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A metagenomics investigation of bacterial communities in gold mine tailings

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Abstract

Gold mine operations release toxic arsenic and other heavy metals into the environment, which can be accumulated in water resources and the food chain. As microbial bioremediation has been a promising method for pollutant removal from contaminated sites, the identification of bacterial communities in arsenic-contaminated resources has recently been in focus. The bacterial communities of tailings dam effluent (TDE) of a gold mine in Iran were analyzed. The bacterial communities were examined using the next-generation sequencing method (Illumina platform) targeting the V3-V4 region of 16S rRNA genes. The 16S rRNA dataset from this study was compared with three arsenic-contaminated groundwater (GW) microbiomes from SRA databases, using the bioinformatics tool QIIME 2. Our findings revealed that the prevalent taxonomic groups observed in all of the samples belonged to *Proteobacteria* (8.06-45.49%), *Bacteroidetes* (1.85-50.32%), *Firmicutes* (1.00-6.2%) and *Actinobacteria* (0.86-5.09%). Metagenomic analyses showed that *Algoriphagus*, *Rhodobacter*, *Anaerospora*, *Limnobacter*, *Halomonas* and *Yonghaparkia* are the main bacterial genera in TDE. Despite the limited similarities in the prokaryotic community of the samples, the most of the retrieved genera of the TDE are unique and the native bacteria of Iran.Conclusions**:** Long-term exposure to arsenic causes changes in bacterial abundance and richness. This resulted in natural selection and expression of the most compatible genes in existing condition. Although there are similarities in some microbial communities of ground waters, but it can be found some native microorganisms, which was adapted to the harsh environment of TDE.

Keywords: 16S rRNA gene, Arsenic, Bioinformatics, Groundwater, Next-generation sequencing, Gold mine waste water

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Introduction

Arsenic (As) constitutes as the most prevalent environmental toxin in aquatic and terrestrial environments. This pollution primarily forms inorganic oxyanions, arsenate (As^{5+}) and arsenite (As^{3+}). As^{5+} is the dominant species in oxygenated surface water, whereas As^{3+} is observed in anoxic or reduced conditions and is more toxic and soluble than As^{5+} (1). Arsenic in ecosystems naturally originates from the environment or anthropogenic sources (2, 3). Mining is the main source of arsenic pollution in the environment (4). Human industrial activities and natural processes such as rock weathering discharge arsenic into the surrounding environment. Arsenic in water and soil can enter the biogeochemical cycle and food chain, accumulate in the human body and pose different risks to human health (5).

Different mechanisms are used by bacteria to neutralize arsenic toxicity including oxidation of As^{3+}, reduction of As^{5+} and methylation and demethylation of arsenical compounds (6-8). Bacteria can change the chemical properties, bioavailability, toxicity, natural behavior, and environmental destiny of arsenic. The expression of genes involved in arsenic metabolism is highly regulated by bacteria and controlled by operons such as *aio*, *ars*, and arr (9, 10). Arsenic-resistant bacteria utilize multiple arsenic-resistance operons for arsenic detoxification in complex and polluted environments (11).

Bacteria are one of the most diverse groups of microorganisms on Earth and less than 1% of bacteria are cultivable. Furthermore, microorganisms coexist with each other and in their habitat, and the pure cultures cannot express the true state of organisms in the natural environment. Culture-independent molecular methods concerning 16S rRNA genes such as next-generation sequencing (NGS) or high-throughput sequencing can effectively introduce the microbial ecology in arsenic bioremediation. (13). The advantages of NGS method are short analysis time, lower sample input requirements specificity and high accuracy. Depending on the type of sequencing method, the result of the analysis can be different, and Illumina and Ion Torrent are the most sensitive sequencing platforms.

Numerous NGS studies have been showed the adverse effects of As and other metalloids on microbial communities in the soil. Shifts in bacterial communities in mine tailing dumps with high heavy metal contents were examined using NGS techniques and shown the *Gammaproteobacteria* was the dominant taxon (15). In arsenic-polluted paddy soils, arsenic reduces the bacterial diversity specially the abundance of *Actinobacteria* and *Acidobacteria* (18). Conversely, as pollution increased relative abundance of *Chloroflexi*, *Betaproteobacteria* and *Bacteroidetes* were increased (12). The bacterial diversity analysis in arsenic-polluted mine tailings has significant impacts on the bacterial communities, and acid-metal tolerant groups were dominants (15-17, 19).

