**Overview of the Diversity of Extremely Saline Soils from a Semi-Arid Region Using 16S rRNA Gene Sequencing: A Case Study of the Sebkhas in Algerian High Plateaus**

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**Abstract**

Sebkha is an Arabic word referring to a closed ground depression temporarily occupied by a salt lake. Very few studies on the composition of the microbial communities from these ecosystems in the Algerian High Plateaus have been carried out. To fill this gap, four sebkhas in the eastern High Plateaus of two different Algerian provinces were probed, in the winter 2020. We employed the 16S rRNA amplicon sequencing to understand the distribution and diversity of prokaryotic communities in these hypersaline soils. Our results indicate that the overall archaeal community in the hypersaline soils was dominated by members of the class *Halobacteria* followed by members of the yet uncultured phyla *Hadarchaeota* and *Nanohaloarchaeota*. Within the bacterial classes, *Alphaproteobacteria* was by far the most frequently recovered in all samples, whereas *Cyanobacteria* phylum dominated in one of the sebkhas. It was evident from the data that *Halorubrum* and *Halapricum* were the most abundant archaeal genera, whilst *Rhodovibrio* and *Limimonas* for

Bacteria, and these were present in all samples. Remarkably, the most abundant OTUs belonging to Archaea affiliated especially to the families *Haloarculaceae* (16.6%) and *Halobacteriaceae* (16.3%).
*Keywords:* halophiles, hypersaline soils, 16S rRNA amplicon, OTUs, Algerian sebkhas

## INTRODUCTION

Hypersaline regions are analogues of the Earth's primitive ecosystems, which are generally inhabited by a limited variety of life forms including aquatic and terrestrial habitats (Vera-Gargallo and Ventosa, 2018). Terrestrial hypersaline ecosystems contain low biomass (Xie et al., 2017). These ecosystems are characterized by an extremely variable overall salinity, which is higher than the salinity of sea water, exceeding 50% (Zhuang et al., 2016). They also differ from other environments in many aspects, such as the ion composition, temperature, pressure, and nutrients. Terrestrial hypersaline habitats are widespread, and include coastal salt marshes and sebkhas, inland salt lakes and deep-sea brine pools, and were more prevalent during past geological epochs (Yakimov et al., 2013). These environments are frequently found in abundance in the desert where the decrease in the water level and the intensity of evaporation lead to an accumulation of salt (McKay et al., 2016). Despite the extreme conditions prevailing in hypersaline habitats, the halophilic microorganisms thrive in these ecosystems (Rodriguez-Medina et al., 2020). Generally, the microbial life in these environments is dominated by *Bacillus, Salinibacter, Haloquadratum* and *Halorubrum* genera, and the candidate division *Nanohaloarchaeota* (Mora-Ruiz et al., 2018). To date, there is limited knowledge of the phylogenetic diversity and the potential microbial processes occurring in hypersaline soil ecosystems (Xie et al., 2017; Vera-Gargallo and Ventosa, 2018). Since only 1–5% of the microorganisms in these type of environments are cultivatable under normal laboratory conditions (Felczykowska et al., 2012), the analysis by 16S ribosomal RNA (rRNA) gene amplicons using next generation sequencing platforms has revolutionised the microbiome research in these environments. This approach allowed us to study the world of microbial communities with unparalleled ease, by improving accuracy and lowering of the cost. Since its discovery, it has been widely used to identify the culturable and unculturable bacterial species from environmental samples and to perform taxonomic analysis.

To improve our knowledge of microbial diversity in hypersaline environments, we aimed to elucidate the structure and composition of the bacterial and archaeal communities present in four different Algerian sebkhas. These sebkhas expand from the coastal areas to the northern Saharan fringes and across

the High Plateaus. Covering this vast region is beyond the scope of one study. Therefore, in this study we focused on the sebkhas in the High Plateaus which are classified as important bird sanctuaries and lack direct contact with the sea. These sebkhas remain poorly described except for few ornithology studies, as only a few microbial explorations have been conducted in the soils. We investigated the composition of microbial communities and their phylogenetic diversity in four distinct soil habitats, from distinct sebkhas using 16S rRNA amplicon sequencing. We present our findings which elucidate the structure of the bacterial and archaeal communities from four hypersaline soils of these Algerian sebkhas.

