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PROFILING THE GENES ASSOCIATED WITH OSMOADAPTATION AND THEIR VARIATION BY SEASONALLY IN TUZ LAKE

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ABSTRACT. Hypersaline environments are one of the extreme habitats in the world. Microorganisms living in a hypersaline environment have developed various molecular adaptation strategies to overcome these extreme conditions. The study aims to investigate the genes associated with osmoadaptation seasonal variation in Tuz Lake by PICRUSt2. Dada2 pipelines were applied for filtering, dereplication, chimera identification, and merging paired-end reads to construct table.qza and rep_seqs.qza files. Therefore, the PICRUSt2 was applied to analyze the metabolic function of archaeal and bacterial diversity in Tuz Lake by using table.qza and rep_seqs.qza files. As a result of metabolic functions based on 16S rDNA amplicon data, the genes related to potassium accumulation played an important role in osmoregulation in Tuz Lake, where the archaea population was dominant. Furthermore, bacteriorhodopsin, halorhodopsin, and sensory rhodopsin functions were determined. The abundance of bacteriorhodopsin and halorhodopsin were increased in summer and spring, respectively.

1. INTRODUCTION

Halophilic microorganisms have developed various molecular mechanisms for adaptation to salt-rich habitats. In general, halophiles use two basic strategies to maintain the osmotic balance between their cytoplasm and environment. First, it is called the salt-in strategy, which is energetically advantageous. The “Salt-in” strategy is a method in which the intracellular salt concentration is kept higher than the environmental osmotic pressure by transporting ions into the cell [1]. It has been observed that halophiles using this method (e.g. *Salinibacter ruber* and *Halobacterium sp.)* have fewer hydrophobic residues and an increase in the synthesis of halophilic protein structures with a predominance of highly acidic amino acids such as aspartic acid. It has been reported that the highly acidic amino acids on the surfaces of halophilic proteins prevent aggregation at a high salt concentration [2, 3]. The second strategy is the accumulation of compatible solutes used by most microorganisms. Most halophilic or halotolerant microorganisms produce or accumulate intracellular small organic compounds (ectoine, trehalose, and sucrose) to maintain osmotic balance in hypersaline environments. Ectoine was first discovered in the haloalkaliphilic photosynthetic sulfur bacterium *Ectothiorhodospira halochloris* but was later found to produce this compound, usually with its 5-hydroxy derivative, by a wide variety of

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halophilic and halotolerant bacteria. It has been reported that ectoine can protect many unstable enzymes and nucleic acids against high salinity, thermal denaturation, and the harmful effects of freezing, thus being important in increasing the shelf life and activity of enzyme preparations [4].

Because of the restricted composition of culture media, it could not reveal all the microbial composition and the dynamic of nutrient cycles. In recent years, the application of next-generation sequencing (NGS) technology to microbial communities has revolutionized environmental microbiological research, allowing cost-effectively both in-depth knowledge and large-scale sequencing of DNA samples without the need for culturing and cloning [5]. As well as sequencing technology, bioinformatics improvements have been advanced by developing powerful new computational tools for practical interpretation and visualization of the taxonomic and functional composition of microbial communities [6]. Nowadays, the prediction of metagenome functions from 16S rRNA gene sequences data by bioinformatics tools like PICRUSt2 allows investigating 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 [7]. Tuz Lake, a thalassohaline lake with a salt rate of 32% (w/v), is one of the best biological systems for studying survival strategies of microbial diversity and their response to environmental factors. There are several studies describing the prokaryotic and eukaryotic microbial diversity in Tuz Lake by NGS [8–11]. However, the characterization of metabolic diversity and seasonal variation of related genes are poorly understood in Tuz Lake. The purpose of the present study is to give insights into how osmoadaptation genes fluctuate in response to environmental parameters by seasonally in Tuz Lake, Türkiye. As per our knowledge, there is no reports showing the seasonal variation of genes associated with osmoadaptation in Tuz Lake.

2. MATERIALS AND METHODS

Water samples were collected from Cihanbeyli and Şereflikoçhisar 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) from November 2018 to January 2020. The water samples were collected from the lake's surface at a depth of 10 cm aseptically in sterile bottles on the same day of every month. Water samples could not be taken in August and September because of drought. The water samples collected from each station have been mixed to create a pool for each month. As a result, water samples covering 13 months, which were November 2018, December 2018, January 2019, February 2019, March 2019, April 2019, May 2019, June 2019, July 2019, October 2019, November 2019, December 2019, and January 2020, were obtained. The water samples were clustered to Fall (October and November), Winter (December, January, February), Spring (March, April, May), and Summer (June, July) to evaluate seasonal variation.