Ecological research on mining waste suggests the richness and diversity of microorganisms have significantly changed in these areas. The short-term effects of heavy metals include decreases in the microbial community. Heavy metals also inhibit the growth of metal-sensitive microbial community. Moreover, prolonged exposure to heavy metals causes natural selection and expression of the most compatible genes with existing conditions (14).

The present study employed high-throughput sequencing technology to investigate the diversity of the bacterial community in the arsenic-polluted tailings dam effluent (TDE) of Zarshuran gold mine (20). The diversity of arsenic-resistant bacteria was analyzed in three arsenic-contaminated groundwater (GW) microbiomes including G3, G6, and G19. The differences in the richness and diversity of bacterial communities were assessed between the TDE and GW samples. Major microbial activities of the arsenic cycle in the TDE were characterized.

Materials and methods

Site description and sample collection

Zarshuran gold mine (36°41'02.7"N, 47°08'05.6"E) is located in West Azerbaijan Province, Iran. For metagenomics analysis, 5 liters of the arsenic-contaminated wastewater from TDE were collected using the sterile polypropylene bottles in June 2018, and straightly transferred to the laboratory.

Wastewater Chemistry

The sampling was performed according to the standard methods of water and wastewater (22), and then its heavy metals were assayed using the polarography method (Metrohm-Model 797 VA). Polarography analysis was performed for arsenic elements in the range of –0.7 till –0.55 V. Simultaneous detection of Ni-Co and Zn-Cu-Pb-Cd was conducted using dropping mercury electrodes in the range of -1.25 till -0.8 V, and -1.25 till +0.05 V, respectively. Solutions were de-oxygenated with a stream of nitrogen gas (1atm).

Genomic DNA extraction and DNA amplification and sequencing

Five liters of TDE wastewater were filtered using 0.22µ sterile nitrocellulose filters (Millipore), and the filters were stored at -80 ◦C for further analysis. The DNA of the samples was extracted using DNeasy PowerWater Kit (Qiagen, Germany), and then stored at -20 ◦C till next processing. The

universal bacterial primers, 27F and 1492R, were used to amplify the full length of 16S rDNA of environmental microbiome. DNA concentration and its purity was checked using NanoDrop 2000 (Thermo, USA) and electrophoresis (agarose1% (w/v)). Approximately 80 ng/µl of the purified PCR product was sent to Macrogen, Inc (Seoul, South Korea) for next amplification, and sequencing. The second PCR amplification was for V3-V4 region of the 16S rRNA genes, which was carried out using the universal 341F/785R primers. The PCR amplicons were sequenced by the paired-end sequencing using Illumina MiSeq (Macrogen, Inc). The raw sequencing data was conceded to the NCBI Sequence Read Archive (SRA), which has accession number PRJNA721938.

Analysis of community by 16S rRNA gene

Read data was assayed using QIIME 2 (https://qiime2.org) as provided by manuals (https://docs.qiime2.org/2019.10/tutorials/moving-pictures/) (23).