## MATERIALS AND METHODS

*Study sites and soil sample collection*

Soil from four Sebkhas (Sebkha Ank-Djemel "ANG", Sebkha Djendli "DJS", Sebkha El-Tarf "ETS", Sebkha Guellif "GFS"; Table 1, Fig. 1) were collected on January 2020 at a depth of 0–10 cm from the High Plateaus in the Northeastern Algeria: province of Batna "DJS", and province of Oum El Bouaghi "ANG", ETS", GFS" (Fig.1). The latter three sebkhas were protected under the auspices of the Ramsar International Treaty for Wetlands of 2004 and have been named Ramsar sites (*rsis.ramsar.org*). These regions are rarely flooded with rainwater and has a typical semi-arid climate, with mild winter and hot and dry summer. Sampling locations were recorded with a GPS. Collected soils from each sebhka were transported to the laboratory in sterile 50 ml falcon tubes in an ice box and immediately frozen at -20˚C.

*Determination of physical and chemical properties*

A portion of the biomass of our samples were centrifuged for 10 min at 10000 rpm. The liquid parts were extracted, diluted according the degree of salinity of each sample using milli-Q water. The diluted liquids were filtered with 0,45 µm syringe filter (Millipore). Ionic composition quantifications were performed at the Research Technical Services of the University of Alicante (Spain) using ion chromatography. Major salt concentrations were calculated from the cations and anions measurements, which were previously normalized according to the dilutions applied for their quantification. The ion concentrations were combined, thus shaping the main salts composition of hypersaline soils, according to the precipitation that experiment in extreme ecosystem. We started with the most important salts (such as

NaCl) and the other salts presence was dependent of the quantity of cations or anions involved in this salt, until the exhausting of the ion concentration. Salinity was measured using a Refractometer. The organic matter was measured with the loss of ignition method in a Muffle Furnace (Nabertherm) as previously described in Font-Verdera et al. (2021).

*DNA extraction*

DNA was extracted according to Högfors-Rönnholma et al. (2018). Eight grams of each soil sample were suspended in 12 ml of sodium phosphate buffer (500 mM $Na_2HPO_4$ and 500 mM $NaH_2PO_4$, pH 7.2), stirred for 5 min at 250 rpm, and chilled for 3 min at 4˚C twice. The resulted slurry was centrifuged in 50 ml falcon tubes at 500 *g* for 15 min. The supernatant was transferred to clean tubes and stored at room temperature in the dark. The pellet was re-suspended again in 12 ml of sodium phosphate buffer and the extraction process was repeated one more. Supernatant from both extractions were pooled for each sample (approximately 30 ml), aliquoted in to 2 ml Eppendorf tubes and centrifuged at 10,000 *g* for 15 minutes. The supernatant, containing the extracellular DNA was discarded and the cell pellets were combined in a single sterile 2 ml tube. The cell pellets were washed with 1 ml sodium phosphate buffer (centrifuged at 10,000 *g* for 15min). The cell pellets were resuspended in 500 µl de TES-lysozyme [50 mM Tris-HCL, 30Mm EDTA, 20 g / 100 ml saccharose, 20 g / ml Lysozyme, pH 8]. The cells were lysed, and DNA was extracted as follows. Sodium Dodecyl Sulfate (10%) was added to the samples and incubated at 37°C for 30 minutes. DNA-containing supernatant was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 12,000 *g* for 5 minutes. The aqueous phase was precipitated with 0.7 volumes of isopropanol and 0.1 volumes of 3 M sodium acetate *overnight* at -20°C. After centrifugation at 14,000 *g* at 4°C for 30 minutes, the DNA was washed with 70% ethanol, dried and dissolved in 50 µl sterile nuclease-free water. Concentration of DNA was quantified using a NanoDrop™ ND-1000 (Thermo Scientific, United States). The extracted DNA samples were stored at -20 °C until further analysis.

*PCR amplification and sequencing of 16S rRNA genes*

Using the extracted DNA from samples as templates V4 variable region of the 16s rRNA was amplified using PCR in a thermocycler (model 2720 Thermal cycler Applied Biosystems, United States) with the following cycling conditions: 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s,

annealing at 50 °C for 60 s and elongation at 72 °C for 90 s. The primers used were 515′F_5' - GTGYCAGCMGCCGCGGTAA- 3' and 806R_5' - GGACTACNVGGGTWTCTAAT- 3' (Caporaso et al., 2011), which amplifies the region of interest in both bacteria and archaea. The 50 µL reaction mixture contained a PCR master mix 25 µl (MyTaq™, Bioline), 2.5µL of template DNA, 2µL of each oligonucleotide primer, and 20.5 µl PCR-grade water. PCR amplicons were examined on a 1% agarose gel in a transilluminator (model *Syngene GBOX systems*). Quality of amplicons were checked with the Qubit 4.0 Fluorimeter (Thermo Fisher Scientic, United States), sequenced at FISABIO Sequencing and Bioinformatics Service (Valencia, Spain) with Illumina MiseqTM technology, 2 x 250 bp paired end run.