2.1. Nucleic acid extraction and 16S rDNA amplicon sequencing

The DNA extraction protocol is same with the previous study [12] which is based on phenol-chloroform method [13]. As summary, 200 ml of water samples for each each month were filtered with 0.22 μm membrane filters and homogenized with liquid nitrogen. Prepared extraction buffer (KCI,Tris-HCI, EDTA) was added to the homogenized filter and centrifuged at 15.000g for 20 min. Then, the supernatant was taken, and RNAse (Thermo Scientific) was 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 15.000g 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 13.000 g. DNA samples were assessed by Qubit DNA Assay (Thermo Scientific Qubit 4.0) and 1% agarose gel electrophoresis. V4 variable region of 16S rDNA was amplified with 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') primers [14] and sequenced using Illumina MiSeq sequencing platform with 2 × 300 bp paired-end protocol by company (BC, Canada).

2.2. Bioinformatics analysis

The quality of reads was evaluated by FastQC [15] tool and the reads were trimmed to the length of 260 nt (Phred score >20) by trim length function. Dada2 [16] pipelines were used to filter, dereplicate, identify chimera, and merge paired-end reads "table.qza" and "rep_seqs.qza" files were created for functional analysis of reads by the PICRUSt2 pipeline [7]. SILVA database "v.132" [17] was used to assign taxonomy with 97% identity thresholds by classify-consensus-blast algorithm in QIIME2. Initially, the table.qza and rep_seqs.qza files were created by the QIIME2 software [18] and converted to BIOM format for subsequence analysis. place_seqs.py command was applied for ASV placement into a reference phylogenetic tree by EPA-NG and gappa based on the Integrated Microbial Genomes database [19]. The castor R package was used for hidden state prediction. hsp.py script was run in PICRUSt2 to predict the copy number of gene families and output the nearest-sequenced taxon index (NSTI) values for each ASV. NSTI scores that were lower than 2.0 were selected for subsequent analyses. metagenome_pipeline.py script was run for inferring the metagenomes of the communities. the --strat_out command was added to the metagenome_pipeline.py script to figure out how each ASV contributes Enzyme Classification (EC) and Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO) numbers. After that, KO numbers were used to infer KEGG pathway levels against the KEGG database by pathway_pipeline.py command. Finally, the description of each functional category was added to the output abundance tables with add_descriptions.py command. STAMP [20] tool was used to analyze the functional profile.

3. RESULTS AND DISCUSSION

In the previous study, we investigated and published the composition of prokaryotic diversity in Tuz Lake by 16S amplicon sequencing [12]. Therefore, in the present study, we used the same water samples for generating predictive functional profiles in Tuz Lake. Total number of reads and ASV were detected as 501,654 and 652, respectively [21]. Also, the number of reads per sample were ranged between 4600-86200 after quality filter. High quality paired end reads were obtained. The mean quality scores of the sequences were found in the range of 32-36 [21].

In our previous study it has been shown that microbial diversity consists of 95% of archaea and %5 of bacteria in Tuz Lake [12]. It was determined that *Haloquadratum, Haloparvum, Halonotius, Halorubrum, Halobellus, Halapricum,* and *Haloarcula* were determined as the most abundant archaeal genera. Moreover, *Salinibacter, Pseudomonas, Arhodomonas, Halorhodospira*, and *Chromobacterium* were the most common bacterial genera [12]. Also, it was reported that the archaeal population in Tuz Lake was composed of the *Euryarchaeota* (96%) and *Nanoarchaeaeota* (4%) phyla [12]. Furthermore, the most abundant bacterial phyla were detected as *Bacteroidetes* (74%) and *Proteobacteria* (25%) (Figure S1).

As a result of predictive functional analysis with the PICRUSt2, the genes related to osmoadaptation, and rhodopsin were revealed and their seasonal variation with environmental parameters was assessed. The precision of the metagenome constructed using PICRUSt2 was confirmed by the nearest-sequenced taxon index (NSTI), suggesting the reference genomes closely correlated to the samples in the analysis. The means of NSTI values were 0.19±0.04 (Table S1). Similar findings were obtained with previously reported microbiome studies including rabbit fecal sample (NSTI = 0.19), soil microbiome from Ohio (NSTI = 0.17), hypersaline microbiome (mean NSTI = 0.23), the cold and the hot deserts soil microbiome (mean NSTI = 0.17), and the rhizosphere microbiome (mean NSTI = 0.23) [22–26].