The microbial diversity composition of TDE wastewater was compared by three arsenic-contaminated GWs. Three ground waters' microbiomes data (G3, G6 and G19) of Rayong in Thailand were accessible. The bacterial diversities had been determined for the V3-V4 region of the 16S rRNA gene (24), and their FASTQ files [G3 (SRR6760375), G6 (SRR6760374), and G19 (SRR6760381)] were accessible in ENA database. The sequence quality control and table construction of the feature were made using DADA2 (p-trunc-len-f 250–p-trunc-len-r 240–p-trunc-q 15–p-trim-lef-f 11–p-trim-lef-r 16) (25). The settings for quality control were calibrated according to the reads' quality distribution over the length of the sequence. These sequences were grouped into the operational taxonomic units (OTUs) based on a 100% similarity threshold. Alpha rarefaction analysis, the taxonomic classification of OTUs, alpha diversity (the number of observed OTUs, Shannon diversity, and Faith's phylogenetic diversity), and beta diversity (Jaccard distance, Bray-Curtis distance, unweighted, and weighted UniFrac distances) were analyzed using QIIME 2. For taxonomic classification, Greengenes 99% OTUs (13_8 release) were utilized as 16S rRNA gene databases (26). The taxonomic classifier was carried out in QIIME 2, originally trained by 341F/785R region of Greengenes registered sequences. The statistical assessment for diversity metrics and creation of principal coordinate analysis (PCoA) schema were calculated. Furthermore, beta diversity metrics were performed through QIIME 2. Sampling depth was set to 98515. The heat map is generated concerning on Qiime taxa filter-table and Qiime feature-table to retain only features which were annotated to the genus level by the most frequent (>300 reads) in datasets.

Results

TDE geochemistry

Arsenic concentration, pH, total organic carbon (TOC), electrical conductivity (EC) and heavy metals concentrations (Zn, Cu, Pb, and Ni) were measured in the TDE. Determination of physicochemical properties, and heavy metal concentration of the TDE wastewater showed the considerable concentration of (µg/l) of zinc (71.5), copper (108.2), lead (59.7), nickel (20.1), cobalt (4.9), cadmium (<0.471), and arsenic (7750). The physicochemical parameters were determined pH 8.02, conductivity 56.98 µS/cm and TDS 36.98 mg/l. Parameters associated with physicochemical properties and heavy metals in the GW samples were adopted from literature (Table 1) (24).

Table 1. The geochemical parameters and sequence information of the samples

		Geochemical parameters of samples				Information of sequences obtained by Illumina sequencing method			
Site	SRA Accession Number	pH	EC (µs cm^{-1})	Total As (µg l^{-1})	Land use	Number of reads	Number of high-quality reads	Number of phyla	Number of genus
TDE	PRJNA721938	8.02	56.98	7750	Mine	98515	12966	10	63
G3	SRR6760375	7.85	496	62.79	Medium community	133557	16026	27	203
G6	SRR6760374	6.36	335	159.76	Agriculture	172242	17574	37	142
G19	SRR6760381	7.40	675.00	56.52	Mine	105532	11109	24	119

Richness and evenness

The Illumina-MiSeq data analysis of the V3-V4 region of bacterial 16S rRNA genes showed a mean Phred quality score of 30 (Phred Q30) in 82.38% of 197,030 paired-end reads. After removing primers and chimeras with an average read length of 250 bp and filtering the length/quality (quality threshold>15), 11109-17547 reads were obtained with a mean frequency of 14418.75. A plateau was observed at a sequencing depth 98515 for Shannon-Wiener curve of all the samples, suggesting the depth adequacy of sequencing (Fig. 1). An increase in the sequence number was also observed on the rarefaction curve of each sample as a function of the OTUs.

In ecology, the rarefaction curve is used as a tool to evaluate species richness. The curve allows the calculation of species richness for a certain volume of specified samples. This curve initially has an exponential growth, in which the most abundant species are found. When the curve reach to the plateau state, rare species are also identified. The larger sample size identify the more species.

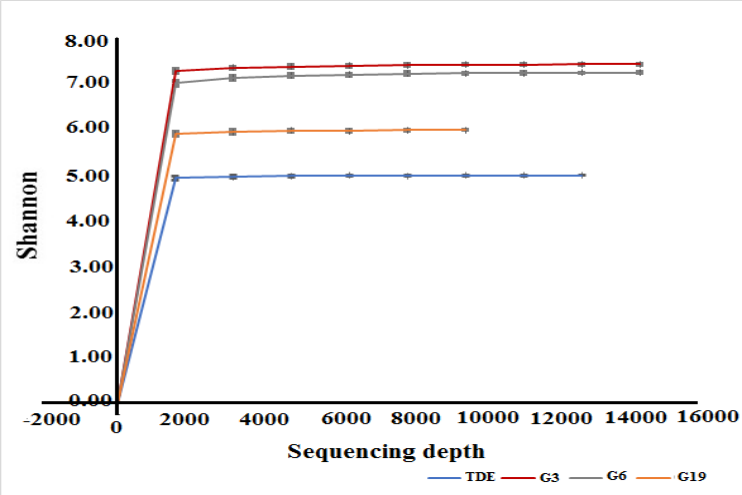

Fig. 1. Shannon-Wiener curves of each sample; these curves were all calculated at 99% level of similarity with Illumina Miseq data for microbiomes of TDE and GW samples.