*16S rRNA sequencing and bioinformatics analyses*

The prokaryotic community composition was analysed using the V4 region of 16S rRNA gene sequences. The sequences were quality-filtered using the Quantitative Insights into Microbial Ecology (QIIME). The following reads were discarded: low-quality reads with a quality score <20; reads shorter than 250 bp; reads with mismatches in the barcode/primer region; reads containing ambiguous bases or any unresolved nucleotides. Potential chimeric sequences were checked and removed processing with the following parameters: *--p-trunc-len-f 280 --p-trunc-len-r 220 --p-trim-left-f 19 --p-trim-left-r 22*, where the forward and reverse reads were further truncated to the length of 280 and 220, respectively, in order to have ~12 overlapping nucleotides. The remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% identity threshold. Representative unique OTUs were aligned using SINA tool using SILVA_138_SSURef_NR99 as a reference. SILVA database taxonomy was utilized for annotations to establish taxonomic levels of OTUs.

All analysis of microbial data was performed in R (Rstudio v4.0.3). All datasets were rarefied to prevent potential bias caused by different sequencing depths. The alpha diversity was calculated using package ampvis2 v2.7.4 and was extracted using the command *amp_alphadiv* to describe the sample complexity (observed OTUs, InvSimpson, Chao1, and Shannon's indices). Venn diagrams were plotted with R package VennDiagram v4.0.5. In addition, the relative abundance of microbial structure was assessed using the rarefied dataset and was calculated for each sample using package phyloseq. The function "*tax_glom*" was used to compare the relative abundance of phyla between different samples, and the function "*transform_sample_counts*" was performed to convert the count data to relative abundance. Stacked bar plots of phyla abundance were plotted using the package ggplot2. Metabolic

profiles of the prokaryotic phyla were predicted based on the data compiled in the FAPROTAX database (Louca et al.,2016) using "microeco" package (v0.2.0, Liu et al., 2021).

## RESULTS

### *General soil properties*

The physical and chemical properties of the sampled soils and information on sampling site are summarized in Table 1. The salinity of the soils were between 18.9% to 26.7%. Lowest NaCl concentration (1.504 M) and conductivity (948 µS/cm) were measured for GFS. Additionally, all samples exhibited trace amounts of $MgSO_4$, $MgCl_2$, KCl, and $CaCl_2$, ranging between 0.064-0.190 M, 0-0.313 M, 0.001-0.037 M and 0.02-0.035 M, respectively. $CaCO_3$ values were comprised between 2.646-1.368M. Noticeably, high concentrations of NaCl, $MgSO_4$ and $CaCO_3$ were detected in ETS and low concentrations in GFS. All sampled soils were generally neutral to subtly basic, with pH values ranging from 7.2 – 8.5. Organic matter was generally lower in GFS sample (8.39%) and increased in the others samples but did not exceed 15% in any samples.

### *Microbial community composition*

The number of sequences per individual site ranged from 155,942 to 161,327. After quality filtering, denoising, and chimera removal, a total of 93,853 rRNA sequences were obtained (34,908, 17,635, 14,926 and 26,384 sequences were acquired from ANG, DJS, ETS and GFS, respectively). These high-quality reads were assembled into 863 OTUs.

To compare the microbial abundance, samples were rarefied to 14,431 reads obtaining a total number of 45,534 sequences for Bacteria (49.23%) and 46,950 sequences for Archaea (50.75%). Our analysis of the hypersaline soils of Algerian sebkhas showed that the prokaryotic community composition was different among all sites (Fig. 2A). Taxonomic distribution indicated that altogether there were 27 different representative phyla, 9 from the bacterial and 18 for the archaeal domains. Members of the archaeal phylum *Euryarchaeota* and of the bacterial phylum *Proteobacteria* dominated almost in all sites. *Alphaproteobacteria* were the most predominant of the *Proteobacteria* (Fig. 2B). *Proteobacteria* represented 63% in DJS, 61% both in ETS and GFS, with the exception of ANG sample (< 32%), that was dominated by *Cyanobacteria* with 48% (Fig. 3). Less than 3% of the total number of sequences of the bacterial fraction were represented by *Firmicutes* OTUs: 10 OTUs were assigned to *Clostridia* and 2

OTUs to the class *Bacilli*. 9.25% were assigned to *Patescibacteria*, representing the classes *Parcubacteria* (<1%), *Gracilibacteria* (2.92%), and *Saccharimonadia* (1.21%), which were exclusively detected only in DJS. Additionally, the number of OTUs classified as *Actinobacteriota* were also only detected in DJS (9%). Other taxa that were identified as a minor taxonomic group in the four samples were *Verrucomicrobiota*, which the higher proportion was detected in ETS (17%) and the lower value was registered in DJS (5%). *Bacteroidetes* phylum was represented by 51 OTUs identified by the lowest number of reads.

