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Seasonal Gene Profiling in Tuz Lake with Regard to Biogeochemical Cycling

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ABSTRACT

Tuz Lake, a thalassohaline lake with a salt rate of 32%, is a unique habitat for a halophilic microbiome. Culture-dependent and independent methods have been applied to identify prokaryotic microbial diversity in Tuz Lake. However, the key processes and genes involved in biogeochemical cycles in Tuz Lake have not been investigated seasonally. The aim of the study is to investigate seasonal gene profiling in Tuz Lake associated with biogeochemical cycling and thereby reveal more about the ecosystem dynamics of this extreme environment. Therefore, the PICRUSt2 tool was applied to analyze the metabolic function of archaeal and bacterial diversity in Tuz Lake. As a result of metabolic functions based on 16S rDNA amplicon sequencing data, it was observed that methane production by H_2 and CO_2 by anaerobic archaea in Tuz Lake was the predominant methanogenesis pathway. It was determined that sulfur oxidation was the dominant sulfur metabolism, while the reductive citric acid cycle was the dominant carbon fixation pathway.

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ÖZET

Tuz oranı %32 olan talassohalin bir göl olan Tuz Gölü, halofilik mikrobiyom için önemli bir yaşam alanıdır. Tuz Gölü'ndeki prokaryot mikrobiyal çeşitliliği belirlemek için kültüre bağlı ve bağımsız yöntemler uygulanmıştır. Tuz Gölü'ndeki prokaryot mikrobiyal çeşitliliği belirlemek için kültüre bağımlı ve bağımsız yöntemler uygulanmıştır. Bununla birlikte, Tuz Gölü'ndeki biyojeokimyasal döngülerde yer alan temel süreçler ve genler mevsimsel olarak araştırılmamıştır. Çalışmanın amacı, Tuz Gölü'ndeki biyojeokimyasal döngü ile ilişkili mevsimsel gen profilini araştırmak ve bu ekstremofilik ortamın ekosistem dinamiklerini ortaya çıkarmaktır. Bu nedenle, Tuz Gölü'ndeki arke ve bakteri çeşitliliğinin metabolik işlevlerini analiz etmek için PICRUSt2 aracı kullanılmıştır. 16S rDNA amplikon dizileme verilerine dayanan metabolik fonksiyonlar sonucunda, Tuz Gölü'ndeki anaerobik arkelerin H_2 ve CO_2 ile metan üretiminin baskın metanojenez yolu olduğu gözlenmiştir. Kükürt oksidasyonu baskın kükürt metabolizması iken indirgeyici sitrik asit döngüsü de baskın karbon fiksasyon yolağı olarak saptanmıştır.

Mikrobiyoloji

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INTRODUCTION

Hypersaline environments are one of the unique and important extreme environments that affect biology, climate, and global geochemistry. The biogeochemical cycles of hypersaline environments differ considerably from those of freshwater and marine conditions, as they are strongly influenced by changes in the physicochemical parameters of the brine as well as the composition of microbial communities due to the fluctuation of salinity (Sternai et al., 2017; Isaji et al., 2019). Hypersaline environments are also described by extreme conditions including high alkalinity, low oxygen concentration, and high UV irradiation as well as high salinity (Ventosa, 2006). Furthermore,

temperature, pH, total organic carbon, oxygen, K^+ and Mg^{2+} levels stand out as critical factors in the taxonomic distribution of microbial communities in hypersaline ecosystems (Zhu et al., 2020; Shi et al., 2021).

With the developing technology, microorganisms can be detected with 16S rRNA and 18S rRNA sequencing by the next-generation sequencing technology without culturing and cloning in the laboratory (Check Hayden, 2014; Başkaya & Kocabaş, 2016; Akyol et al., 2017). The prediction of metagenome functions from 16S rDNA gene sequences data by bioinformatics tools like PICRUSt allows investigation of the metabolomes of complex microbial communities with high precision and confidence at a high taxonomic resolution besides a much lower cost compared to metagenomic sequencing (Douglas et al., 2020). Microorganisms have a role in the biogeochemical cycles of carbon (C), nitrogen (N), phosphorus (P), and sulfur (S) in ecosystem dynamics. Thus, they regulate most biosphere activities associated with fluxes of greenhouse gases, such as CO_2, CH_4, and N_2O. The metagenomic approach gives an opportunity to unveil the composition and functional profile of microorganisms in extreme environments to understand their significant role in biogeochemical cycles.

Tuz Lake is one of the extreme environments with a salt rate of 32% (w/v) and a thalassohaline lake close to neutral pH. Tuz Lake is a class A wetland and is important for protecting biological diversity in Turkey. Hypersaline ecosystems such as salt lakes, sea salts, and salty soils are composed of high salt concentration, the most important life-limiting factor for microorganisms. In studies conducted with extremophile microbial communities living in salt lakes, it has been revealed that the existence of functional microbial groups is involved in the cycles of carbon, nitrogen, sulfur and other elements. The investigation of the biogeochemical cycle that interacts with microbial diversity and primary productivity provides the basis for understanding such ecosystems (Shi et al., 2021). The aim of the study is to investigate biogeochemical cycles and environmental factor relationships by seasonally and thereby reveal more about the ecosystem dynamics of this extreme environment. Picrust2 tool was used to determine functional profile of microbiome in Tuz Lake, including nutrient-cycling processes, as well as their seasonal fluctuation with environmental parameters in the period of November 2018 to January 2020.

MATERIALS and METHOD

The water samples were obtained from Cihanbeyli and Sereflikochisar regions of Tuz Lake at three different points of both sites (38°46`33``N-33°14`59``E, 38°45`20``N-33°13`50``E, 38°45`25``N-33°15`6``E, 38°47`1``N-33°26`42``E, 38°46`34``N-33°28`25``E, 38°45`50``N-33°27`6``E). Conductivity, temperature, dissolved oxygen, humidity, and pH values were measured while collecting the samples. Water samples were taken aseptically and delivered to the laboratory at 4°C in the period of November 2018 to January 2020 except in August and September due to drought.