In addition to the seasonal variation, water samples of November and December, taken in both 2019 and 2018, were compared to make year-based comparisons of metabolic functions, and no statistically significant difference was observed.

3.1. Osmoadaptation

Microorganisms overcome the osmotic stress by two mechanisms: KCl accumulation, which requires heavy modification of the enzyme content of the cell, or organic compatible solute deposition, which requires less proteomic modification and allows adaptation to different salt concentrations [27].

As a result of functional genes analysis with PICRUSt2, trkA/B/G/H genes related to potassium accumulation [28] were found predominant (Figure 1). The genes related to the potassium accumulation were predominated in *Haloarchaea,*

which uses a bio energetically efficient "salt in" strategy to move K+ and Cl- ions into the cell and Na+ ions out. High intracellular K+ concentration is maintained by active K+ transport [29]. Trk genes were primarily associated with *Halapricum*, *Haloparvum*, and *Salinibacter* (Table 1). Furthermore, the genes encoding the low-affinity K transporter TrkA/H, driven by the membrane potential [28], were detected. ATPase-K-KdpA/B/C genes were also found (Figure 1). It was seen that TrkA/H genes were higher in spring than in winter. Furthermore, the sequences related to TrkA, which was the most abundant functional gene associated with osmoadaptation, were determined the lowest proportion in December and January (Figure 1 and 2).

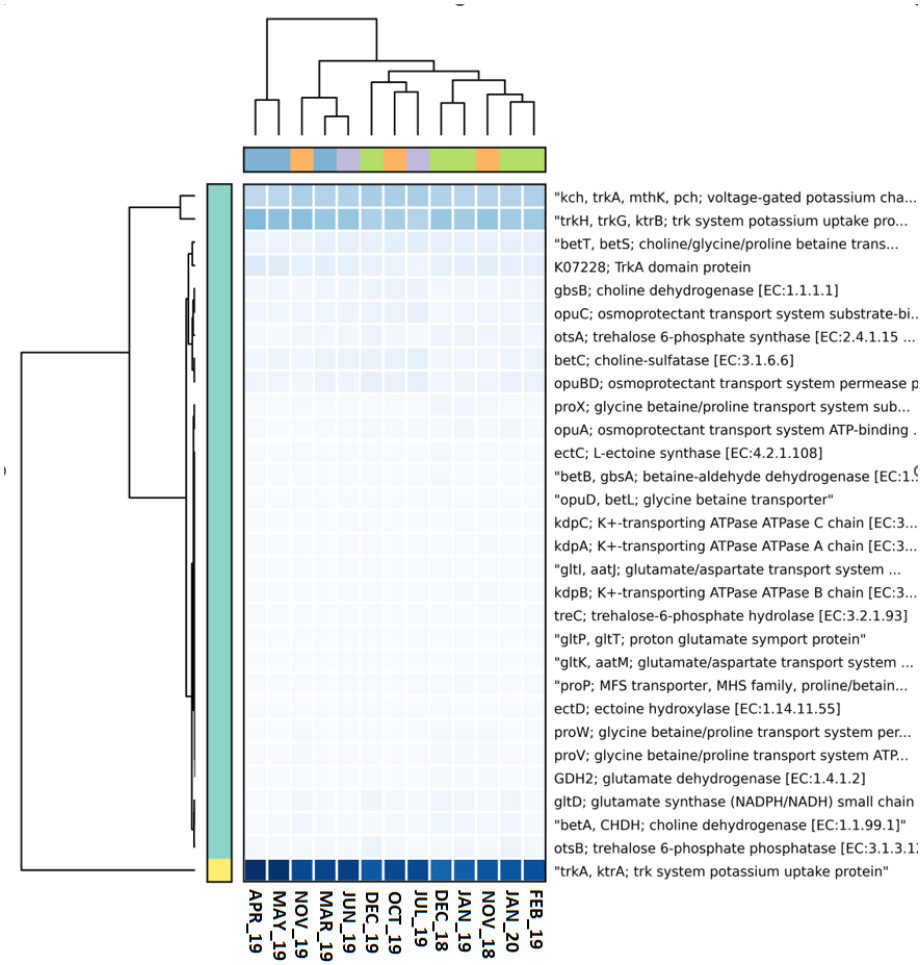


FIGURE 1. The changes of functional genes involved in environmental stress adaptation with heatmap graph by months (Spring:Blue, Fall:Orange, Summer:Purple, Winter:Green).