Bacterial diversity

The QIIME2 data analysis showed 1191 OTUs in four samples, suggesting that 99.94-100% of the reads were assigned to bacteria and 0-0.06 % to archaea. Forty-five bacterial phyla and 441 bacterial genera were retrieved from the samples. Archaeal sequences were affiliated to *Euryarchaeota*, *Parvarchaeota*, and *Crenarchaeota* which detected only in GW samples.

Common sequences of the prevalent taxonomic groups in all the samples were affiliated to *Proteobacteria* (8.06-45.49%), *Bacteroidetes* (1.85-50.32%), *Firmicutes* (1.00-6.2%), *Actinobacteria* (0.86-5.09%), *Planctomycetes* (0.05-9.37%), and *Cyanobacteria* (0.6-2.71%) (Fig. 2). As the main phylum (3 out of 4), *Proteobacteria* accounted for 62.21% of total valid features.

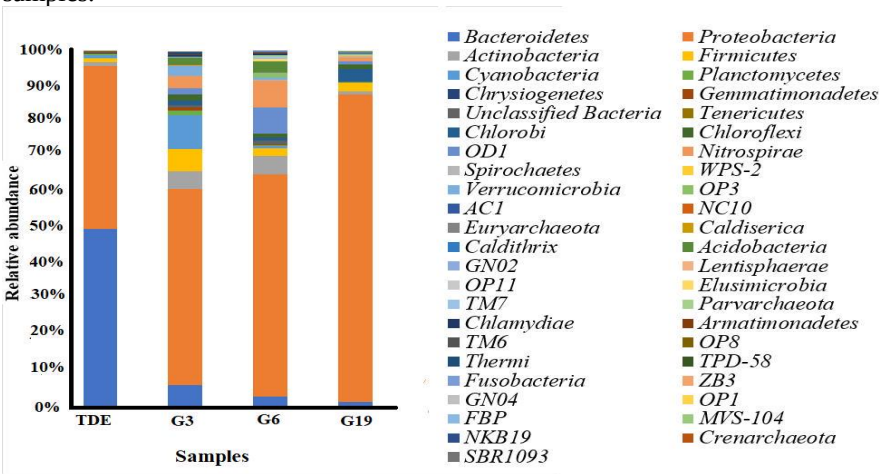

Fig. 2. The taxonomic composition at the level of phylum

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The TDE primarily comprised *Bacteroidetes* (50.3%), *Proteobacteria* (45.49%), *Actinobacteria* (1.14%), and *Firmicutes* (1.08%), which was inconsistent with the G3 sample containing phyla *Proteobacteria* (54.93%), *Cyanobacteria* (9.37%), *Bacteroidetes* (6.46%), and *Firmicutes* (6.2%). A shift was observed in the G6 sample and four abundant phyla comprised *Proteobacteria* (62.38%), *Nitrospirae* (7.6%), *OD1* (7.3%), and *Actinobacteria* (5.08%). *Proteobacteria* (86.06%) followed by *Chlorobi* (3.57%), *Firmicutes* (2.3%), and *Bacteroidetes* (1.85%) were the most abundant phyla in the G19 sample collected from areas surrounding the mine.

