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METAGENOMICS (16S AMPLICON SEQUENCING) AND DGGE ANALYSIS OF BACTERIAL DIVERSITY OF ACID MINE DRAINAGE

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ABSTRACT

Acid mine drainages (AMDs) are characterized as low pH water containing metal, which is the best-known habitat for the microorganisms described as acidophiles. Most of these members do not survive above pH 4.0; therefore they are acidophilic. Because of these metabolic properties of acidophilic microorganisms, AMD is accepted as a model system in biogeochemical studies on iron and sulfur cycles for the analysis of microbial ecology.
In this study, microbial diversity of the acid mine drainage in Çan (Çanakkale) was investigated by using culture-independent techniques including Denaturing Gradient Gel Electrophoresis (DGGE) and high-throughput sequencing.
A total of 105,593,810 and 115,689,952 bases read were performed. Following sequencing, raw data were proceeded in "Quantitative Insights Into Microbial Ecology" (QIIME 1.9.1). *Acidiphilium*, *Acidocella* and *Acidosoma* were genus obtained by bioinformatic analysis. The highest rate belonged to *Acidiphilium* for each water sample. *Acidiphilium cryptum*, *Acidithiobacillus* sp. and *Acidibacter ferrireducens* were determined according to DGGE analysis.

Keywords: acidophiles, diversity, metagenomics, 16S amplicon sequencing

INTRODUCTION

The best documented artificial water area associated with mining is acidic, metal-rich water, commonly called as "acid mine drainage" (AMD) (Johnson and Hallberg, 2003). AMD is the best-known habitat for the acidophilic microorganisms and characterized by low pH (Chen et al., 2016) and heavy metal etc. (Johnson, 1998). The processed water from deep and surface levels of coal/metal ore mines includes the microbial load of mines near environment. This water has highly variable contaminations depending on such factors including the structures of the ore body and associated geological layers, climate and mining engineering constraints. This drainage has soluble iron and sulfide in high concentration. On the other hand, trace elements including barium, cadmium, copper, manganese, molybdenum, nickel, lead, selenium and zinc in acid mine drainage are in a lower concentration than iron due to three main reasons (Johnson, 2003). First of all, heavy metals and metalloids have been organized as sulfide minerals and accelerate microbial oxidative dissolution. Secondly, solutions with rich acidic ferric iron occur after pyrite dissolves aggressively. Finally, numerous metals are more soluble in acidic waters (Johnson, 2003).
Microbiota in acidic and metal rich environments has substantial variety. Many of these members are acidophilic and do not survive above pH 4.0 (Johnson, 1998). Therefore, acidophilic microorganisms isolated from acidic environments (pH<3) have both metabolic and phylogenetic diversities (Johnson, 2003; Johnson and Hallberg, 2003; Johnson and Hallberg, 2005; Oren, 2010). Acidophilic microorganisms are classified as anaerobic, aerobic and facultative according to use of oxygen and are classified as phototrophic, chemotrophic, heterotrophic according to nutrition synthesis (Oren, 2010; Chen et al., 2016). Microbial species growing in acidic environments can use iron, sulfur, nitrogen, carbon and oxygen in their metabolisms. Sub-cycling of these elements was described in some starting studies. Different models were shown for only iron and sulfur cycling in remarkable examples. These studies were carried out by biomolecular and culture-dependent methods and more recently omics approaches (Méndez-García et al., 2015).
Acidophiles have evolved by developing adaptations to the extreme conditions and these adaptations have effectively accelerated acid production process (Chen et al., 2016). For this reason, they have a vital role in the conservation of energy and material flow in the ecosystem/ microenvironment. AMD environments are ideal targets for biogeochemical studies on iron and sulfur cycles as model systems for quantitative analysis of microbial ecology and community function (Navarro et al., 2013; Johnson, 2014; Chen et al., 2016;Gumulya et al., 2018).
Metagenomics was firstly used by Handelsman and co-workers (Handelsman et al., 1998) for analysis of function and sequencing of microbial genomics extracted from environmental samples. The shotgun metagenomics have the full recruitment capacity of most of the genomes present in an environmental sample. This creates the biodiversity profile associated with a functional analysis of known and unknown organism species. With shotgun metagenomics, answers were given to the question of who is present in the environmental community, what they are doing functionally and how they interact in ecological location. Nowadays, the metagenomics approach of a marker gene in which the related genetic polymerase chain reaction is used. The marker gene metagenomics in which the respective genes are located is used for the fingerprint or taxonomic distribution of the conserved 16S rRNA gene region. This approach has received considerable attention recently in microbial diversity studies (Oulas et al., 2015).
Jones and coworkers (2012) have investigated the community, physiology and biogeochemistry of biofilm via metagenomics, rRNA methods and lipid geochemistry. They have found *Acidithiobacillus thiooxidans* (>70) as dominant species, and *Thermoplasmatales* represented with small population in low level (Jones et al., 2012).
Chen and colleagues (2015) have studied metagenomic and metatranscriptomic analysis of microbial diversity in AMD. They found *Acidithiobacillus, Leptospirillum* and *Acidiphilium* with high transcriptional activities as a result of taxonomic analysis. They have investigated microorganisms which are adapted to the AMD environment by regulating the expressed genes contained in multifunctional activities, especially low pH and other activities in organism-wide comparative analyses (Chen et al., 2015).
In another study, Liljeqvist *et al.,* (2015) have studied a diversity of microorganisms equipped for growth at low temperatures and acidic pH in AMD in Kristineberg, Sweden. They have done metagenomic analysis of the biofilm and planktonic fractions and found that microorganism to be similar to the psychrotolerant acidophile, *Acidithiobacillus ferrivorans*. Metadata showed *Acidithiobacillus* species, an *Acidobacteria*-like species, and a *Gallionellaceae*-like species. Furthermore, they have predicted genes which encode functions