Nucleic Acid Extraction and 16S rDNA Amplicon Sequencing

The DNA extraction by the phenol-chloroform method in the previous study was applied (Doğan & Kocabaş, 2021). Firstly, the samples were filtered with 0.22 µm membrane filters and homogenized with liquid nitrogen. Extraction buffer was added to homogenized filter and centrifuged at 15000 g for 20 min. After the supernatant was taken and RNAse added, Rnase was inactivated by keeping it at 37 °C for two hours. Phenol: chloroform: isoamyl alcohol (25: 24: 1), pH: 8 was added and centrifuged at 15000g for 15 min. 3M sodium acetate solution was mixed with the supernatant and kept overnight at -20 °C to precipitate nucleic acids. The pellet was washed with 70% ethanol and dissolved in 10 mM Tris (pH, 8) after final centrifugation at 13000 g. V4 variable region of 16S rDNA was amplified by 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') primers. Illumina MiSeq platform was applied for 16S rDNA amplicon sequencing with 2 × 300 bp paired-end protocol.

Bioinformatics Analysis

Dada2 pipelines were applied for filtering, dereplication, chimera identification, and merging paired-end reads. Reads were trimmed to the length of 260 nt by --p-trunc-len-f/r function of Dada2 based on the quality control (Phred score >20). QIIME2 tool was used for bioinformatics analysis of raw sequencing reads and constructed table.qza and rep_seqs.qza files for functional analysis of sequences by the Picrust2 pipeline (Bolyen et al., 2019).

Picrust2 pipeline was applied for the functional gene analysis (Douglas et al., 2020). First of all, the table.qza and rep_seqs.qza files generated by the QIIME2 tool were converted to BIOM format. place_seqs.py commend was used ASV (amplikon sequence variant) placement into a reference phylogenetic tree by EPA-NG and gappa. Hidden state prediction was applied using the castor R package. hsp.py script runs in Picrust2 to predict the copy number of gene families and output the nearest-sequenced taxon index (NSTI) values for each ASV. The enzyme Classification (EC) and KEGG databases were used to construct predictive metagenome. NSTI scores higher than 2.0 were removed due to not having a representative genome in the reference phylogenetic tree. metagenome_pipeline.py script was run to infer

the metagenomes of the communities. Furthermore, the --strat_out option was added to the metagenome_pipeline.py script for the stratified output file, a long-format table representing how ASVs contribute KOs and ECs. pathway_pipeline.py command was run to infer KEGG pathway levels against the KEGG database. Moreover, add_descriptions.py commend was run for adding the description of each functional id to the output abundance tables. Finally, results were analyzed with STAMP tool (Parks et al., 2014).