At each sample site, the *Euryarchaeota* were the most abundant, accounting for 81% in GFS, 79% in DJS, 75% in ANG, and 59% in ETS of the total archaeal sequences (Fig. 3). *Euryarchaeota* mostly comprised of the class *Halobacteria*, and the families *Haloarculaceae* (16.62%) and *Halobacteriaceae* (16.3%). The four phyla *Hadarchaeota, Nanoarchaeota, Nanohaloarchaeota,* and *Thermoplasmatota* together accounted for 19% and 41% of the total archaeal sequences among all samples. The highest proportion of sequences belonging to the phylum *Hadarchaeota* within the class *Hadarchaeia*, were detected in ETS (18%), and the lowest in ANG (3%) samples. The highest abundance for *Nanohaloarchaeota* was registered in ANG (10%) followed by ETS (7%) whereas *Nanoarchaeota* was represented with 7%, 6%, 3%, and 2% in ANG, ETS, GFS, and DJS, respectively. Other phyla did not exceed 2% across all samples. In summary, microbial community distribution is remarkably diverse among bacteria compared to the archaeal fraction, which was mainly monopolized by phylum *Euryarchaeota* in all samples.

*Predominant prokaryotic genera*

Of the 863 total OTUs identified in all samples there were 85 OTUs, which constituted at least 0.1% of relative read abundance each (Fig. 4) in a single sample and 229 OTUs were distributed among 16 genera affiliated to both domains, with a relative sequence abundance of more than 1% each of the total communities in the four soil samples (Fig. 4 and Fig. 5). In the archaeal domain, *Euryarchaeota* displayed a substantial diversity, and was by far the most dominant phylum with most detected genera in all samples, representing 23 genera. Only 12 genera were dominant with a relative read abundance of more than 1% (Fig. 4). These were *Halapricum* (6.4% - 24.5%), *Halorubrum* (4.9% - 13.2%), *Halodesulfurarchaeum* (4.3% - 13.7%), *Halococcus* (4.9% -10.3%), *Natronomonas* (3.5% - 5.8%) and *Haloplanus* (2.4% - 5.2%). The remaining archaeal genera detected were *Haloarcula* (0.5% - 7.4%),

*Halobellus* (1% - 2.8%), *Halovenus* (1.6% - 4.2%) and *Halonotius*, which was represented with 4.9% in ANG and 4.7% in GFS, not detected in sample ETS. The genera *Halomicroarcula* and *Halorubellus* were found with abundances <1.8% among all samples. Some genera were found in lower abundance of <1% in only one sample, such as *Halarchaeum* (0.6% of abundance), that was detected only in DJS (Fig. 4). Additionally, the genera *Haloquadratum* and *Halorientalis* were present in all samples, with the exception of ETS with lower abundances. *Halobacterium* was detected in ETS and GFS with 0.6% - 0.1% of relative abundances, respectively. However, the bacterial fraction was much less diverse in terms of abundance than that of Archaea. *Rhodovibrio, Limimonas, Desulfitibacter* and *Salinibacter* were the 4 most representative genera with relative read abundances of more than 1%, at least in each individual sample. *Rhodovibrio* represented relative sequence read abundances of >25%, except in the ETS sample (Fig.5). Similarly, *Limimonas* represented between 5.4% – 13.5% and *Desulfitibacter* (within *Firmicutes*) between 1.1% – 3.8% of the reads. The genus *Salinibacter* displayed a lower relative abundance ranging from 0.1% to 1.2%. All other genera, including *Desulfovermiculus, Altererythrobacter, Enhydrobacter* and *Erythrobacter* displayed a very lower relative abundance (<0.9%) overall and were not detected in all samples in our study. Interestingly, most of the above mentioned bacterial genera were abundant in the ANG sebkha except for the genus *Limimonas*, which was least abundant in ANG (5.4%). Some genera were detected in only one sample and were absent in the others with a relative abundance <0.2% such as *Altererythrobacter, Erythrobacter* and *Salinimicrobium* which were detected only in DJS, and *Enhydrobacter* which was detected only in ETS. We speculate that this may be due to the lower abundance of these genera in these soils and therefore below the detection threshold of the 16S rRNA sequencing.

*Soil prokaryotic community diversity*

The microbial diversity within each sample was estimated using Shannon and Simpson diversity index (Table 2). Shannon index ranged between 4.28 and 5. Simpson evenness remained stable across all samples, ranging only within 0.95 - 0.98. The Inverse Simpson index was highly variable among the different sites, from 21.99 (ANG) to 95.46 (ETS).

