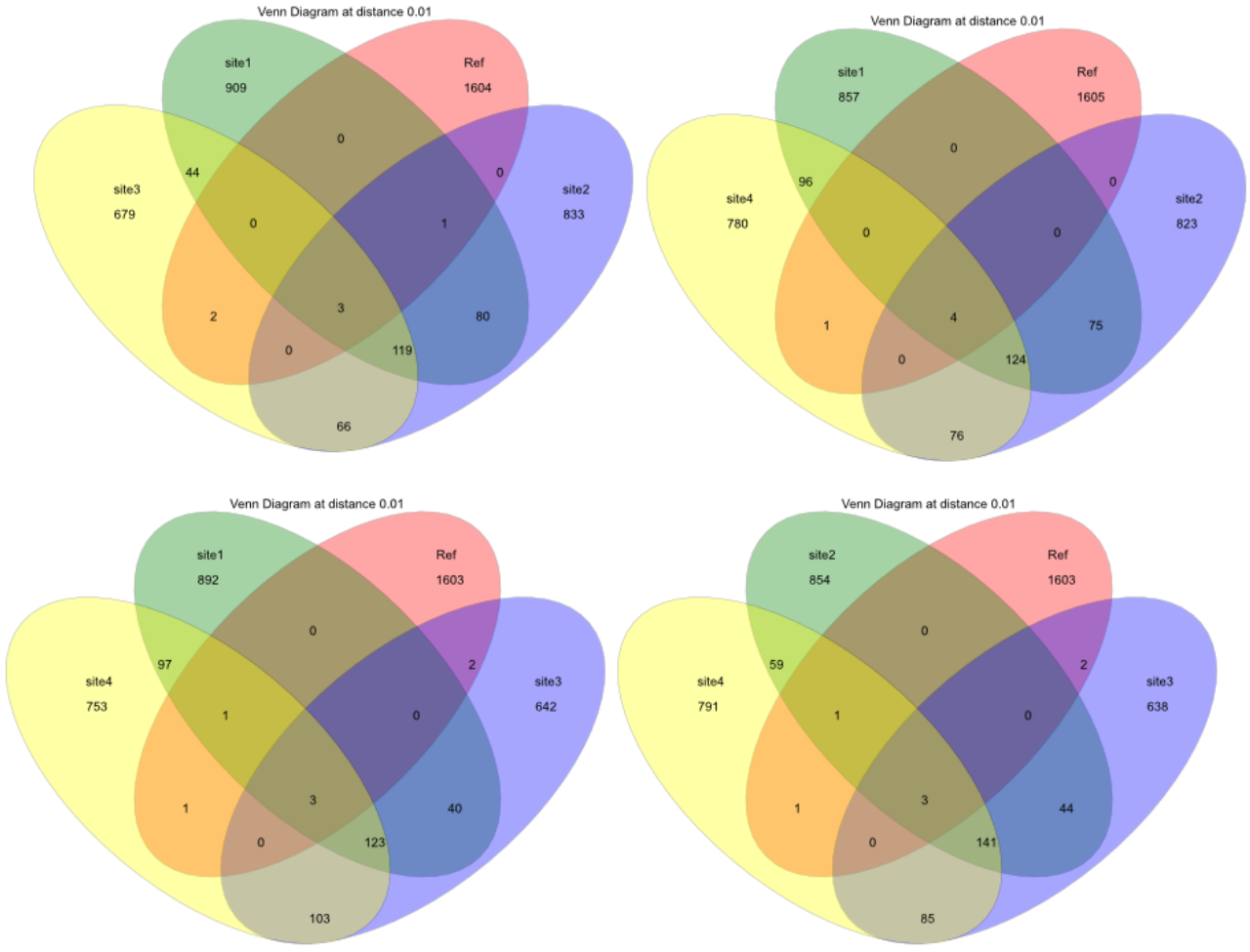

Fig. 2: Venn diagram for bacterial OTUs (at a distance of 0.01)