A total of 441 genera were obtained from all the samples, including 203 from G3, 142 from G6, 119 from G19, and 63 from the TDE. Table 2 presents the dominant bacterial genera among the four samples (≥1% of total OTUs). Richness slightly decreased at the genus level in G3, G6, G19, and TDE samples. Six dominant genera were retrieved from the TDE sample, 178 from G3, 8 from G6, and 6 from G19. Members of *Erythrobacteraceae* (10%), *Rhodobacteraceae* (1%) and *Beijerinckiaceae* (1.08%), and order of *Sphingobacteriales* (1.02%) observed in the TDE sample were not identified at genus or family levels, suggesting unknown or unclassified bacteria in the TDE samples.

A higher abundance of over 1% of the total sequences in the TDE sample was observed only in six genera, including *Algoriphagus* (*Bacteroidetes*), *Rhodobacter* and *Anaerospora* (*Alphaproteobacteria)*, *Limnobacter* (*Betaproteobacteria)*, *Halomonas* (*Gammaproteobacteria),* as well as *Yonghaparkia (Actinobacteria),* as a unique dominant genus in the TDE sample. The most abundant genera included *Algoriphagus (*45%), *Rhodobacter* (12.7%), and *Limnobacter* (5.3%).

The present research found significant differences between the TDE and GW samples in terms of their microbial community structure. *Pseudomonas* and *Acinetobacter* in G3 and *Acinetobacter* in G19 were highly abundant in the low-arsenic GW sites. The vast majority of reads in G6 were related to an unidentified genus. The rarely-observed bacteria included *Methylosinus* in all the samples, *Pseudomonas* in TDE, G3, and G19 and *Bacillus* and *Nocardioides* in TDE, G3, and G6. The samples were; however, different in terms of their abundant genus. Fig. 3 shows the heat maps of the main feature (>300 reads) in datasets.

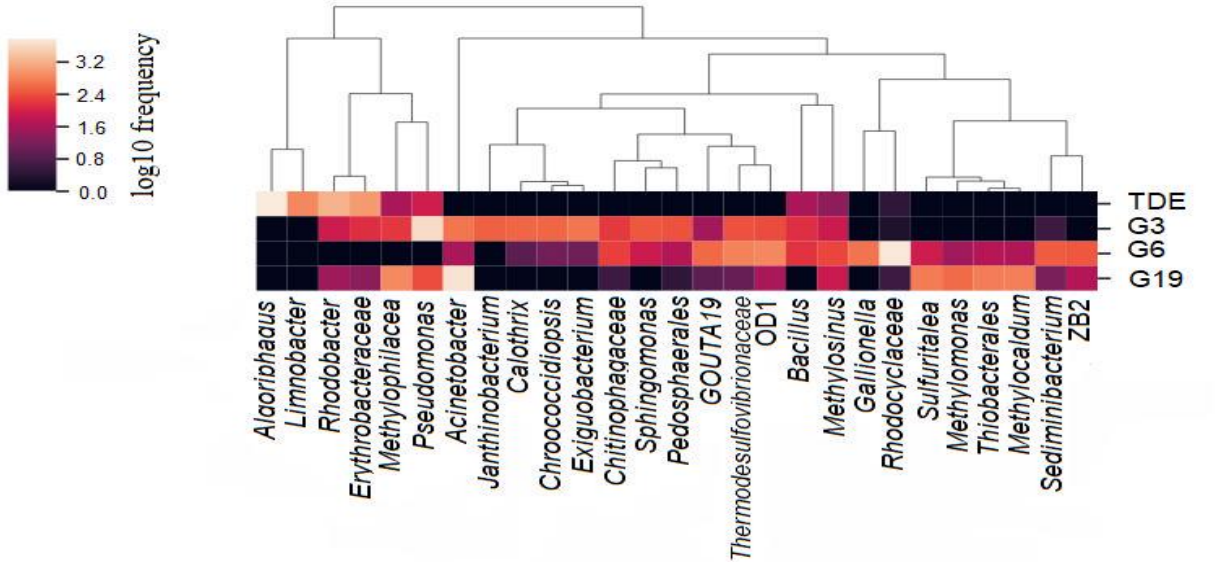

Fig.3. Heat map of the most frequent feature (>300 reads) in datasets (TDE, G3, G6, and G19). Darker and lighter colors represent lower or higher frequency, respectively.