Among the 863 OTUs observed in the four soil samples, only 34 were found common within them (Fig. 8). The highest number of 359 OTUs were detected in ANG (41.59% of the total OTUs) followed by GFS (319 OTUs, 36.96%), DJS (291 OTUs, 33.71%) and ETS (248 OTUs, 28.73%). Moreover, ANG

also included the highest number of unique OTUs (190 OTUs), closely followed by ETS (186 OTUs). Samples GFS and DJS displayed 142 and 141 unique OTUs respectively.

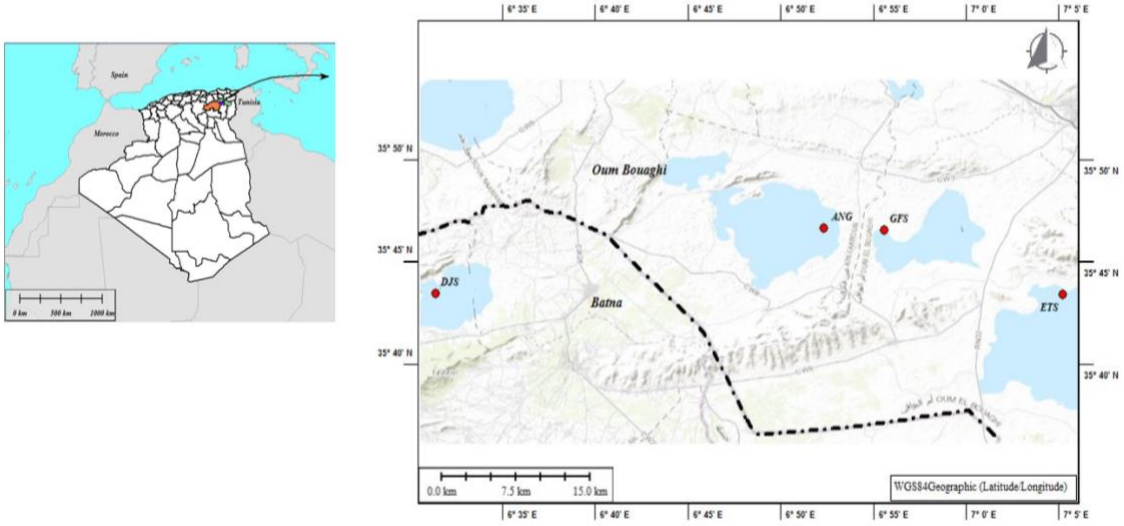


**Fig. 1.** Sampling locations of different sebkhas in the High Plateaus in Algeria: ANG, sebkha Ank-Djemel; DJS, sebkha Djendeli; ETS, sebkha El-Tarf; GFS, sebkha Guellif.

**Table 1.** Soil physical, chemical and geographical properties.

| **Properties** | **ANG** | **DJS** | **ETS** | **GFS** |
|---|---|---|---|---|
| **pH** | 7.2 | 8.0 | 7.8 | 8.5 |
| **Salinity (%)** | 24.5 | 26.7 | 18.9 | 25.7 |
| **EC(µS/cm)** | 1,130 | 1,260 | 1,023 | 948 |
| **NaCl (M)** | 2.725 | 2.847 | 3.275 | 1.504 |
| **CaCO3 (M)** | 1.391 | 2.052 | 2.646 | 1.368 |
| **MgSO4 (M)** | 0.064 | 0.158 | 0.190 | 0.146 |
| **MgCl2 (M)** | 0.00 | 0.167 | 0.230 | 0.313 |
| **KCl (M)** | 0.004 | 0.037 | 0.033 | 0.001 |
| **CaCl2 (M)** | 0.035 | 0.029 | 0.025 | 0.02 |
| **% OM** | 14.673 | 14.454 | 13.510 | 8.396 |
| **Altitude (m)** | 826 | 870 | 834 | 830 |
| **Latitude** | 35,7770556° | 35,7236044° | 35,7224167° | 35,7752778° |
| **Longitude** | 6,8724444° | 6,5241975° | 7,0872222° | 6,92725° |
| **Date of soil sampling** | Jan,2020 | Jan,2020 | Jan,2020 | Jan,2020 |
| **Province** | Oum Bouaghi | Batna | Oum Bouaghi | Oum Bouaghi |

