

Journal Pre-proofs

Original article

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PII: S1319-562X(23)00286-3
DOI: <https://doi.org/10.1016/j.sjbs.2023.103841>
Reference: SJBS 103841

To appear in: *Saudi Journal of Biological Sciences*

Received Date: 4 September 2023
Revised Date: 2 October 2023
Accepted Date: 15 October 2023

Please cite this article as: N. Elshafey, M. A. I. Mansour, H.A. Hamedo, M.E. Elnosary, N. Hagagy, A. Ahmed Al-Ghamdi, R. María Martínez-Espinosa, Phylogeny and Functional Diversity of Halophilic Microbial Communities from an Thalasso Environment, *Saudi Journal of Biological Sciences* (2023), doi: <https://doi.org/10.1016/j.sjbs.2023.103841>

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Phylogeny and Functional Diversity of Halophilic Microbial Communities from an Thalasso Environment

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Abstract

The El-Rawda solar saltern, located in North Sinai, Egypt, is formed through the process of water evaporation from the Bradawil lagoon. This evaporation leads to the precipitation of gypsum, halite minerals, and salt flats, which subsequently cover the southern and eastern areas of the lagoon. This study employed the shotgun metagenomic approach, the illumine platform, and bioinformatic tools to investigate the taxonomic composition and functional diversity of halophilic microbial communities in solar saltern. The metagenomic reads obtained from the brine sample exhibited a greater count compared to those from the sediment sample. Notably, the brine sample was primarily characterized by an abundance of archaea, while the sediment sample displayed a dominant abundance of bacteria. Both samples exhibited a relatively low abundance of eukaryotes, while viruses were only found in the brine sample. Furthermore, the comparative analysis of functional pathways showed many important processes related to central metabolism and protein processing in brine and sediment samples. In brief, this research makes a valuable contribution to the understanding of very halophilic ecosystems in Egypt, providing insights into their microbial biodiversity and functional processes.

Keywords: shotgun metagenomic; saline soil; brine; North Sinai; halophiles; solar saltern, Bardawil lagoon.

1. Introduction

Saltern crystallizer ponds provide a very beneficial setting for researching halophilic microorganisms in their natural environment. All around the world, salterns are primarily found in inland coastal tropical and subtropical regions. Regardless of location, saltern brines with identical chemical compositions and global salt concentrations have similar microbial populations (Javor, 2002; Oren 2002a, 2009). Most of the microorganisms in these ponds have been discovered to be natural sources of intriguing compounds with prospective uses in biotechnology Martinez et al. (2022).

Microorganisms that exist in diverse extreme environmental niches have been classified as thermophiles, psychrophiles, piezophiles, xerophiles, acidophiles, alkaliphiles, and halophiles. The ability of living forms to thrive in challenging environments has drawn the interest of the scientific community due to the prospective applications of their bioactive compounds in various sectors such as medicine, agriculture, bioenergy, and other industries. Halophilic microorganisms are distinguished by their ability to thrive in

saline environments, making them the predominant living forms in such conditions. According to the study conducted by Abaramak et al. (2020), For this reason, interest in the characterization of hypersaline ecosystems is increasing with the aim of identifying new species. Thus, research contributes to knowledge of these ecosystems and their microbial communities and sheds light on the design and scaling up of biotechnological processes. Within this context, this work is focused on characterizing a non-explored salty environment in Egypt. Lagoon of Bardawil, this shallow, hypersaline lagoon (salinity 50.9%) covers a significant stretch of Sinai's Mediterranean shore. Two artificial and one natural inlet connect the surface area, which has a surface area of about 600 km². Gypsum and mineral halite are precipitated because of lagoon water evaporation (Embabi, 2018). A unique class of microorganisms that can with stand and/or require high salinities, high temperatures, and harsh sun irradiation are supported by the high salt concentrations in these habitats Jamadar et al. (2016). Microbes that can survive in this type of salty environment are known as halophilic and halotolerant microorganisms. Halophiles belong to all three domains: *Archaea*, *Bacteria*, and *Eukarya* and include representatives of a wide range of physiological types adjusted to high salt concentrations (Aljohny, 2015). Extreme halophiles, in general, are members of the archaeal groups *Halobacteriaceae* and *Haloferacaceae*. They thrive in media with 2.5–5.2M NaCl and make up most communities in environments with high salt concentrations, while the majority of the moderately halophilic bacteria thrive at 0.5–2.5M NaCl. In salty ponds, *Salinibacter* species are widespread and thrive best in conditions containing less than 2.5 m NaCl Anton et al., (2022), McGenity and Oren, 2012; Ventosa et al., (2012) and Oren, 2016. A few species of green algae, including *Dunaliella*, yeasts, such as *Hortaea werneckii*, filamentous fungi, like *Wallemia chthyophaga*, and protozoa, like *Halocafeteria seosinensis*, are the best salt-adapted eukaryotes, whereas halophilic Eukaryotes are less common in salted ponds Gunde-Cimerman et al. (2018). Microbiologists have been studying environmental metagenomes for the past few decades in order to characterize ecological aspects based on the abundance of uncultivable microbiota and identify taxa Pramanik et al. (2015) and Mangrola et al. (2015). Numerous studies have been conducted on the cultural aspects of extremophiles, as well as various culture-independent techniques employed to determine their existence, monitor their physiological processes, understand their biochemical characteristics, and investigate their interactions with the surrounding environment. Rampelotto, 2013 and Merino et al. (2019). Metagenomics is a cutting-edge technology for studying the genetically rich