Table 2. The bacterial genera were retrieved in the arsenic-contaminated TDE and GW samples.

Phylum	Class	Genus	TDE	G3	G6	G19		
Proteobacteria	*Alpha-*	*Anaerospora*						
		Bradyrhizobium						
		Brevundimonas						
		Erythromicrobium						≥10%
		Parvibaculum						
		Reyranella						
		Rhodobacter						≥5%
		Rubellimicrobium						
		Sphingomonas						
	Beta-	*Aquaspirillum*						≥3%
		Gallionella						
		Limnobacter						
		Janthinobacterium						≥2%
	Gamma-	*Acinetobacter*						
		Halomonas						
		Lysobacter						≥1%
		Methylocaldum						
		Methylomonas						
		Methylococcus						<1%
		Pseudomonas						
		Psychrobacter						
		Rheinheimera						
	Deltaproteobacteria	*Geobacter*						
Actinobacteria	*Actinobacteria*	*Yonghaparkia*						
Bacteroidetes	*Cytophagia*	*Algoriphagus*						
	Flavobacteriia	*Chryseobacterium*						
	Saprospirae	*Sediminibacterium*						
Cyanobacteria	*Nostocophycideae*	*Calothrix*						
		Scytonema						
	Oscillatoriophycideae	*Chroococcidiopsis*						
	Synechococcophycideae	*Leptolyngbya*						
Firmicutes	*Bacilli*	*Exiguobacterium*						
	Clostridia	*Staphylococcus*						
		Clostridium						
Nitrospirae	*Nitrospira*	*GOUTA19*						
		LCP-6						

Bacterial community structure

The TDE and three GW samples were divided into two groups according to PCoA. The first group including the TDE sample was characterized by a high concentration of arsenic (7.75 mg/l). The other group included three GW samples (G3, G6, and G19) with low arsenic concentrations (62.79 µg/l, 159.76 µg/l, and 56.52 µg/l, respectively) (Table 1). The average numbers of the observed alpha diversity indices of Pielou's evenness and Faith's phylogenetic diversity were not significantly different (P=0.18, Kruskal-Wallis test). The beta diversity analysis (unweighted) also showed insignificant differences between the TDE and GW samples in terms of OTUs and diversity of bacterial population (PERMANOVA, P= 0.276, 999 permutations). Fig. 4 shows the principal coordinates of Jaccard and Bray-Curtis distance as the qualitative and quantitative measures of community dissimilarity.

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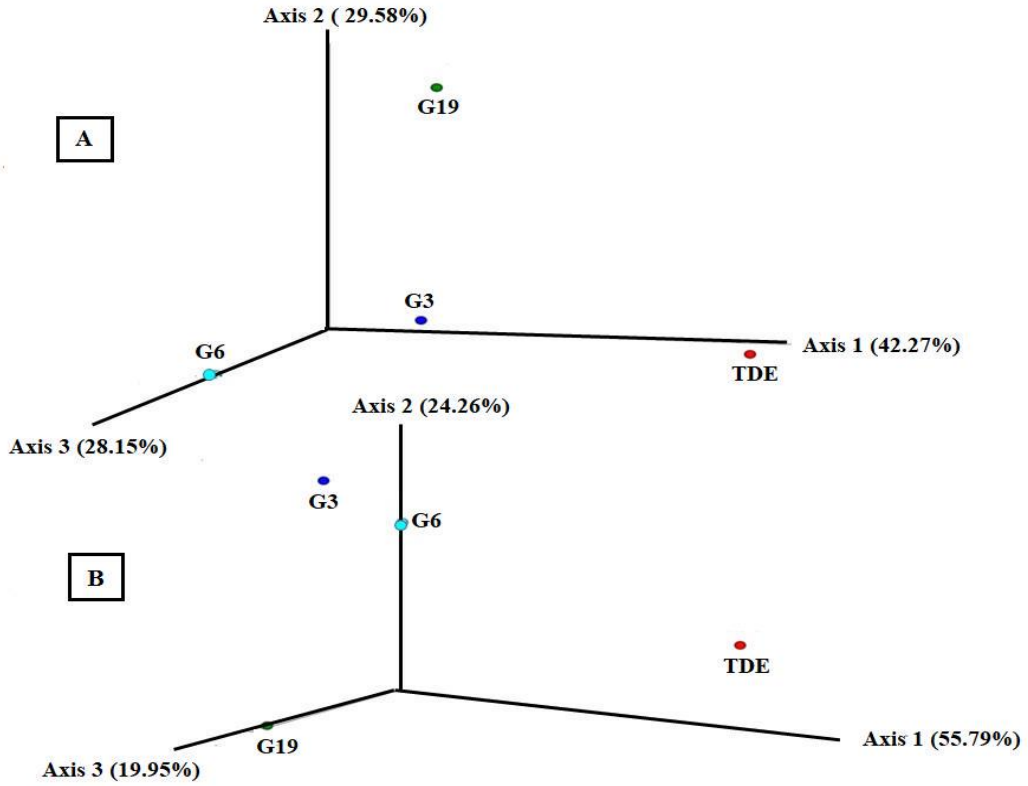