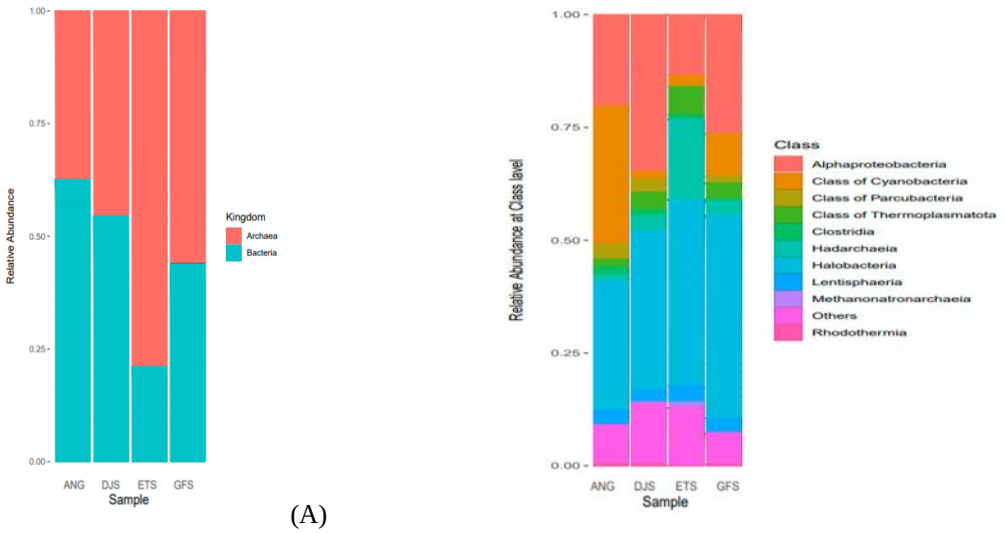


**Fig. 2.** Relative abundance of OTUs in each soil sample at the kingdom and class levels. (A) Relative abundance of Bacteria and Archaea kingdoms. (B) Relative abundance of the 10 most abundant classes.

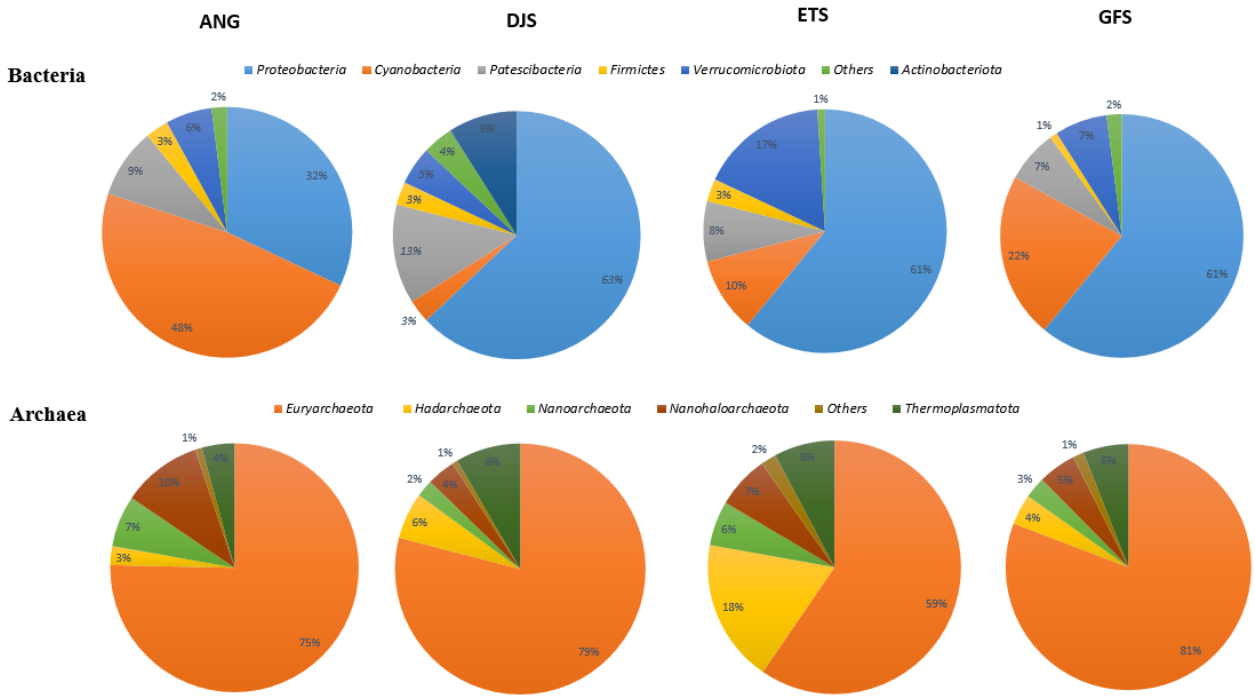


**Fig. 3.** Taxonomic distribution at the phylum level for both kingdoms Bacteria and Archaea in all samples of hypersaline soils. *Patescibacteria* is considered a superphylum (Tian et al. 2020).