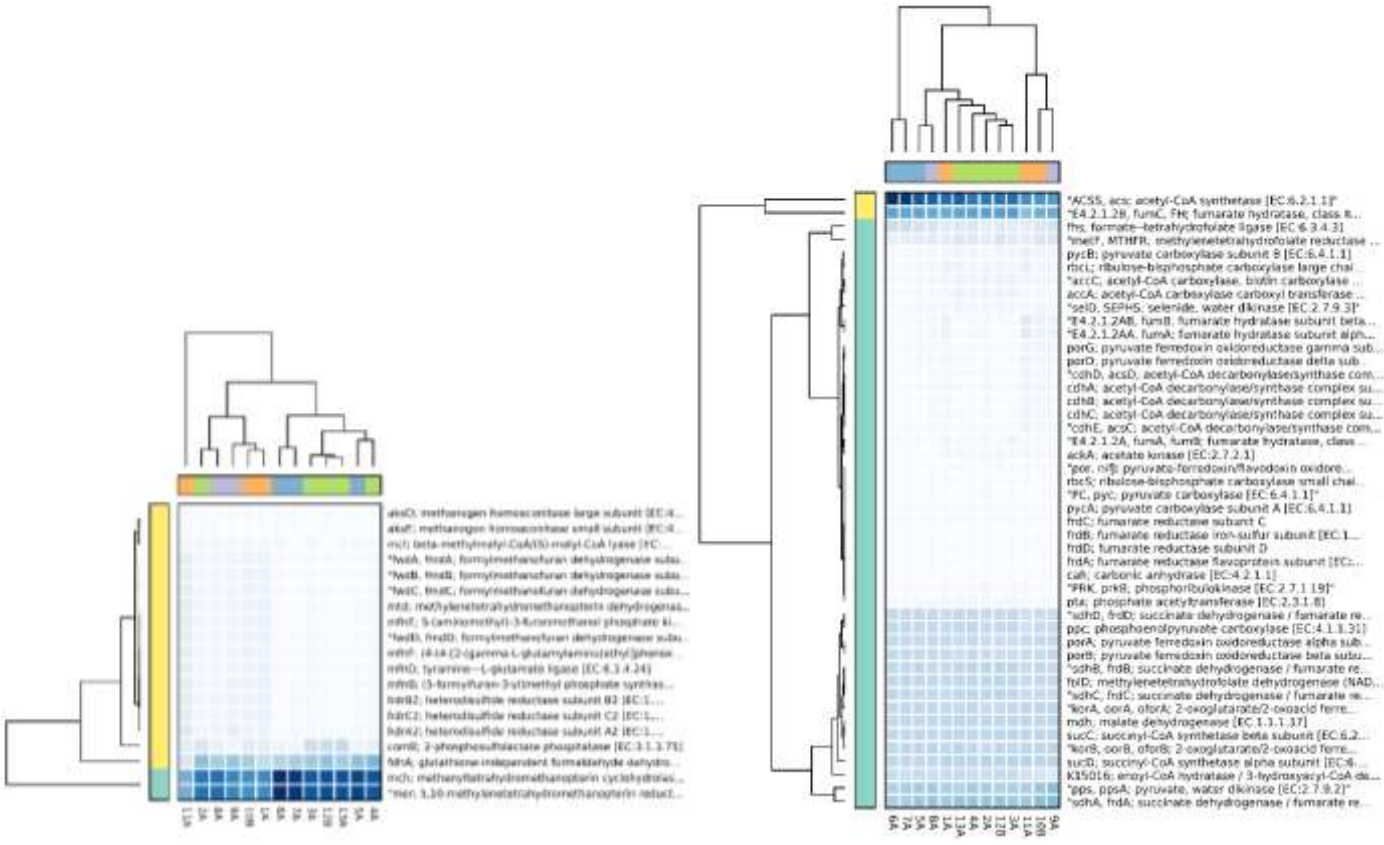
RESULTS and DISCUSSION

The previous study, it was investigated and published the composition of prokaryotic diversity in Tuz Lake by 16S rDNA amplicon sequencing (Doğan & Kocabaş, 2021). In the present study, the same water samples were used to generate functional profiles in Tuz Lake.

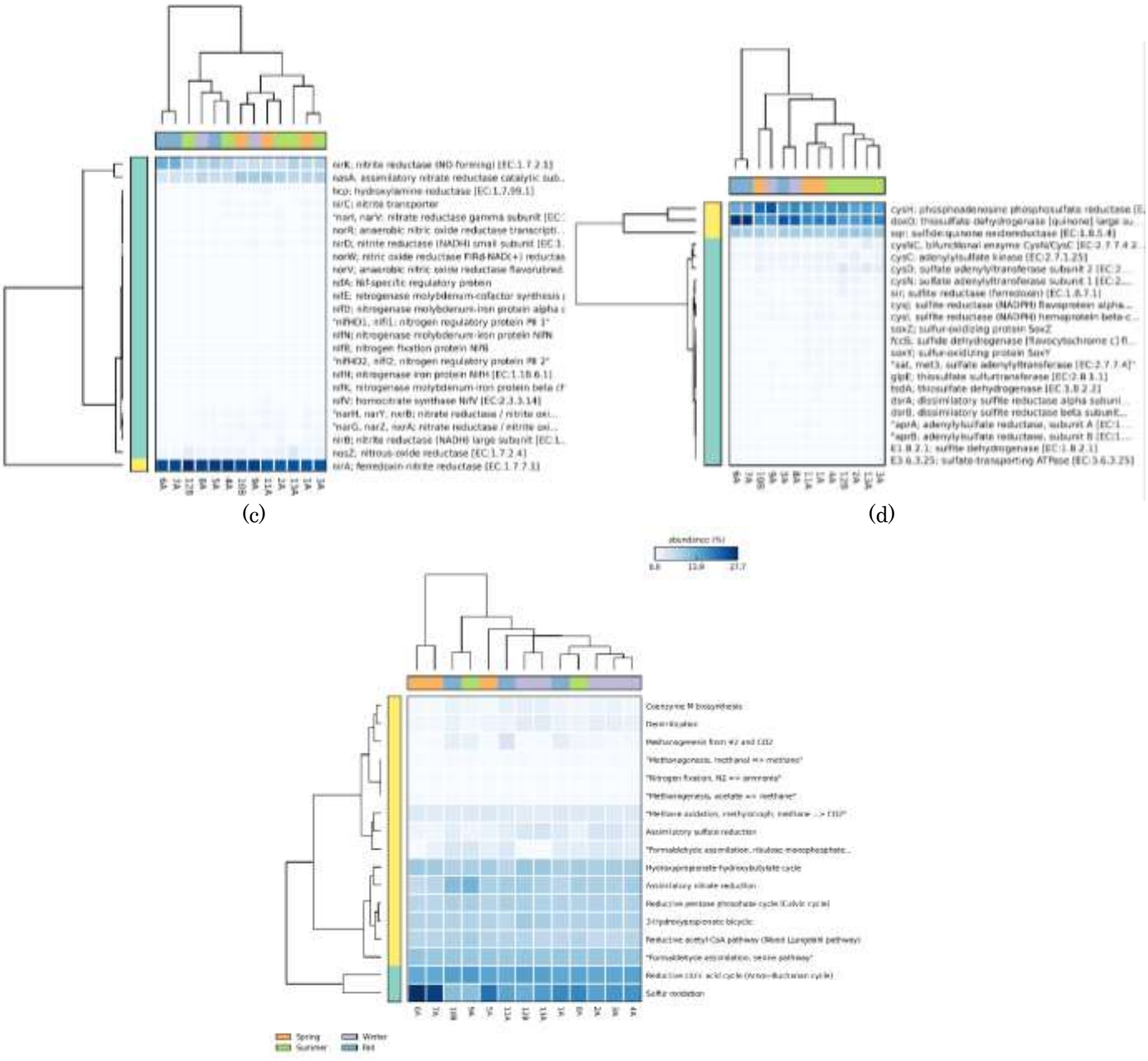
As a result of predictive functional analysis with the PICRUSt2 tool, functional genes related to biogeochemical cycles were found in the samples, and their seasonal changes with environmental parameters were investigated. The accuracy of the metagenome constructed using PICRUSt2 was tested via the nearest-sequenced taxon index (NSTI), which reflects the presence of reference genomes closely related to the samples in the analysis. The means of NSTI values were found as 0.19 ± 0.04. This result appears similar to previously reported microbiome studies: hypersaline microbiome (mean NSTI = 0.23 ± 0.07), from the cold deserts of the McMurdo Dry Valleys of Antarctica, and the hot deserts of the Southwestern United States soil samples (mean NSTI = 0.17 ± 0.02), and the rhizosphere microbiome (mean NSTI = 0.23 ± 0.02) (Langille et al., 2013; Zeng et al., 2015; Lopes et al., 2016; Yuan et al., 2016). Carbon fixation (ko00720), sulfur (ko00920), nitrogen (ko00910), and methane (ko00680) metabolism and genes involved in these metabolisms were determined monthly (Figure 1).