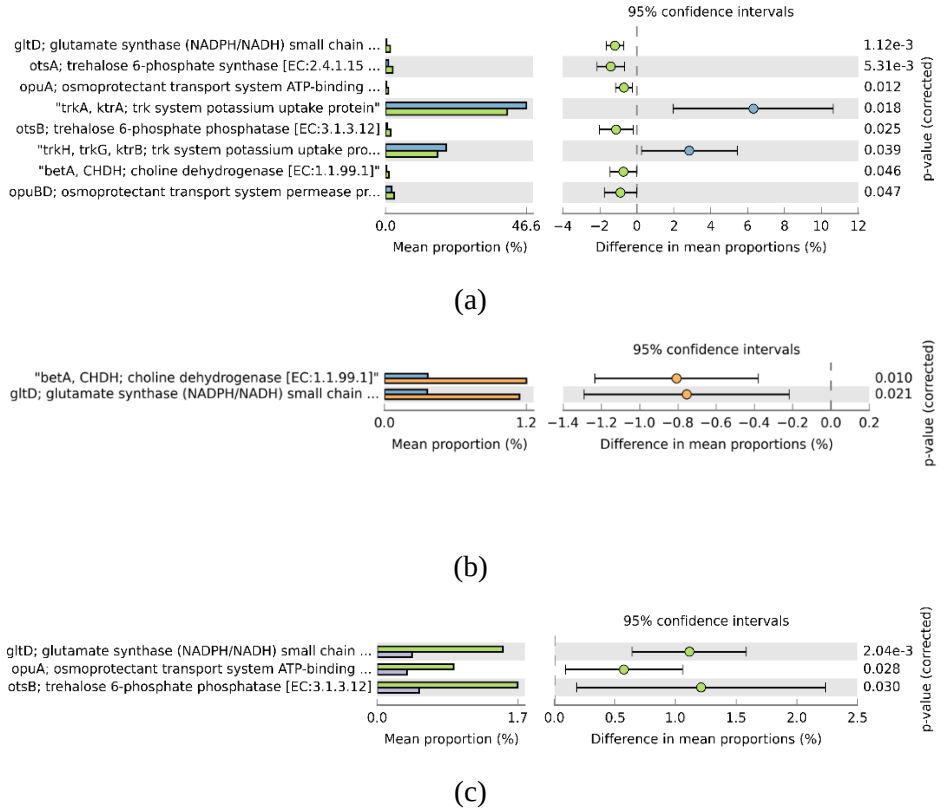


FIGURE 2. Analysis of seasonal variation of the relative abundances of functional genes involved in environmental stress adaptation using the Stamp program and Welch t-test (95% confidence interval, $p < 0.05$) (a) Spring and Winter (b) Spring and Fall (c) Winter and Summer (Spring:Blue, Fall:Orange,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

It is more favorable to take osmolytes from the environment instead of their biosynthesis in the cell. Therefore, bacteria and archaea encode multiple osmoregulation-related carrier proteins to take up osmolytes with high affinity [30]. In Tuz Lake, various genes encoding osmolytes transport proteins were revealed, too. BetT (choline transport)/betS (glycine betaine/proline betaine) and opuC were detected as the most abundant functional genes related to compatible solutes accumulation strategy. However, there was no statistical difference between betT/betS and opuC genes between the seasons. In addition, pro X/P/V/W (glycine betaine/proline/betaine transport) and glt (glutamate/aspartate transport) transport genes were also observed (Figure 1). OpuA, OpuBD, and OpuC are high-affinity ABC transporters, OpuD belongs to the betaine-choline-carnitine-transporter (BCCT) family, while the proline transporter OpuE is a member of the sodium-soluble-symport (SSS) family [28]. OpuC is responsible

for the intracellular transport of many compatible solutes, including choline and glycine betaine [31]. It has been reported that ABC transport proteins responsible for betaine transport were transcriptionally increased during the high salt adaptation of *Desulfovibrio vulgaris* [32]. However, present study opuA and opu BD were elevated in winter (Figure 2a and 2c).