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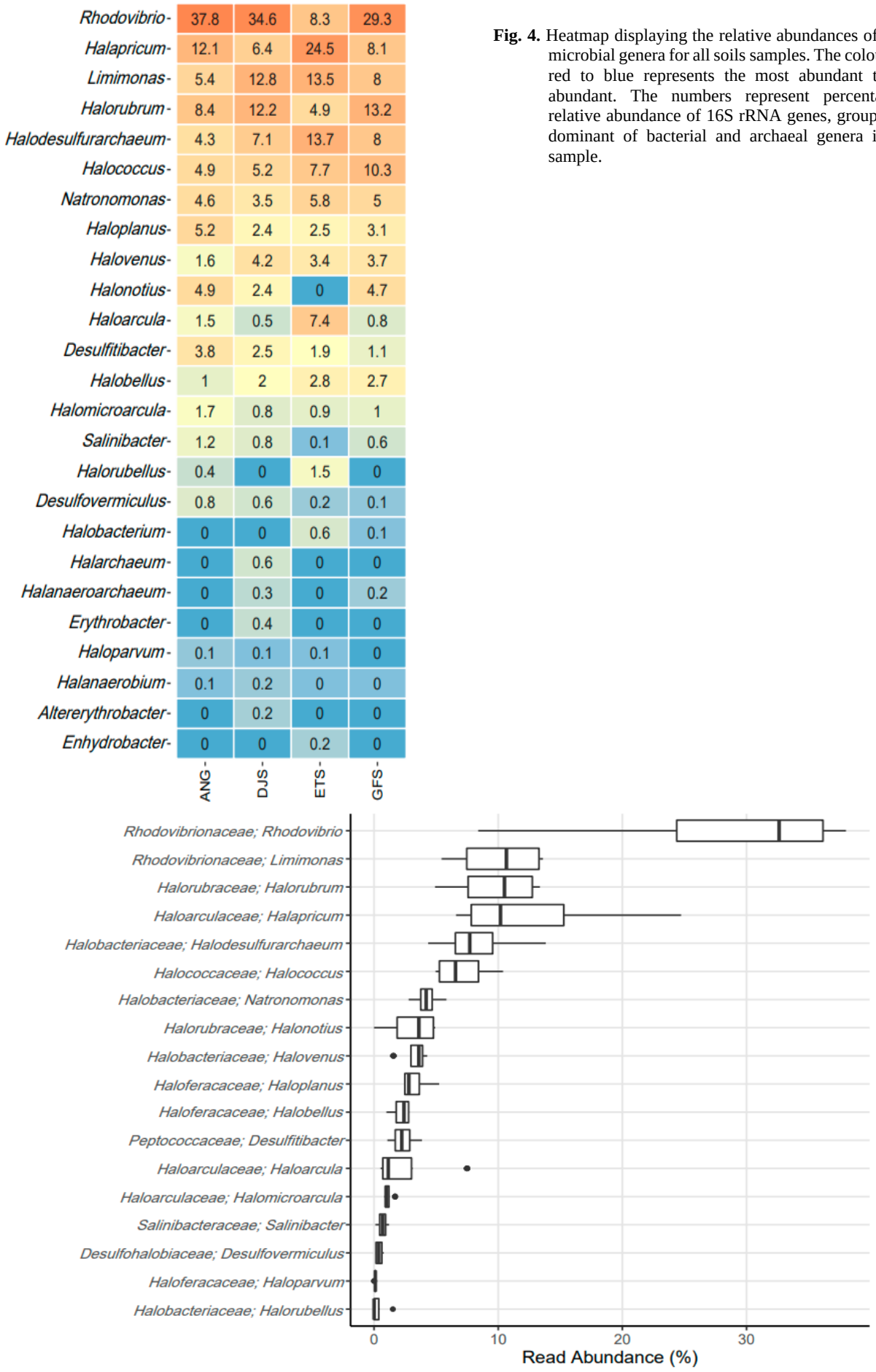


**Fig. 4.** Heatmap displaying the relative abundances of top 25 microbial genera for all soils samples. The colour from red to blue represents the most abundant to least abundant. The numbers represent percentage of relative abundance of 16S rRNA genes, grouping the dominant of bacterial and archaeal genera in each sample.

**Fig. 5.** Boxplot for average abundance of the top 18 major genera with their families in the hypersaline soils from different sebkhas (x-axis = Read abundance is displayed on a log-scale, vertical bold line = the boxplot depicts the median, horizontal lines = minimum and maximum value; and dots = outliers).