Methane Metabolism

Three types of methanogenic pathways, including H_2 and CO_2 to methane (M00567), methanol to methane (M00356), and acetate to methane (M00357), were recognized in the samples. The sequences related to the M00356 and M00357 pathways were very rare and observed only in February (Figure 1d). It was observed that the seasonal variation of methane metabolism was statistically significant. The mer gene associated with hydrogenotrophic methanogenesis was also detected at a high proportion and was found at a higher proportion in spring than in autumn and winter (Figure 2a and b). The abundance of fwdA/B/C/D genes related to this pathway also increased during the summer and fall when the temperature was high, and the dissolved oxygen level decreased (Fig. 1a, Fig. 2a)



(a) (b)



(c) (d)

(e)

Figure 1. Change of functional genes related to biogeochemical cycles by months with heatmap graph (a) Methane metabolism (b) Carbon fixation metabolism (c) Nitrogen metabolism (d) Sulfur metabolism (Spring: Blue, Fall: Orange, Summer: Purple, Winter: Green) (e) Metabolic pathways related to energy metabolism. (1A: November 2018, 2A: December 2018, 3A: January 2019, 4A : February 2019, 5A : March 2019, 6A : April 2019, 7A : May 2019, 8A : June 2019, 9A: July 2019, 10B: October 2019 , 11A : November 2019, 12B: December 2019, 13A : January 2020).

Şekil 1. Isı haritası grafiği ile biyojeokimyasal döngülere ilişkin fonksiyonel genlerin aylara göre değişimi (a) Metan metabolizması (b) Karbon fiksasyon metabolizması (c) Azot metabolizması (d) Kükürt metabolizması (İlkbahar:Mavi, Sonbahar:Turuncu, Yaz:Mor, Kış: Yeşil) (e) Enerji metabolizması ile ilgili metabolik yollar. (1A : Kasım 2018, 2A : Aralık 2018, 3A : Ocak 2019, 4A : Şubat 2019, 5A : Mart 2019, 6A : Nisan 2019, 7A : Mayıs 2019, 8A : Haziran 2019, 9A: Temmuz 2019, 10B: Ekim 2019, 11A : Kasım 2019, 12B: Aralık 2019, 13A : Ocak 2020).

(Doğan & Kocabaş, 2021). The mfnB/D/E/F genes, which take part in the biosynthesis of methanofuran, the carbon carrier cofactor group involved in the first step in reducing CO_2 to methane, were found high abundance in fall and summer (Figure 1a and Figure 2a). Anaerobic hydrogenotrophic methanogen *Methanococcus* can convert CO_2 and H_2 into valuable cleaner energy fuel (CH_4) (Goyal et al., 2016). Also, sequences related to the aks genes involved in methane

metabolism were found to be associated with *Methanococcus* (Table 1).

Methane oxidation was observed in very little abundance in October and November (Fig. 1d). The pmob (methane monooxygenase subunit B) gene, involved in the aerobic oxidation of biogenic methane, was not detected in the samples. Therefore, it was thought that the oxidation of methane to CO_2 may occur by using different electron acceptors such as sulfate, nitrate/nitrite, or Fe(III)/Mn(IV) instead of O_2. In addition, methanotrophs and methylotrophs can oxidize methane to CO_2 for biosynthesis and further energy source. Also, formaldehyde is converted to C_2 or C_3 compounds in two ways: the serine pathway (M00346) and the ribulose monophosphate pathway (M00345). The serine pathway was found to be more abundant than the ribulose monophosphate pathway (Fig. 1d). In general, methanogenesis genes were not detected at a high proportion in the samples. It was stated that methanogens and sulfate-reducing bacteria share H_2 as the same substrate (Shi et al., 2021). In addition to the low proportion of organic matter and minerals in the hypersaline environment, this competition mechanism may also limit the habitat of methanogens in Tuz Lake.

Table 1. Taxonomic units associated with the functional profiles of biogeochemical cycles
Çizelge 1. Biyojeokimyasal döngülerin fonksiyonel profilleriyle ilişkili taksonomik birimler

Sample	Function	Taxon	Taxon abundance	Taxon relative abundance	Genome function number	Taxon function abundance	Taxon relative function abundance
4A	K16792	*Methanococcus*	30	0.03	1	30	0.03
12B	K00266	*Salinibacter*	454	11.7	1	454	11.7
13A	K00266	*Chitinophagales; uncultured bacterium*	136	1.9	1	136	1.9
11A	K01601	*Natronomonas*	362	1.8	1	362	1.8
12B	K01601	*Halorhodospira*	31	0.8	2	62	1.6
1A	K00380	*Halorhodospira*	481	2.0	1	481	2.03
1A	K17226	*Halorhodospira*	481	2.0	1	481	2.03
6A	K11180	*Desulfovermiculus*	7	0.01	1	7	0.01

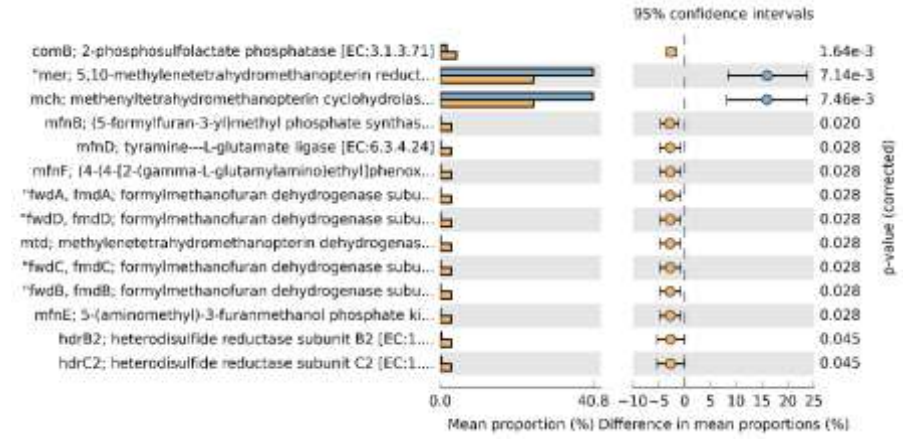


Figure 2. Analysis of seasonal variation of the relative abundances of functional genes involved in methane metabolism using the Stamp program and Welch t-test (95% confidence interval, p <0.05) (a) Spring and Fall (b) Spring and Winter (Spring: Blue, Fall: Orange, Summer: Purple, Winter: Green)