related to pH homeostasis and metal resistance related to growth in acid mine water (Liljeqvist et al., 2015).
The aim of this study was to show the microbial diversity of AMD in Çan, Turkey with culture-independent methods including DGGE and metagenomics (16S amplicon sequencing). In addition to the fact that there are few studies with a metagenomics approach for determining acidophile microbial diversity in the world, this study will constitute as the first metagenomics data to literature concerning acidophilic microorganism diversity in Turkey. We investigated the population structure in water samples from acid mine drainage and found that microorganism with heterotrophic character according to metadata. Similar results have been exhibited via DGGE analysis.

MATERIALS AND METHODS

Sampling area

Water samples were collected from the surface of the AMD in Çan, Turkey (March, 2017). The sampling area was located at 39° 58' 7" N, 26° 51' 55"E (Figure 1). Two samplings of this area were performed and the pH values of samples were measured.

Figure 1 Sampling area on Turkey map

Chemical analysis of samples

Water samples were sent to commercial measurement services (Acme Analytical Lab. Ser. Co. Ltd., Ankara, Turkey) to analyze the metal and semi-metal contents of samples. ICP-MS (PerkinElmer, ELAN 9000) was used to determine concentrations of elements.

Total DNA extraction and metagenomics analysis

GTTP membrane filters (0,22 µm pore size, Merck Millipore, Germany) were used to collect the microorganisms. Two water samples were filtered by vacuum pump. These filtrates were broken up to into small pieces and total DNA was extracted using UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, USA) according to the manufacturer's instructions. The DNA was analysed by 1% agarose gel electrophoresis and imaged by Gel DocTM XR+ System with Image LabTM Software (BIO-RAD). The DNA concentrations were measured by NanoDrop™ 2000/c Spectrophotometers (Thermo Scientific, USA).
DNA from these water samples were also sent to a commercial sequencing service (BM Software Consultancy and Lab. System Co. Ltd. Ankara, Turkey) for metagenomic analysis. The analysis was 16S Ribosomal RNA Gene Amplicons by the Illumina MiSeq System. The target in NGS analysis was V3-V4 region amplified with the 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) primer pair for prokaryotes. 2.5 µL of microbial environmental DNA was used as a template for amplification of V3-V4 region. The reaction was performed in a total volume of 25 µL including 12.5 µL of 2x KAPA HiFi Hot Start ReadyMix, 5 µL of each Amplicon PCR Primers (1 µM). The reaction conditions comprise of the initial denaturation step at 95 °C for 3 minutes, 25 cycles at 95 °C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 minutes then final extension step at 72°C for 5 minutes. AMPure XP beads were used to purify the free primers and primer dimer species in the amplicon product. Index PCR attaches the Illumina Sequencing adapter via Nextera XT Index Kit. N719 (GCGTAGTA) - N515 (TTCTAGCT) (index sequence for Ç1) and N719 (GCGTAGTA) – N516 (CCTAGAGT) (index sequence for Ç2) index primers were used in amplification with 8 cycles. AMPure XP beads were again used to clean up the final library. Validation process was performed on Bioanalyzer DNA 1000 chip. Sequencing was practiced by the Illumina Miseq platform (Illumina Miseq, 16S Metagenomic Sequencing Library Preparation Part # 15044223 Rev. B).