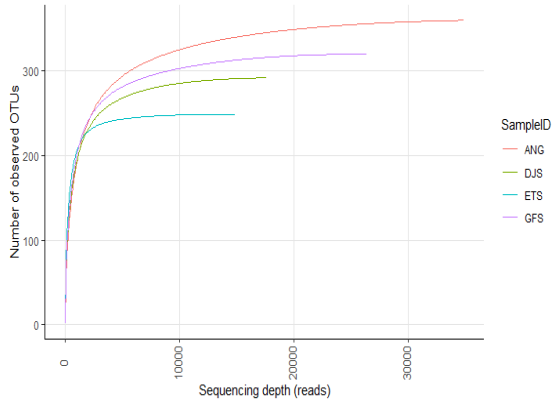


**Fig. 6.** Rarefaction curves indicating the observed number of OTUs based on sequencing depth in all samples of hypersaline soils ANG, DJS, ETS, and GFS.

**Table 2.** Richness and diversity indices of prokaryotes for the four hypersaline soils from different sebkhas.

| Sample ID | Observed OTUs | Shannon | Simpson | InvSimpson | Chao-1 | ACE |
|---|---|---|---|---|---|---|
| **ANG** | 360 | 4.28 | 0.95 | 21.99 | 361.28 | 363.70 |
| **DJS** | 292 | 4.62 | 0.97 | 43.73 | 292.33 | 293.35 |
| **GFS** | 320 | 4.72 | 0.98 | 52.51 | 320 | 320 |
| **ETS** | 249 | 5.00 | 0.98 | 95.46 | 249 | 249 |

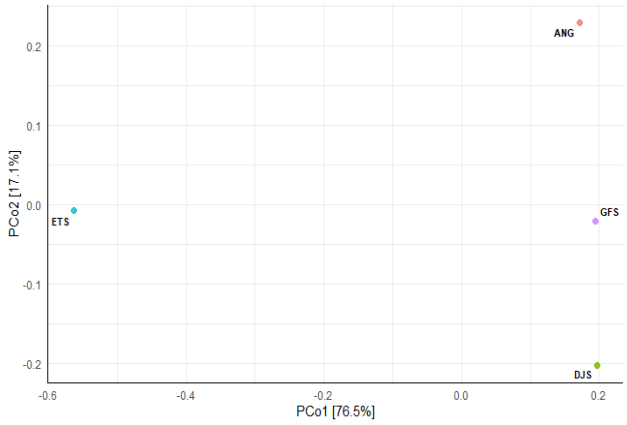


**Fig. 7.** Principal Coordinates Analysis (PCoA) based on the Bray-Curtis dissimilarity of all samples from hypersaline soils. Principal Components (PCs) 1 and 2 explained 76.5% and 17.1% of the variance, respectively.

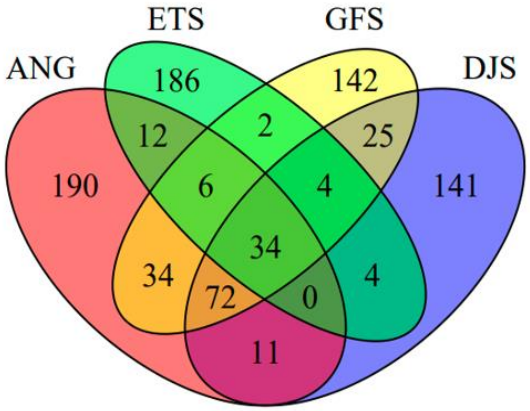


**Fig. 8.** Venn diagram showing the unique and shared OTUs in all soil samples. ANG, sebkha Ank-Djemel; DJS, sebkha Djendeli; ETS, sebkha El-Tarf; GFS, sebkha Guellif.

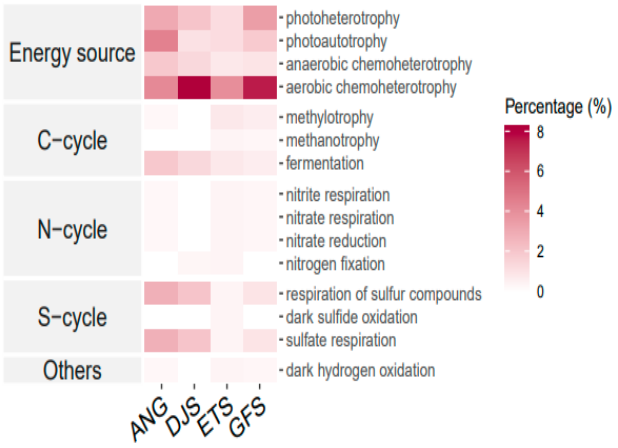


**Fig. 9.** Difference in functional pathway prediction using microeco package between the 16S RNA data of hypersaline soils and published metagenome shotgun sequencing data.


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