Şekil 2. Stamp programı ve Welch t-testi (%95 güven aralığı, p <0,05) kullanılarak metan metabolizmasında yer alan fonksiyonel genlerin göreceli bolluklarının mevsimsel değişiminin analizi (a) İlkbahar ve Sonbahar (b) İlkbahar ve Kış (İlkbahar : Mavi, Sonbahar: Turuncu, Yaz: Mor, Kış: Yeşil)

Carbon Fixation Metabolism

The reducing citric acid cycle (Arnon-Buchanan cycle) (M00173), the reducing acetyl-CoA pathway (Wood-Ljungdahl pathway) (M00377), 3-hydroxypropionate cycle (M00376), the hydroxypropionate-hydroxybutyrate cycle (M00375) and the reductive pentose phosphate cycle (Calvin-Benson cycle) (M00165) were determined for carbon fixation metabolism. The reductive citric acid cycle was observed as the highest carbon fixation pathway (Fig. 1d). The seasonal difference related to carbon fixation was statistically significant and the highest proportion in the spring.

The reductive citric acid cycle reverses the energy-producing oxidative TCA cycle. Instead of oxidizing acetyl-CoA and producing ATP, reducing CO_2 by consuming ATP forms carbon compounds. CorA/B (2-oxoglutarate ferredoxin oxidoreductase), mdh (malate dehydrogenase), fumA/B/C (fumarate hydratase), frdA/B/C/D (fumarate reductase), porA (pyruvate ferredoxin oxidoreductase), icd (isocitrate dehydrogenase), acnA (aconitate hydratase) and sucD/C (succinyl-CoA synthetase) genes were detected related to the reductive citric acid cycle (Fig. 1b). No seasonal differences were observed in the sequences associated with the korA/B gene, one of the key enzymes of this cycle. While all enzymes related to the cycle were present, ATP citrate lyase, one of the key enzymes, was not detected in the samples. However, citryl-CoA lyase enzyme was detected, which enables the degradation of citryl-CoA to acetyl-CoA and oxaloacetate.

Moreover, an incomplete reductive TCA cycle was also observed in the samples. In this cycle, acetyl-coA is converted to oxoglutarate. Some hydrogenotrophic, autotrophic, and methylotrophic methanogens have been reported to use the incomplete reductive TCA cycle (Ferrer et al., 2012). The reductive TCA cycle and incomplete reductive TCA cycle in Tuz Lake might be related to anaerobic hydrogenotrophic methanogens *Methanococcus* detected in Tuz Lake samples (Doğan & Kocabaş, 2021).

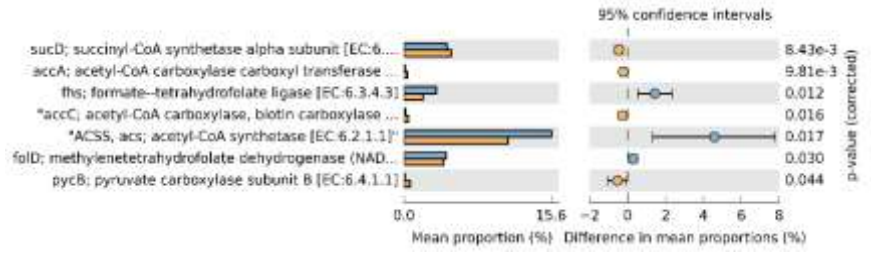
The reductive acetyl-CoA pathway is used in CO_2 fixation by acetogen as well as anaerobic sulfate-reducing microorganisms and methanogens. The sequences related to the reductive acetyl-CoA pathway were changed seasonally and were highest in summer (p-value, 0.045). cdh A/B/C/D/E and fhs (formate tetrahydrofolate ligase) genes, the key enzymes related to the reducing acetyl-CoA pathway (Wood-Ljungdahl pathway), were determined (Fig. 1b). CdhC/D genes encode the corrinoid iron-sulfur protein component of the complex responsible for reactions in the methyl branch of the WL pathway, while acsAB encodes the catalytic component responsible for CO_2 reduction and acetyl-coA synthesis (McGonigle et al., 2021). In addition, the folD (methylenetetrahydrofolate dehydrogenase) was also detected in all samples (Fig. 1b). WL is the most energy-efficient pathway in anaerobic acetogens to convert CO_2 to formate and acetyl-CoA. With this advantage, acetogens are accepted as an important industrial source for producing biofuels and chemical products by fermentation (Song et al., 2020). The WL pathway has been reported to be an important component of the metabolic process in a wide variety of anaerobic prokaryotes, including numerous uncultured bacterial phyla. Most *Acetothermia* genomes have been noted to be capable of autotrophic growth using the reductive WL pathway (Youssef et al., 2019). The *Acetothermia* phylum, which was reported to use the WL pathway, was also detected in Tuz Lake samples (Doğan & Kocabaş, 2021). Acs (acetyl-CoA synthetase), which was highly observed in the samples, was responsible for the oxidation of acetate (Fig. 1b). Ferrer et al. (Ferrer et al., 2012) stated that acetyl-CoA synthetase is related to the methanogenic *Euryarchaeota*.

Moreover, syntrophic acetate oxidizers, some sulfate-reducing bacteria, and acetylastic methanogens have been reported to reverse the WL pathway (Vavourakis et al., 2018). It was thought that this enzyme might be related to the anaerobic members of *Euryarchaeota*, which was the most abundant phylum in Tuz Lake (Doğan & Kocabaş, 2021). The WL pathway was the most prevalent metabolic pathway after the reductive TCA cycle as a part of carbon fixation metabolism in Tuz Lake (Fig. 1d).