Bioinformatics analysis of metagenomics raw data

Raw data were processed with QIIME 1.9.1 (http://qiime.org/) by entering the specific commands in Linux. Mapping files including sample names, barcode sequence, linker primers sequences, description and any information about sampling area were prepared. Libraries were processed together using QIIME version 1.9.1 (Caporaso et al., 2010a). Poor-quality sequences were discarded based on Phred score, primer mismatches, divergence from expected amplicon length (298 base pairs) using the default settings in QIIME (split_libraries_fastq.py). Sequences were clustered into *de novo* operational taxonomic units (OTUs) at 97% similarity using QIIME's pick_de_novo_otus.py command. Singleton OTUs (defined as OTUs represented by only a single representative sequence across all samples) were discarded. Representative sequences from each OTU were aligned using PyNAST (Caporaso et al., 2010b) and classified according to the Greengenes taxonomy (version gg_13_8; McDonald et al., 2012). After the quality filtering process, 61,372 valid sequences remained and were used in subsequent analysis. Then the read-pair merging approximately 75% of the reads were remained.

Denaturating gradient gel electrophoresis (DGGE) analysis

DGGE PCR products were amplified using total DNA as a template in reaction mix. 341F-GC and 907R pair of primers were used for Bacteria domain respectively as forward and reverse primer to amplify products in 2xMaster Mix (Ampliqon, Denmark). Touchdown PCR was used in DGGE analysis. PCR was implemented in 5 min at 94 °C, 1 min at 65 °C, 3 min at 72 °C for first cycle; next 9 cycles were performed with decreasing 1 °C in annealing step per every cycle. The next 20 cycles was implemented in 1 min at 94 °C, 1 min at 55 °C, 3 min at 72 °C. The last cycle was implemented in 1 min at 94 °C, 1 min at 55 °C, 10 min at 72 °C. DGGE PCR products were run into 1% Agarose Gel Electrophoresis and imaged by Gel DocTM XR+ System with Image LabTM Software (BIO-RAD). 16S PCR products from agarose gel were cleaned up using GeneJET™ Gel Extraction Kit (Fermentas).
DGGE analysis was performed with D-Code System (BioRad) according to modified protocols describing by Muyzer and coworkers (Muyzer et al., 1993). The samples were loaded into DGGE gels containing polyacrylamide with a urea-form amide gradient being from 40 % to 60%. The gel was run at 90 V for 18 h.
Each of bands were cut from the gel and put in the new tubes. DGGE lysis buffer was added on the bands in the tubes which were incubated horizontally at 37 °C. Once incubation, DGGE Re-PCR were performed using liquid section as a template. The PCR products were loaded in gel and run at 100 mV in 70 minutes. The products given as positive bands were sent to commercial sequencing service (BM Software Consultancy and Lab. System Co. Ltd., Ankara, Turkey) for DNA sequence analysis. Chromatograms were analyzed via 4Peaks and the sequences of each band were aligned via BLAST n Nucleotide (nr/nt databank). The accession numbers were provided by submitted the information of uncultured environmental DNA sequences to Submission Portal (https://submit.ncbi.nlm.nih.gov/subs/genbank/).

RESULTS

Metal contents of sampling area

pH values were measured in water samples belonging to Ç1 and Ç2 as 3.0 and 2.9, respectively. Concentrations of metal contents in these samples were mentioned in Table 1. Concentrations of the metals mentioned in the table are given as ppb or ppm. When the obtained data were analyzed, it can be seen that there were dominantly chemicals such as aluminum (Al), boron (B), calcium (Ca), cobalt (Co), iron (Fe), sulfur (S), strontium (Sr), potassium (K), magnesium (Mg), zinc (Zn), manganese (Mn) and sodium (Na) in water samples. These chemicals and their concentrations were indicated as bold in previously mentioned table.