The genes responsible for choline synthesis in the samples are pcs (phosphatidylcholine synthase [EC:2.7.8.24]), betT/betS (choline/glycine/proline betaine transport protein), betC (choline sulfatase [EC:3.1.6.6]), betaA (choline dehydrogenase [EC:1.1.99.1]) was observed in all months. The main function of choline in most species is to form a precursor metabolite for glycine betaine biosynthesis [33]. Most bacteria produce glycine betaine through the oxidation of choline with products of two genes encoding betB and betA, respectively [30]. While functional genes related to proline, glycine betaine such as proX, betaS/T/C were found at high proportion, genes for ectoine metabolism such as ectC (L-ectoine synthase [EC:4.2.1.108]) were observed at a very low proportion (Figure 1). A bioinformatics study showed that ectoine biosynthesis was predominantly found in bacteria and only in a few archaea [30]. In Tuz lake samples, genes encoding the ectoine synthase were predominantly observed in *Halorhodospira* (Table 1). In Tuz Lake, the sequences related to glycine betaine were found to be more abundant than the sequences associated with ectoine. In the metagenomic research conducted in the hypersaline Santa Pola saltern, it has been reported that genes required for betaine synthesis and especially betaine and choline transport were found in various halotolerant bacteria and cyanobacteria species [34]. In addition, it was stated that very few sequences related to ectoine transport and trehalose biosynthesis were obtained [34]. Trehalose, a glucose disaccharide, is found in a wide variety of microorganisms and helps to protect many biological structures against the stresses generated by typical extreme conditions such as drought, high temperature, and hyperosmotic conditions in saline environments [35]. In the current study, otsA (trehalose 6-phosphate synthase) and otsB (trehalose 6-phosphate phosphatase) genes were observed at a high level (Figure 1). OtsAB uses UDP-glucose and glucose-6-phosphate precursors for trehalose metabolism. Also, trehalose is synthesized as a nitrogen-free alternative to nitrogen-containing glycine betaine and ectoine, especially at low nitrogen concentrations [34]. It has been reported that the nitrate concentration of Tuz Lake was lower in winter than in spring [36]. The present study showed that genes related to trehalose metabolism were generally higher in winter. In addition, the genes related to trehalose synthesis were predominantly linked to *Halonotius* and *Salinibacter* (Table 1). Furthermore, it was reported that trehalose concentration decreased with increasing NaCl concentrations [37]. The sequences related to trehalose metabolism might be increased in the winter because of the low temperature and salinity [12,36].

The gltD (glutamate synthase) gene, involved in glutamate synthesis, was also detected at a high ratio (Figue 1 and 2). It has been reported that the synthesis of

this osmolyte is mostly found in bacteria, especially in the *Salinibacter* sequences [38]. In present study, the gltD gene was associated with the most abundant bacterial genus *Salinibacter*, as well *as Chitinophagales*; uncultured bacterium. Moreover, the gene encoding glumate dehydrogenase was primarily observed in the bacterial genera *Marinobacter* and *Halorhodospira* (Table 1).

TABLE 1. Taxonomic units associated with functional profiles of osmoregulation.

Month	Function	Taxon	Taxon abundance	Taxon relative abundance	Predicted copy number of function per taxon	Taxon function abundance	Taxon relative function abundance
November -2018	K15371	*Halorhodospira*	481	2.0	1	481	2
December -2018	K15371	*Marinobacter*	238	1.2	2	476	2.5
November -2018	K06720	*Halorhodospira*	481	2.0	1	481	2
February -2019	K06720	*Nitrococcaceae; g__uncultured*	416	0.5	1	416	0.5
April -2019	K04642	*Haloparvum*	5761	9.0	1	5761	9
February -2019	K04641	*Haloquadratum*	3717	4.8	2	7434	9.6
December -2018	K03499	*Halapricum*	505	13.0	5	2525	65.0
April -2019	K03499	*Haloparvum*	5761	9.0	6	34566	53.9
April -2019	K03498	*Haloparvum*	5761	9.0	3	17283	27.0
December -2018	K03498	*Salinibacter*	454	11.7	2	908	23.3
May-2019	K03499	*Haloparvum*	5074	8.7	6	30444	51.7
March -2019	K00697	*Halonotius*	313	0.5	1	313	0.5
April -2019	K00697	*Salinibacter*	269	0.4	1	269	0.4
May-2019	K03499	*Haloparvum*	5074	8.6	6	30444	51.7

Finally, when the seasonal variations of the glt (glutamate), bet (glycine betaine), ots (trehalose) and ect (ectoine) genes were assessed, it was seen that they were generally high proportion in winter (Figure 1 and 2). Therefore, the sequences related to the osmolyte accumulation might be increased in the winter because of the low temperature and salinity [12,36]. Furthermore, it was also stated that *Haloarchaea* typically prefers the salt-in strategy at high salt concentrations to maintain osmotic balance. Thus, organic solutes such as simple sugars, glycerol, and amino acids are taken into the cell are probably consumed as substrates [39].