Furthermore, the microbiome study in the Red Sea revealed that the reductive TCA cycle and WL metabolism were higher at low oxygen levels and high salinity (Michoud et al., 2021). Hypersaline environments are characterized by high salt concentration and low oxygen levels. In Tuz Lake the reductive TCA cycle and WL pathway were detected as the dominant carbon fixation metabolism (Fig. 1d).

The hydroxypropionate-hydroxybutyrate cycle identified in *Archaea* is an important carbon fixation pathway in the global carbon cycle (Ferrer et al., 2012). This pathway was also revealed in Tuz Lake as carbon fixation metabolism (Fig. 1d). The sequences related to the accC/A (acetyl-CoA carboxylase) gene catalyzing the synthesis of 3-hydroxypropionate by fixing CO_2 in the 3-hydroxypropionate cycles were identified (Fig. 1b). It was observed that the change in the sequences related to this gene was statistically significant and elevated in winter when the conductivity was high, the temperature was low, and therefore the dissolved CO_2 ratio might be high (Fig. 3) (Doğan & Kocabaş, 2021). In addition, these two cycles were seasonally changed and were observed in the highest proportion in winter (p-value < 0.05).

The RbcL/S (ribulose 1,5-biphosphate carboxylase) and PRK (phosphoribulokinase) genes, the key enzymes in the reducing pentose phosphate cycle, were found as a result of functional analysis (Fig. 1b). It has been stated that ribulose-1,5-biphosphate carboxylase is not only used by photoautotrophs to fix CO_2 in the reductive pentose phosphate cycle but also different RubisCO forms are found in ecologically and evolutionarily diverse chemoautotrophic bacteria (Alfreider & Tartarotti, 2019). It has been reported that environmental CO_2 concentration has a function in the regulation of RbcL/S gene. Furthermore, the genes responsible for regulation allow the microorganism to respond rapidly to environmental fluctuations in CO_2/O_2 concentrations. RubisCO (rbcL/cbbL) enzyme in Tuz Lake is similar to Type I RubisCO enzymes, which are common in plants, autotrophic and chemoautotrophic bacteria (Alfreider et al., 2017). They also reported that these genes were related to *Thiohalorhabdaceae* and *Halothece-like cyanobacteria* species (McGonigle et al., 2021).



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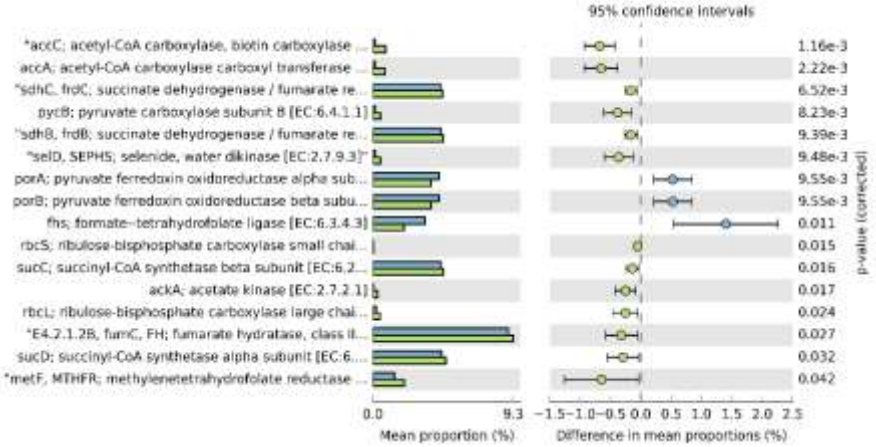

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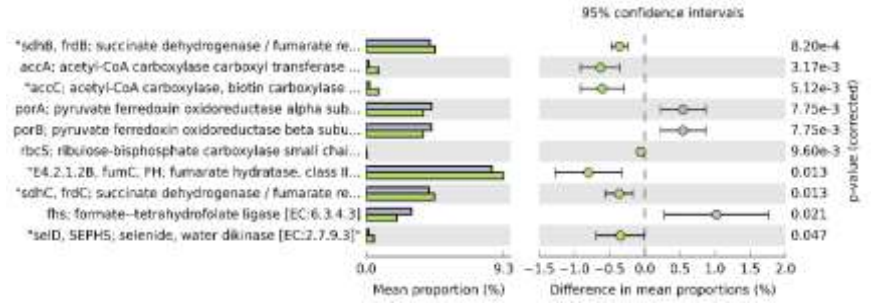

(c)

Figure 3. Analysis of seasonal variation of the relative abundances of functional genes involved in carbon metabolism using the Stamp program and Welch t-test (95% confidence interval, p <0.05) (a) Spring and Fall (b) Spring and Winter (c) Summer and Winter (Spring:Blue, Fall:Orange, Summer:Purple, Winter:Green)

Şekil 3. Stamp programı ve Welch t-testi (%95 güven aralığı, p <0,05) kullanılarak karbon metabolizmasında yer alan fonksiyonel genlerin göreceli bolluklarının mevsimsel değişiminin analizi (a) İlkbahar ve Sonbahar (b) İlkbahar ve Kış (c) Yaz ve Kış (İlkbahar:Mavi, Sonbahar:Turuncu, Yaz:Mor, Kış:Yeşil)

Members of the *Cyanobacteria* and autotrophic *Proteobacteria* also conducted the reductive pentose phosphate cycle in Tuz Lake. It was thought that anoxygenic phototroph *Halorhodospira*, which was highly observed in the Tuz Lake microbiome, has genes involved in sulfur oxidation and RubisCO, to be

effective in CO_2 fixation (Doğan & Kocabaş, 2021). Moreover, it was detected that *Natronomonas* and *Halorhodospira* had the highest abundance of RubisCO genes (Table 1). Biomass production based on CO_2 assimilation with the reductive TCA cycle is more energy favorite than the reductive pentose phosphate cycle (Alfreider et al., 2017). As a result of the analysis, the reductive TCA cycle was dominant over the reductive pentose phosphate cycle and all other carbon fixation pathways in the samples (Fig. 1d).