Table 1 Metal analysis of water samples from acid mine drainage

METAL	Ç1	Ç2
AL (PPB)	20423	15211
B (PPB)	966	989
CA (PPM)	326.2	324.6
CO (PPB)	549	532
FE (PPB)	10965	<10000
K (PPM)	11	12
MG (PPM)	108	105
MN (PPB)	28618	28296
NA (PPM)	219	219
S (PPM)	565	538
SR (PPB)	2273	2275
ZN (PPB)	1040	843

Total DNA extraction

Concentrations of DNA from Ç1 and Ç2 were respectively found as 25.9 ng/µL and 26 ng/µL. The absorbance ratio between 260 nm and 280 nm was shown to measure protein contamination. This value must be greater than or equal to 1.8. The A260/280 ratios of DNAs from Ç1 and Ç2 were 1.99 and 2.02, respectively.

Processing of Raw Data

During the analysis, total 104,865,386 read bases (bp) of total 349,928 reads were carried out for the Ç1 sample. In the same procedure, total 114,909,694 read bases (bp) of total 383,426 reads were performed for the Ç2 sample (Table 2). After analyses of two environmental water samples, there was no record in domain Archaea but domain Bacteria had 90.5% of total reading. Approximately 9.5% of remaining of the total reading was unassigned. Results could be given separately at the levels of phylum, class, order, family, genus and species. The information expressed in a column graphs could be considered meaningful. Proteobacteria (38.60%), Actinobacteria (16.80%) and Acidobacteria (8%) were the phyla of Bacteria have the most members than other phyla for the Ç1 water sample (Figure 2).

Table 2 Raw data Stats (Sample ID : Sample name; Total read bases : Total number of bases sequenced; Total reads:Total number of reads; GC(%) : GC content; AT(%) : AT content; Q20(%) : Ratio of reads that have phred quality score of over 20; Q30(%) : Ratio of reads that have phrased quality score of over 30)

SAMPLE ID	TOTAL READ BASES (BP)	TOTAL READS	GC(%)	AT(%)	Q20(%)	Q30(%)
Ç1	105,593,810	350,810	55.424	44.58	83.569	74.753
Ç2	115,689,952	384,352	55.579	44.42	83.486	74.787

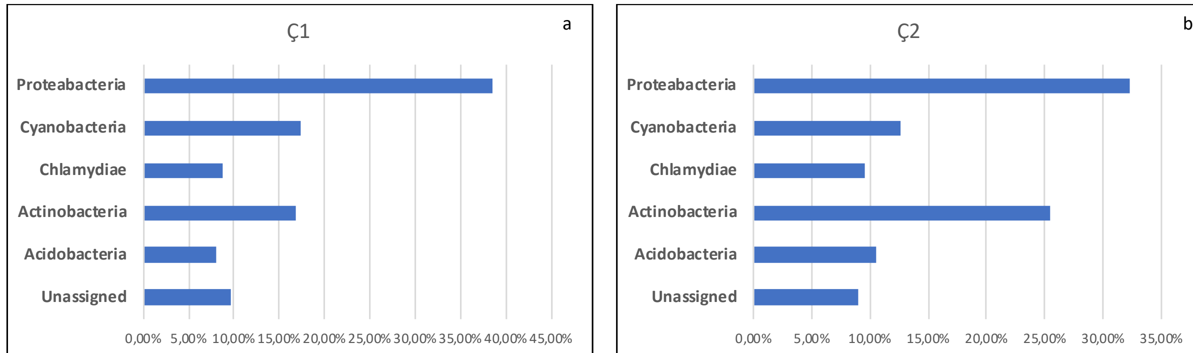

Figure 2 Graphical view at phylum level of metagenomics data. a) phyla of Ç1 b) phyla of Ç2