In Tuz Lake, trkA were found the most abundant genes related to K+ uptake. The functional genes related to the intracellular osmolyte accumulation strategy predominantly increased in winter and fall, while trkA genes were found in high abundance in spring and summer (Figure 1 and 2). Moreover, the genes encoding potassium uptake proteins showed similar fluctuations with the archaeal population that increased in the summer and spring [12].

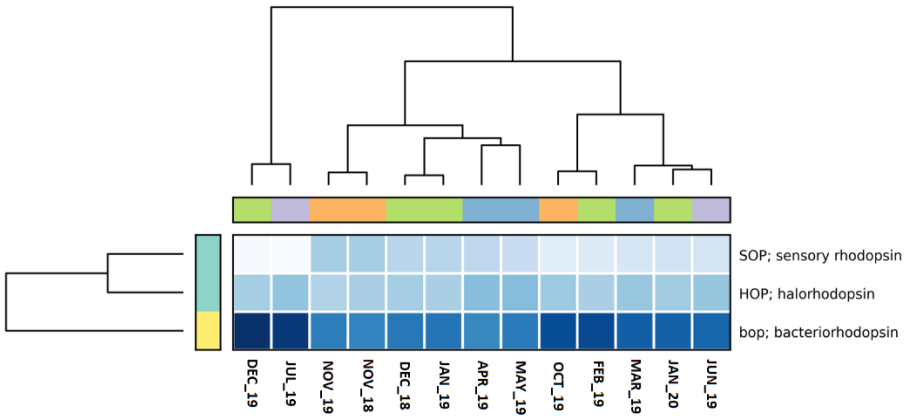
Finally, when the gene profile associated with osmoadaptation was assessed in principal component analysis (PCA) graph, it might be said that although there is no complete seasonal separation, a roughly grouping was observed in the spring and winter seasons (Figure S2).

3.2. Rhodopsin

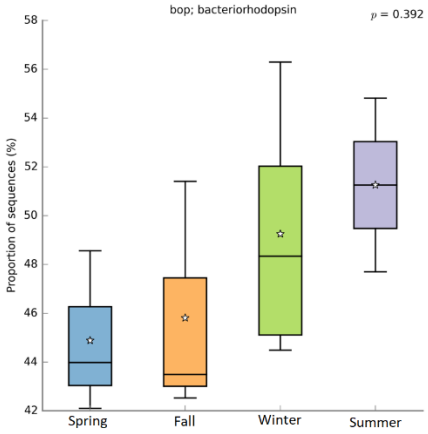
Bacteriorhodopsin (K04641), halorhodopsin (K04642), and sensor rhodopsin (K04643) functions were observed in Tuz Lake (Figure 3a). Light is widely used as an energy source by rhodopsins in hypersaline environments [40]. Bacteriodopsin is an integral membrane protein that acts as a light-driven proton pump. The ion gradient produced across the membrane is then converted into chemical energy (ATP). Halorhodopsin is a bacteriorhodopsin-like retinal protein but an inwardly directed electrogenic chloride ion pump rather than an outwardly directed proton pump [41]. It was reported that along the salinity gradient, an increase in the number of sequences related to bacteriorhodopsins as well as sensory rhodopsins and halorhodopsins was observed, and it was stated that this was due to the more abundant species at higher salinities, *H. walsbyi* and *S. ruber* [42]. In Tuz Lake, the abundance of bacteriorhodopsin and halorhodopsin was increased in summer and spring, respectively. Also, bacteriorhodopsin was observed as the predominant rhodopsin gene (Figure 3a, 3b and 3c). In addition, the genes related to halorhodopsin and bacteriorhodopsin was primarily assigned to *Haloparvum* and *Haloquadratum*, respectively (Table 1). Archaea also use these structures for protection against high salinity and harmful light [43].