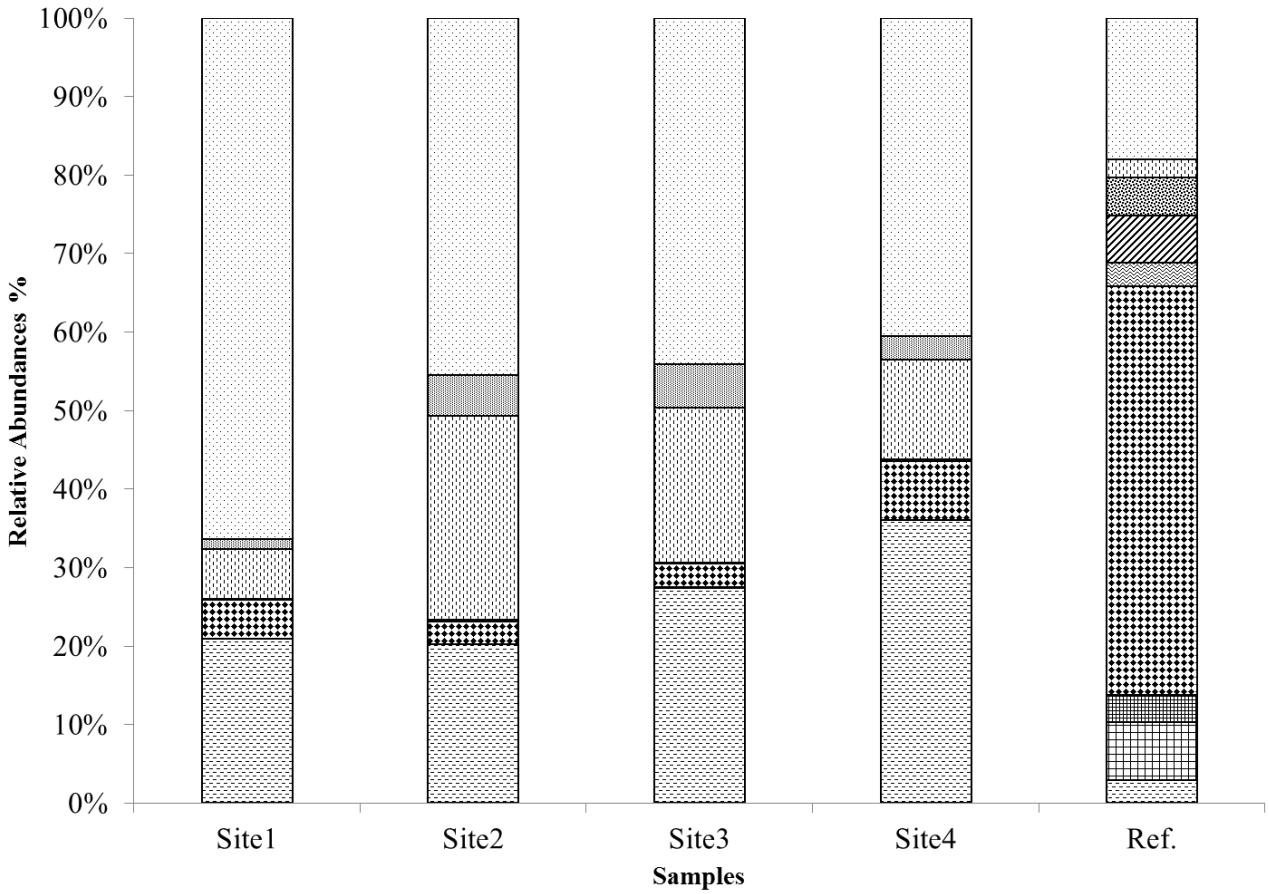

Fig. 3: Distribution of detected bacterial phyla based on the 16S rRNA clone libraries created from tannery contaminated four sites and one reference site. ▨, Bacteroidetes; ⊞, Chloroflexi; ▩, Cyanobacteria; ⊞, Actinobacteria; ▧, Nitrospirae; ▨, Acidobacteria; ▩, Gemmatimonadetes; ▨, Firmicutes; ▩,Synergistetes; ▨, Proteobacteria.

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