The results for the Ç2 water samples were similar as results of Ç1. Proteobacteria (32.30%), Actinobacteria (25.50%) and Acidobacteria (10.50%) were phyla including the most members for Ç2 water sample. Proteobacteria in metadata of water samples has three classes at the taxonomic level named as Alphaproteobacteria, Betaproteobacteria *and* Gammaproteobacteria. Actinobacteria and Acidobacteria have a taxonomic class being Acidimicrobiia and Acidobacteriia, respectively. Acidobacteriales, Acidomicrobiales, Ellin329, Rhodospirillales, *Methylophilales, Legionellales* and Xanthomonaales were found at the level of taxonomic order. Acidobacteriaceae, Acidimicrobiaceae and Acetobacteraceae were encountered most strikingly at the level of taxonomic family. *Acidiphilium* was the only identified genus taxon in all results with highest percentage. *Acidosoma* and *Acidocella* were found at a considerable amount following the genus *Acidiphilium* (Figure 3). No detailed description at the species level has been obtained. *Proteobacteria* in Ç1 AMD water sample has members of Alphaproteobacteria (30.40%), Betaproteobacteria (4.60%) *and* Gammaproteobacteria (3.70%) classes and Ç2 AMD water sample has member of Alphaproteobacteria (25.40%), Betaproteobacteria (2.60%) and Gammaproteobacteria (4.40%) classes. 15.10% of total reading is *Acidiphilium* and 18.80% of this genus is in microorganisms of Ç1 and 11.80% of this genus is in microorganisms of Ç2.

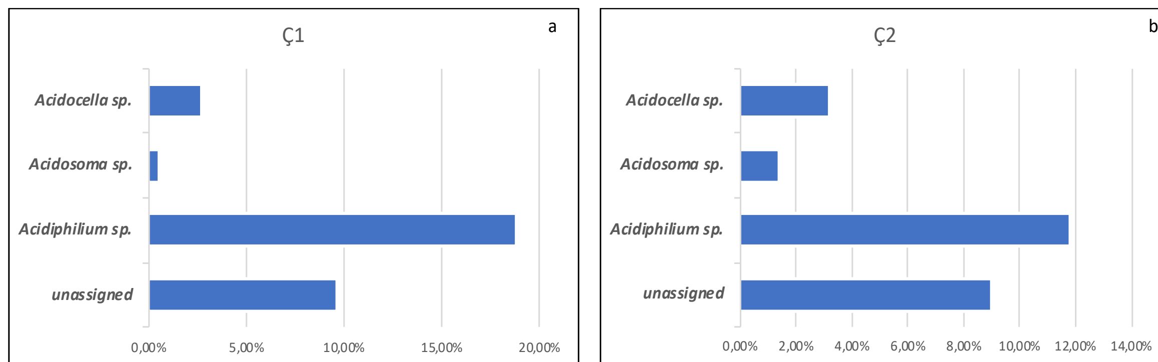

Figure 3 Graphical view at genus level of metagenomics data. a) genera of Ç1 b) genera of Ç2

DGGE analysis

The Figure 4 shows four DGGE bands having of each environmental water samples. The bands were indicated with arrow and numbers. Arrows 1 and 2 indicate the Ç1 sample's diversity and arrows 3 and 4 indicate the Ç2 sample's diversity. According to BLAST alignment, Ç1-1 and Ç1-2 were described as an uncultured bacterium and uncultured *Acidiphilium cryptum* in Ç1 sample DGGE profile. Furthermore, Ç2-1 and Ç2-2 were described respectively uncultured bacterium and uncultured *Acidithiobacillus* sp in Ç2 sample DGGE profile.

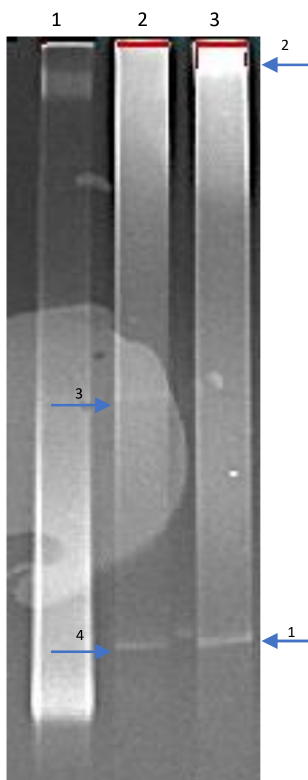

Figure 4 DGGE gel of environmental samples, 1: acidophilic microorganism BY16, 2:Ç2, 3:Ç1. Arrow 1 and 2 belong to sample Ç1; arrow 3 and 4 belong to sample Ç2.