Halorhodopsin regulates intracellular osmotic pressure by carrying chloride ions into the cell. It also plays a role in transporting betaine into the cell for osmoregulation [35]. Moreover, the increase in ion concentration may also be a factor, as the conductivity is high during these months. The sequences related to the sensor rhodopsin genes were observed at the lowest proportion in summer while at the highest ratio in fall (Figure 3d). The experiment was conducted under microoxic conditions with *Halobacterium sp.* NRC-1 showed that three of the four rhodopsin types were expressed to harness solar energy and support periods of phototrophic growth. It has also been observed that sensory rhodopsin I (SRI) directs the phototaxis of the cell to wavelengths of light, where both bacteriorhodopsin and halorodopsin can be efficiently absorbed from solar energy and used most efficiently [44]. In the experiment with extremely

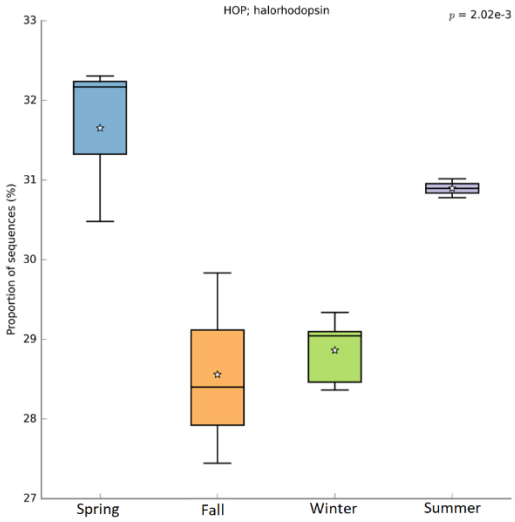
halophilic *Salinibacter*, SRI is synthesized only under low oxygen stress (like the proton pump BR and chloride pump HR) and mediates the absorption of the orange light that drives the proton pumps; it has been also determined that SRII is produced in high oxygen conditions and as a photophobic response to blue light when the cell bioenergetics are activated by the respiratory chain [43]. No statistical difference was observed between the seasons in the sequences related to sensory rhodopsin (Figure 3d). However, the sequences associated with sensory rhodopsin (SOP) function were observed as the lowest proportion in the summer (Figure 3d). SOP could be increased to direct bacteriorhodopsin and halorhodopsin to absorb solar energy efficiently.



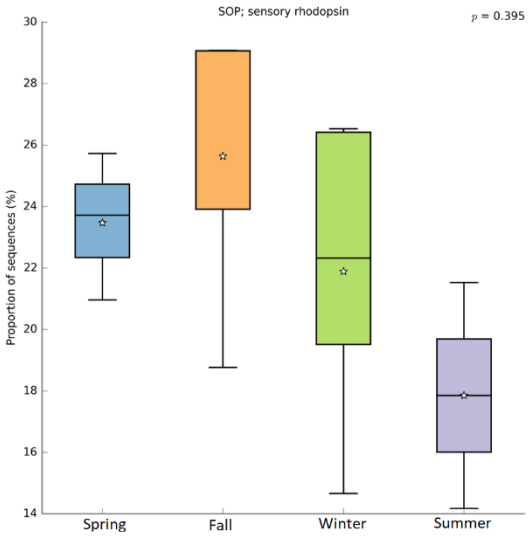
(a)


bop; bacteriorhodopsin
p = 0.392
Proportion of sequences (%)
58
56
54
52
50
48
46
44
42
Spring
Fall
Winter
Summer

(b)


HOP; halorhodopsin
p = 2.02e-3
Proportion of sequences (%)
33
32
31
30
29
28
27
Spring
Fall
Winter
Summer

(c)



(d)

FIGURE 3. (a) Change of functional genes related to rhodopsin by months with heatmap graph (b) Seasonal variation of bacteriodopsin (c) Seasonal variation of halorodopsin (d) Seasonal variation of sensor rhodopsin (Spring:Blue, Fall:Orange, Summer:Purple, Winter:Green). ANOVA statistical test and Tukey-Kramer post-hoc test was applied by Stamp software (95% confidence interval, $p < 0.05$).

4. CONCLUSIONS

The genes related to osmoadaptation, and rhodopsins in Tuz Lake were analyzed, and its seasonal variation was investigated. The sequence associated with K+ uptake genes seems to play an essential role in osmoregulation in Tuz Lake, where the archaeal population was dominant with 95% abundance. Bacteriorhodopsin, halorhodopsin, and sensory rhodopsin functions were observed as rhodopsin functions. The abundance of bacteriorhodopsin and halorhodopsin was increased in summer and spring, respectively. Also, bacteriorhodopsin was observed as the predominant rhodopsin gene. These functional profiles in Tuz Lake might be valuable sources for future ecological and biotechnological research. Moreover, predictive functional profile could be beneficial to indentify candidate microorganisms for commercially significant genes. Further studies are required to reveal the regulation mechanisms of osmoadaptation in halophiles response to environmental parameters.