Nitrogen Metabolism

Nitrogen is an essential element for all microorganisms and is required to synthesize essential cellular components such as amino acids and nucleotides (Hu et al., 2014; Kuypers et al., 2018). While assimilatory nitrate reduction (M00531) and denitrification pathways (M00529) play an important role in the nitrogen cycle in Tuz Lake, nitrogen fixation was found to be negligible (Fig. 1d). Although statistically significant differences were observed in the genes associated with nitrogen metabolism, no difference was observed in total nitrogen metabolism seasonally.

Marinobacter and *Arhodomonas* genera belonging to the *Gammaproteobacteria* group have been noted to perform assimilatory nitrate reduction (Sorokin et al., 2014). It was seen that these species, which were also found in Tuz Lake, contributed to nitrate reduction (Doğan & Kocabaş, 2021). The nif gene responsible for nitrogen fixation was found at very low abundance in February and April (Fig. 1c). The low level of nitrogen fixation is thought to belong to the *Geitlerinema* cyanobacteria, which is capable of nitrogen fixation, observed as a result of taxonomic analysis (Doğan & Kocabaş, 2021). No pathway information was found regarding nitrification and anammox. A metagenomic research performed by Fernández et al., in a hypersaline environment were found a significant number of assimilatory nitrate and nitrite reduction genes were found while no nitrification related genes determined (Fernández et al., 2014). The 13 genes most abundantly associated with the nitrogen cycle were identified, including assimilatory nitrate reduction and denitrification (Fig. 1c). The sequences related to nir, nos, nar, and nor using nitrate as an electron acceptor for denitrification have been identified. Nitrate reductases are mainly involved in anaerobic nitrate respiration and denitrification (Feng et al., 2014). It was determined that the nar G/H gene increased in fall (Fig. 4a and b). The sequences associated with nosZ were also higher in fall than in spring (Fig. 4a). The low amount of dissolved oxygen in the fall may have caused an increase in the number of those genes. Microorganisms, capable of denitrification, prefer nitrogen instead of oxygen as a terminal electron acceptor during respiration (Robertson & Groffman, 2015). *Halomonas*, which can grow anaerobically and perform denitrification, has been encountered in the samples (Doğan & Kocabaş, 2021). The nirA and nasA genes, which catalyzes the reduction of nitrite to ammonium, which is used in assimilatory nitrate reduction, were also detected at an elevated level in the samples (Fig. 1c). Assimilatory nitrate reductases (Nas) are cytoplasmic enzymes usually stimulated by nitrate or nitrite while inhibited by ammonium. They also use NAD(P)H or ferredoxin as electron donors (Feng et al., 2014). The sequences associated with the NasA gene was exhibited statistically significant differences and increased in the fall (Fig. 4). Also, the NasA gene showed high abundance in summer based on the heatmap graph (Fig. 1c). Kırkağaç et al. (Kırkağaç et al., 2017) also stated that the amount of nitrate and nitrite in the spring and summer was higher than in the winter in Tuz Lake. When available, most microorganisms favorably use ammonium to avoid reducing nitrate to ammonium, an energy-requiring process (Maier, 2015). It has been reported that genes related to nitrate assimilation are increased when ammonia and other forms of nitrogen fixation are limited, and nitrate is present (Shapleigh, 2009).

Sulfur Metabolism

The conversion of sulfur in the environment is mainly dependent on microbial activities. Microorganisms use the reduction and oxidation of inorganic sulfur compounds to generate and conserve biochemical energy (Wasmund et al., 2017). The sulfur cycle in Tuz Lake consists of assimilatory sulfate reduction and sulfur oxidation. Although no dissimilatory sulfate reduction pathway was found, the dissimilatory sulfide reductase gene (dsr) and adenylylsulfate reductase (apr) were observed at very low abundant in February (Fig. 1d). While there was no statistically significant difference between the seasons related to total sulfur metabolism, there were monthly differences in the related genes. In addition, it was observed that sulfur oxidation varied seasonally and was highest in spring (p-value, 0.006).

The cys and sat genes responsible for assimilatory sulfate reduction were increased in winter (Fig. 5). In addition, SoxY/Z (sulfur-oxidizing protein), tsdA (thiosulphate dehydrogenase), and glpE (thiosulfate sulfurtransferase) genes were found as a part of sulfur oxidation (Fig. 1d). Sox genes were determined to be higher in spring and winter (Fig. 5). High oxygen and conductivity (Doğan & Kocabaş, 2021) may affect the increase of the sequences associated with these genes. The increase in the conductivity may also affect the amount of thiosulfate, sulfite, and sulfide used as substrate in the Sox system. In addition, the sulfur:quinone oxidoreductase (sqr) gene, responsible for sulfur oxidation, was also detected as a high

proportion (Fig. 1d). It has been stated that sulfur-oxidizing bacteria can obtain high energy from the oxidation of sulfide/thiosulfate to sulfate with high salt concentrations (Sorokin et al., 2014). Sulfur oxidation was observed in a very high proportion in the samples (Fig. 1e). In addition, the genes related to sulfur metabolism were found to be primarily associated with *Gammaproteobacteria*. Cys and sox genes were predominantly associated with *Halorhodospira*, and the dsr gene, which was observed in very low abundance, was associated with *Desulfovermiculus* (Table 1).