The found results and accession numbers were given in Table 3. The microbial diversity was showed with phylogenetic tree (Neighbor Joining Method) constructed by using MEGA 7.0 and a non-relative species (*Methylobacter bovis* L20839) to see true distance of species was used in this tree (Figure 5). The percentage matrixes of these sequences constructed by Muscle Software are given in Table 4. *Acidibacter ferrireducens* strain MCF85 from Ç1 and Ç2 showed approximately 97.3% percentage identity. *Acidiphilium cryptum* and *Acidithiobacillus* sp. had 74.20% percentage identity.

Table 3 DGGE bands and accession numbers

DGGE Bands	*Accession Numbers*	*BLAST/n*	*Analyzed Base Number*
Ç1-1	MH729781	Uncultered *Acidibacter* sp.	591 bp
Ç1-2	MH735136	Uncultured *Acidiphilium cryptum*	640 bp
Ç2-1	MH735137	Uncultered *Acidibacter* sp.	593 bp
Ç2-2	MH735145	Uncultured *Acidithiobacillus* sp.	592 bp

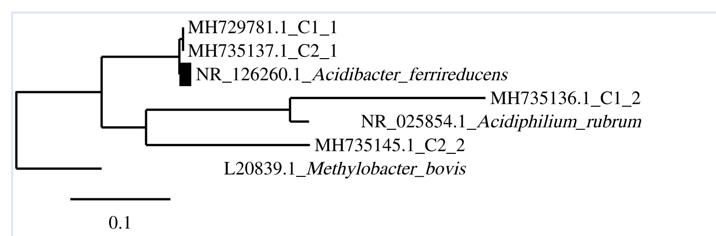

Figure 5 The phylogenetic tree of DGGE bands

Table 4 Percent Identity of DGGE bands sequence

	Ç1-2 (MH735136)	*Ç2-2 (MH735145)*	*Ç1-1 (MH729781)*	*Ç2-1 (MH735137)*
Ç1-2 (MH735136)	100.00	74.20	75.22	75.27
Ç2-2 (MH735145)	74.20	100.00	80.34	80.03
Ç1-1 (MH729781)	75.22	80.34	100.00	99.15
Ç2-1 (MH735137)	75.27	80.03	99.15	100.00

DISCUSSION

Metagenomic studies have been increasing day by day. This approach enables to evaluate better microbial diversity of extreme environments. In this study, Acidobacteria, Proteobacteria, Actinobacteria were detected as dominant phyla according to metagenomic analysis. Other studies utilizing 16S amplicon sequencing of diversity of acidophilic microorganisms have also been reported. Miller and colleagues reported results concerning 16S amplicon sequencing (Illumina Miseq platform; V3-V4 region amplification) of samples in AMD from Colorado. They have revealed Proteobacteria, Actinobacteria, Chlorofexi, and Firmicutes phyla with higher relative abundance from AMD samples (Miller et al., 2018). On the other hand, Aguinaga and colleagues have published an article about 16S gene amplicon sequencing of sediments from AMD. In this study, Protebacteria has been found as dominant phylum in this area. Bacterioideles, Acidobacteria, Cyanobacteria and Actinobacteria respectively higher relative abundance from AMD area (Aguinaga et al., 2018).

AMD waters were characterized as potentially toxic metals including trace and semi-metal, therefore a lot of metal ions in these water samples are expected. It has been known that mining waters are characterized by these metal ions. There were different metals in this study, but iron, phosphorus, lead and manganese were the highest value in analysis.

DGGE analysis showed similar results to metagenomic analysis. *Acidibacter* sp. *Acidiphilium cryptum* and *Acidithiobacillus* sp. were the names of bands defined in profile. Another different result is the presence of *Acidithiobacillus* sp. not being seen in the metagenomic analysis. It is known that *Acidithiobacillus* sp. produces energy by oxidizing the Fe(II) to reduction of O_2 (White et al., 2016). The Fe ion content of Ç2 sample has rather high value in other metal contents. In other studies, data related DGGE analysis has been reported by researches. Gonzalez-Toril and colleagues analyzed the microbial community from AMD in Tinto River by using molecular ecology techniques including DGGE approach. 16S rDNA DGGE-PCR amplification was performed with 341F and 907R primer pair for *Bacteria* and 344F and 915R primer pair for *Archaea*. They found members of different phylogenetic groups including γ-Proteobacteria, α-Proteobacteria, Nitrospira, Actinobacteria and Thermoplasmata in the result of analysis. The population structure contained *Acidithiobacillus* sp., *Acidithiobacillus ferrooxidans, Leptospirillum ferrooxidans, Acidiphilium* sp., *Ferrimicrobium acidiphilum, Ferroplasma acidiphilum* (Gonzalez-Toril et al., 2003). Hamamura and colleagues have studied bacterial communities related to hydrocarbon seep in soils in other study. They have performed experiments by molecular techniques including clone library and DGGE profile. They performed 16S rDNA DGGE PCR amplification with 1070F and 1392R primer pairs for *Bacteria*. According to DGGE analysis they described as *Acidocella* sp, *Acidiphilium acidophilum,* Acidobacteriaceae isolate (Hamamura et al., 2005).