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Author Contribution Statements SŞD: designed the study, performed the wet-lab work and analyzed the data, wrote and reviewed the manuscript; AK: designed the study and applied for funding.

Declaration of Competing Interests The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

TABLE S1. Weighted_NSTI values of the samples.

Samples/Month	Weighted_NSTI values
NOV_18	0.18
DEC_18	0.17
JAN_19	0.16
FEB_19	0.15
MAR_19	0.17
APR_19	0.23
MAY_19	0.22
JUN_19	0.18
JUL_19	0.16
OCT_19	0.18
NOV_19	0.27
DEC_19	0.17
JAN_20	0.16

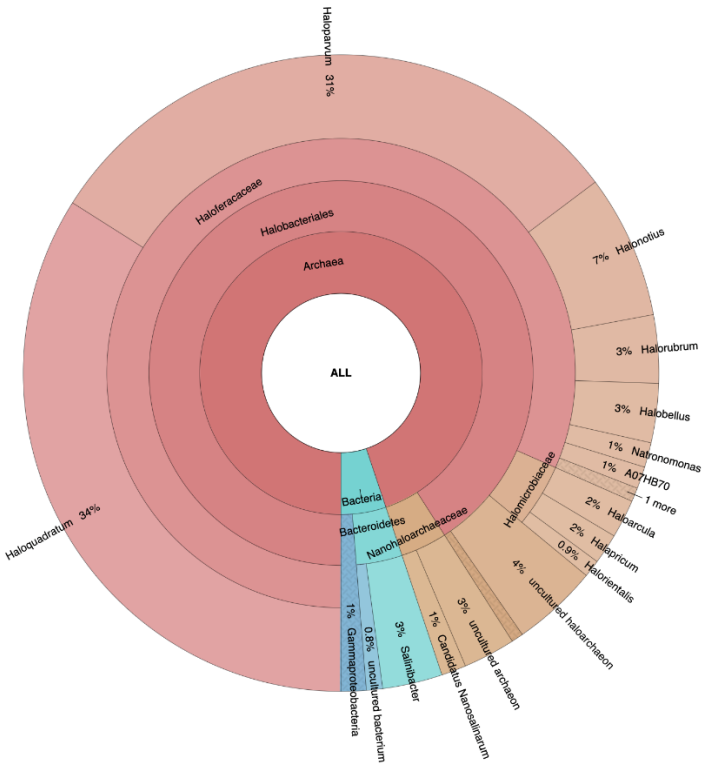

FIGURE S1. The composition of archaea and bacteria in Tuz Lake [12].

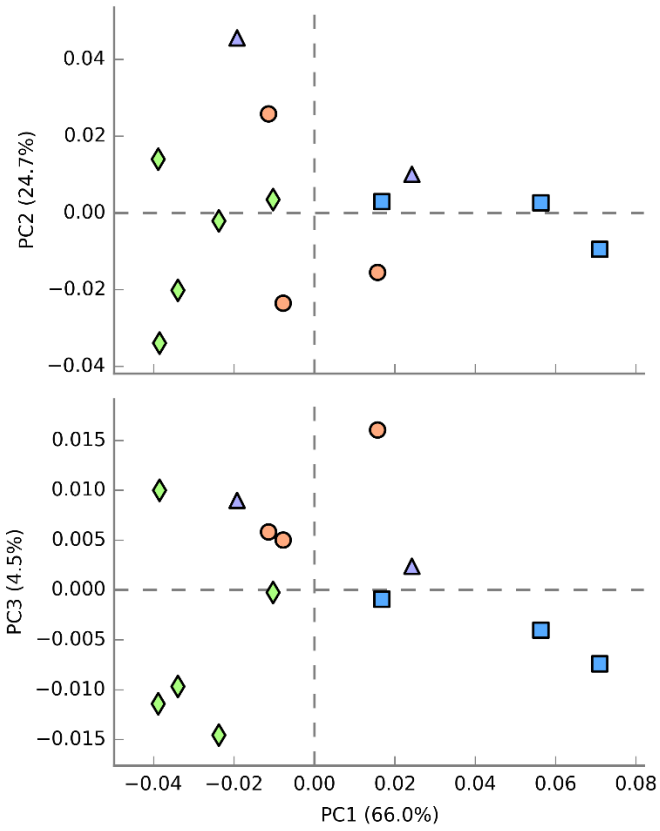

FIGURE S2. PCA plot representing metabolic functions related to osmoadaptation (Spring:Blue, Fall:Orange, Summer:Purple, Winter:Green)