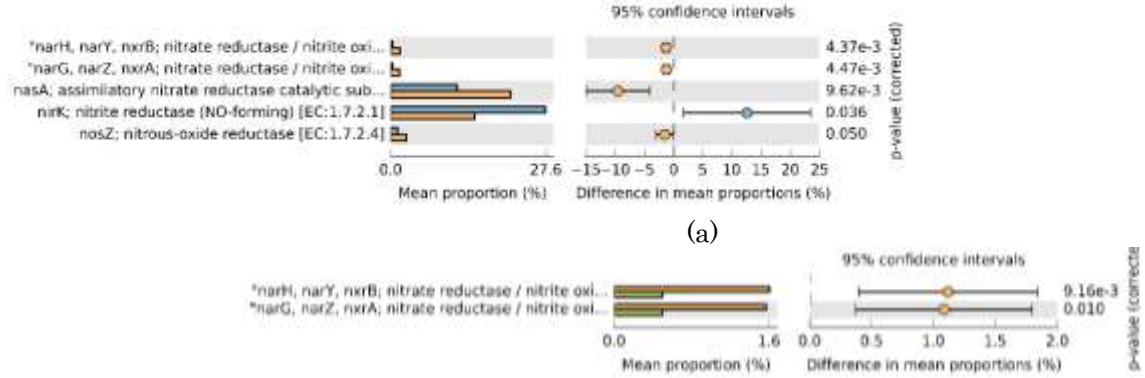

Figure 4. Analysis of seasonal variation of the relative abundances of functional genes involved in nitrogen metabolism using the Stamp program and Welch t-test (95% confidence interval, p <0.05) (a) Spring-Fall (b) Spring-Winter (Spring: Blue, Fall: Orange, Summer: Purple, Winter: Green)

Şekil 4. Stamp programı ve Welch t-testi (%95 güven aralığı, p <0,05) kullanılarak nitrojen metabolizmasında yer alan fonksiyonel genlerin göreceli bolluklarının mevsimsel değişiminin analizi (a) İlkbahar-Sonbahar (b) İlkbahar-Kış (İlkbahar : Mavi, Sonbahar: Turuncu, Yaz: Mor, Kış: Yeşil)

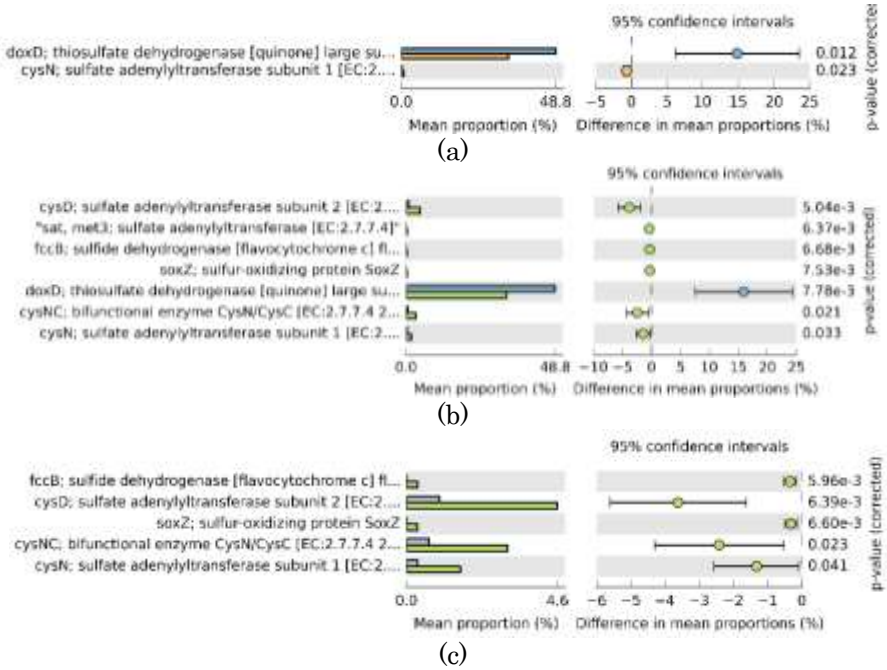

Figure 5. Analysis of seasonal variation of relative abundances of functional genes involved in sulfur metabolism using Welch t-test (95% confidence interval, p <0.05) with Stamp program (a) Spring-Fall (b) Spring-Winter (c) Summer -Winter (Spring:Blue, Fall:Orange, Summer:Purple, Winter:Green)

Şekil 5. Stamp programı ve Welch t-testi (%95 güven aralığı, p <0,05) kullanılarak sülfür metabolizmasında yer alan fonksiyonel genlerin göreceli bolluklarının mevsimsel değişiminin analizi (a) İlkbahar-Sonbahar (b) İlkbahar-Kış (c) Yaz-Kış (İlkbahar: Mavi, Sonbahar: Turuncu, Yaz: Mor, Kış: Yeşil)

CONCLUSION

Genes related to biogeochemical cycles in Tuz Lake were analyzed, and their seasonal variation was investigated. It was noted that methane production by H_2 and CO_2 by anaerobic archaea in Tuz Lake was the dominant methanogenesis pathway. The abundance of genes related to this pathway also increased during the summer and fall. The reductive citric acid cycle, reductive acetyl-CoA pathway, 3-hydroxypropionate cycle, hydroxypropionate-hydroxybutyrate cycle producing acetyl-CoA, and reductive pentose phosphate cycle were determined as pathways associated with carbon fixation. Furthermore, it was seen that carbon fixation was statistically significant and had the highest proportion in the spring. The reductive citric acid cycle was determined as the predominant carbon fixation pathway. While assimilatory nitrate reduction and denitrification pathways were found as part of the nitrogen cycle, nitrogen fixation was negligible. No pathway information was detected linked to nitrification and anammox. Although statistically significant differences were observed in the genes associated with nitrogen metabolism, no difference was observed in total nitrogen metabolism seasonally. Furthermore, it was determined that the sulfur cycle consisted of assimilatory sulfate reduction and sulfur oxidation. Also, the genes responsible for assimilatory sulfate reduction were increased in winter. The results showed that metabolic pathway and functional genes profiles were changed by seasonally in Tuz Lake. It was thought that the changes in environmental parameters depending on the seasons might affect the genes associated with these pathways. These results related to the seasonal changes of functional profiles in Tuz Lake might be valuable sources for future ecological and biotechnological research in extremophile environments.

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Contribution of Authors

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Conflict of Interests and Ethical Statement

None.

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