Fig.4. PCoA analysis of arsenic polluted water, (A) PCoA plots of unweighted-unifrac. Each point indicates an individual sample and clustering of the points means the similarity in the components of OTUs among those samples. (B) PCoA plots of weighted-unifrac.

Discussion and conclusion

The main types of minerals in Zarshuran gold mine area are orpiment (As_2S_3) (> 60%), realgar (As_2S_4), and arsenopyrite (FeAsS) (21), which are soluble in alkaline solutions. Different methods such as cyanidation are used in gold mining. This leads to the dissolution of orpiment mineral ores in alkaline solutions, which releases arsenic species in TDE (21). Mine tailings contain limited organic substances (27), and microbiological activities are influenced by the type and chemistry of metal(loid)s (28). The present results confirmed previous findings suggesting *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Acidobacteria*, *Gemmatimonadetes*, and *Planctomycetes* constitute the main phyla in gold mine tailings contaminated with arsenic oxyanions [16, 27].

The TDE was less abundant in diversity (10 groups) at the phylum level than the GW samples, which can be explained by the high concentrations of arsenic, cyanide, antimonite, and other toxic heavy metals and the limited number of available electron donors and acceptors in the TDE (29). This low bacterial diversity in the TDE is consistent with previously reported findings (30-32). A low bacterial diversity was observed in the acidic drainage of the mine, which contained arsenic concentrations as high as 750-2700 mg/l.

The feature analysis of the NGS library showed *Proteobacteria* with a high frequency in all the samples irrespective of the arsenic components, which can be explained by the resistance or the tolerance mechanisms of *Proteobacteria* (12). Numerous studies found *Proteobacteria* to be the main phylum (33-35). The present study found *Bacteroidetes* to be the main phylum in the TDE. Bacterial communities isolated by Riley et al. from a river in South Africa contained different contaminants, including arsenic, chromium, nickel, and uranium. The type and abundance of bacteria at phylum level depending on concentrations of contaminants were significantly lower downstream compared to upstream sites. *Firmicutes*, *Acidobacteria*, *Cyanobacteria*, and *Verrucomicrobia* were prominent at downstream sites, while *Bacteroidetes* was more frequently observed in the contaminated areas (36). The high frequency of *Bacteroidetes* can be attributed to the harsh environment of the TDE and resistance or tolerance of this group of bacteria. Investigating the susceptibility of 105 strains of *Bacteroidetes* to heavy metal ions (As, Ag, Ni, Co, Pb, Cd, Cr, and Hg) found all strains of *Bacteroidetes* to be multiple

resistant (36). *Bacteroides vulgatus* ATCC 8482 is highly resistant to As^{5+} and methyl arsenate. The arsenical-inducible transcriptional unit (*ars* operon) in the genome of *B. vulgatus* ATCC 8482 includes eight continuous genes. Furthermore, *arsR* and *arsDABC* genes of this operon play a key role in the detoxification of arsenic (37). About 0.2% of the sequence reads of the OTUs also belonged to the unclassified bacterial taxa, which showed unknown microorganisms in this TDE sample.