According to metagenomics results, microbial diversities of these water samples have heterotrophic character because described genera with the most percentage are *Acidiphilium* and *Acidocella.* Among this genera, *Acidiphilium* shows heterotrophic and acidophilic character from *α-Proteobacteria* (Johnson 2007). *Acidocella* is heterotroph genus like *Acidiphilium* but this genus could tolerate less extreme acidity and heavy metal concentration than *Acidiphilium* (Johnson 2007). With reference to metagenomic data in this study, it was found that *Acidiphilium* had more members than *Acidocella.* Aytar *et al.* took samples from same location during March 2012. They studied using culture-dependent and culture-independent methods. They have also isolated heterotroph acidophilic microorganisms from Çan (Aytar et al., 2015). Aytar and colleagues have reported the presence of *Ferroplasma*-like prokaryotes according to the results of T-RFLP and qPCR analyses. qPCR generated semiquantitative data for genera of some of the iron-oxidizing acidophiles isolated and/or detected, suggesting the order of abundance was and *Ferroplasma> Leptospirillum>Acidithiobacillus* in Çan AMD. They described *Acidiphilium* spp.; *Acidithiobacillus* and *Leptospirillum* spp. according to terminal restriction fragment analysis. In the same study, strains of *Leptospirillum ferriphilum*, *Acidicapsa ligni*, and *Acidiphilium rubrum* were isolated from Çan AMD using culture-dependent technique (Aytar et al., 2015). Previous results and given results in this paper are compatible. There is no finding information about *Acidithiobacillus* and *Leptospirillum* spp in this study. It may be due to change of pH, because pH value of this area was reported as 2.8 in March 2012. Besides, increase of organic matter may occur. It is thought that difference of metal contents may cause a decrease in microbiological diversity. In March 2012, it was reported that aluminum, chromium, and copper were greater in Çan AMD (Aytar et al., 2015), but in this study, iron, phosphorus, lead and manganese were found greater than other metals or semi-metals.

Acidophilic microorganisms can survive in high heavy metal concentrations through active metal resistance system in their adaptation mechanisms. It is thought that undefined resistance mechanisms will contribute to current acidophile metal resistance mechanisms (Dopson et al., 2014).

The resistance against to some heavy metals has been reported in *Acidiphilium* species. The arsB gene was found by hybridization technique in some acidophiles containing *Acidiphilium* and *Acidocella*. *Acidiphilium symbioticum* has three plasmids contributing to resistance against metal ions of Zn (II) and Cd (II). Moreover among acidophiles, *Acidiphilium multivorum, Acidocella aminolytica*

and *Acidocella* strain GS19 have resistance to Ni (II) ion (Bhowal and Chakraborty 2015). The metagenomic data also reveals *Acidiphilium* and *Acidocella* as dominant genera in samples. As (arsenic), Zn (zinc), Cd (cadmium) and Ni (nickel) were found as considerably higher concentrations than reference values in this study.

Another study reported that the copper resistance caused the morphological variety in cells of *Acidiphilium symbioticum*. Acidophiles have tolerated high copper concentrations (>100 mM). Furthermore, acidophiles living in mining environment could better survive and grow in presence of copper than acidophiles in other environments (Dopson and Holmes, 2014).

CONCLUSION

Microbial diversity of AMD in Çan was studied by a metagenomic approach, contributing as a metadata to literature with this first study from Turkey. This group of extremophiles has vital importance in laboratory and industrial applications due to of surviving in low pH and having active metabolism optimum in low pH.

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