Algoriphagus, *Rhodobacter*, and *Limnobacter* are the most abundant genera in the TDE sample. *Algoriphagus* was identified in shallow aquifers (38) and sediments (39) with high arsenic levels using metagenomics methods can reduce As^{5+} to As^{3+} using nitrate, acetate, sulfide, and Fe(II) as electron donors.

As facultative anaerobes and photosynthetic bacteria, purple non-sulfur bacteria (PNSB) are flexible in carbon source utilization and resist to As^{5+}, As^{3+}, and other heavy metal ions. Different resistance mechanisms used by many PNSB and several other bacteria include conversion of metals to a reduced toxic state through modifying their oxidation state, arsenate reduction, arsenite methylation pathways (11, 40, 41) and efflux of heavy metals out of the cells (42). Under anaerobic conditions, *Rhodobacter* can respire A^{+5} and oxidize certain carbon sources for hydrogen photofermentation (43). Certain *Rhodobacter* species can generate exopolysaccharides with arsenic and lead chelating activities (44). The high abundance of *Rhodobacter* in the harsh conditions of the TDE showed the potential of this organism for both bioremediation and use of effluents contaminated with arsenic and other heavy metals as a substrate.

As a sulfur-oxidizing bacterium, *Limnobacter* was the third abundant genus in the TDE sample, suggesting a feasible practical biogeochemical sulfur cycle in this area (45). The abundance of *Limnobacter* in the TDE of Zarshuran mine ores containing sulfide minerals is normal. This genus comprises species such as *Limnobacter litoralis* (46) and *Limnobacter thiooxidans* (47) which can oxidize thiosulfate. Although this study did not investigate thiosulfate, *Limnobacter* spp. can play a role in sulfur or thiosulfate oxidation.

Anaerospora is a genus with sulfur and/or iron-reducing potential. *Anaerospora hongkongensis* can perform ferrihydrite reduction and anaerobic oxidation of ammonium driven by sulfur redox cycling (48). Observing this group of bacteria is normal, considering the use of ammonia as an oxidizing agent for sulfur-bearing minerals in gold mining. This genus can be also involved in the arsenic cycle given that it was identified in paddy soil bacterial communities at different heavy metal levels (49). The bacteria belonging to the *Yonghaparkia* genus and isolated from microbial communities in a uranium mine (Athabasca Basin, Canada) (50), a gold mine (Linglong, China) (51), and the black shale (China) (52) can contribute to the biogeochemical cycles of S and Fe.

As an arsenic-resistant gamma-proteobacterium, *Halomonas* sp. was frequently observed in both the TDE sample and arsenic-contaminated environments. *Halomonas* sp. strain GFAJ-1 was found to use arsenic as an alternative to phosphorus to survive under phosphate deficiency conditions (53). The arsenate reductase and arsenite oxidizing activities of *Halomonas* sp. play key roles in arsenic biogeochemical cycles (54). *Halomonas* species contain *arxA* gene and can contribute to *arx*-dependent arsenite oxidation or couple As^{3+} oxidation with nitrate reduction (55). The increased exopolysaccharide production observed in *Halomonas* sp. isolated from the rhizosphere under arsenic stress plays a key role in arsenic sequestration (56). In line with literature, the present study found *Pseudomonas* and *Acinetobacter* isolated from aquifers with high arsenic levels to be involved in the arsenic cycle, including arsenic resistance, As^{5+} reduction and As^{3+} oxidation (57, 58).

This study was conducted on the bacterial diversity and community structure of a gold tailings dam. Metagenomics analyses showed *Algoriphagus*, *Rhodobacter*, *Anaerospora*, *Limnobacter*, *Halomonas*, and *Yonghaparkia* to be the main bacterial genera. Despite the limited similarities in the prokaryotic communities, the most retrieved genera in the TDE are unique, and we retrieved the native bacterial genus of Iran. The present study suggested the key role of functional bacteria in the geochemical cycles of heavy metals, which helps with the scientific management of the bioremediation and ecological risk assessments of gold mine tailings.

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