resources of uncultured microbiota without the use of conventional culture-dependent techniques. To determine the true microbial composition of those environments, it is based on the premise of direct DNA isolation from special environmental samples containing a variety of microbiota (Ngara and Zhang, 2018) and Ahmad et al. (2019). Next Generation Sequencing (NGS) has also made targeted metagenomics more accessible (Amrane and Lagier, 2018). Understanding the interactions between microbial populations and their habitats is another benefit of metagenomic study. Metagenomics and bioinformatic analysis can thus be used to mine massive metagenomic datasets and identify overarching trends that control microbial ecosystems. The initial hypothesis assumed is that bacterial and archaeal cells respond differentially to physicochemical parameters of brine and sediment, which can be manifested in variations in the number of total cells and different composition of the microbial communities. We are aware of no reports of metagenomic community profiles of halophiles from these salterns. As a result, this work uses metagenomics, which gives an overview of microbial communities' diversity, distribution, and functionality, to reveal for the first-time new insights into the community structure of native microbiota from the North Sinai solar saltern.

2. Material and Methods

2.1. Sampling and Site description

In January 2021, sediment and brine samples were collected from a solar saltern, North Sinai, Egypt (Figure 1), located at 31°02'26.5"N 33°21'19.6"E. This solar saltern is in the southern part of the Bardawil lagoon's and it is used for NaCl production from seawater described as Elrawda saltern. Brine samples were taken from 3 to 5 cm below the surface and placed in sterile Pyrex bottles with a capacity of 1000 ml. 500g of sediment samples were taken from red, pink, or white precipitate and placed in sterile plastic bags before being transported to the lab under cooling condition at -4°C. They were immediately frozen at -80°C upon arrival at Arish University for DNA extraction. Physicochemical characterization of sediments and brines as pH and Electrical Conductivity (EC) were measured in the field by using pH and conductivity probes V. tech, pH/conductivity and temperature meter, Taiwan. The concentration of ions like Ca²⁺, Mg²⁺, Cl²⁻ was measured by atomic absorption spectrophotometer, Model Perkin Elmer 2380 Szép et al. (2017 and 2019). The salinity of brines and sediments were determined in the chemistry department,

science faculty, Suez Canal University, according to procedures described by Trussellet al. (1989).

2.2. Metagenomics analysis:

Metagenomics is a culture-independent method that allows the identification and characterization of organisms from all kinds of samples. Whole-genome shotgun sequencing analyses the total DNA of a chosen sample to determine the presence of micro-organisms from all domains of life and their genomic content. Importantly, the whole-genome shotgun sequencing approach reveals the genomic diversity present but can also give insights into the functional potential of the micro-organisms identified Pérez-Cobas et al. (2020). Following the manufacturer's recommendations, the genomic DNA was extracted from soil samples using a DNeasy Power Soil Kit (QIAGEN, Germany) Nguyen et al. (2021). Using a customized protocol and the DNA Isolation Kit, 100 ml of brine sample was passed through a sterilized 0.45 m filter membrane to extract the DNA. After collecting the filter, 300 l of Meta-Lysis Solution (2X) and 1 l of Proteinase K were added to the tube. The Nextera XT DNA library preparation kit was used to mix by vortexing the library, in accordance with the manufacturer's instructions. Finally, equal volumes of normalized libraries and pooling libraries were merged in a single tube. After pooling, the library pool was diluted and heat-denatured in accordance with the manufacturer's instructions at the genomic research program, Children's Cancer Hospital, Egypt 57357 (Illumina), before the libraries were loaded for the sequencing run.

2.2. Bioinformatics analysis

The quality of generated raw reads was inspected using Fast QC and Trimoraic to trim reads with a quality lower than 20 and trimming adaptor sequences Bolger et al. (2014). Taxonomy classifications were performed on trimmed reads using Kraken2 wood et al. (2019) against PlusPFP collection retrieved from (<https://benlangmead.github.io/aws-indexes/k2>). This collection contains archaea, bacteria, viral, plasmid, human, protozoa, fungi, and plant data. Taxonomy classification data were visualized by Pavian Florian et al. (2020). Also, trimmed reads were aligned against NCBI RefSeq database Pruitt et al. (2005) using diamond aligner V0.9.30 Buchfink et al. (2015) with default parameters followed by enrichment analysis using MEGAN V6.21.7. Huson et al. (2016).

2.3. Statistical analysis

The data were examined with Minitab 19 and shown on graph pad 8. *P* values of 0.05 were regarded as significant. *Post hoc* analysis of all group interactions was performed using the Tukey test for pairwise and one-way ANOVA comparisons. Letters are used to represent the outcomes of *post-hoc* analysis, with separate letters denoting significant differences across different groups and groups that share the same letter, indicating non-significant differences.

The relation between physicochemical parameters and microbial community was measured using the correlation method by means of graph pad 8. A *P* value of 0.05 and Pearson factor *r* from -1 to 1.

3. Results

3.1. Physico-chemical analysis

From the results of the physico-chemical analysis, it can be concluded that hypersaline features characterize the studied solar saltern. The pH ranged from 6.06 to 7.48 in brine and sediment, respectively, and salinity recorded was higher in brine than in sediment samples, 29% and 12%, respectively. The conductivity value was higher in brine (13840 ds m⁻¹) than those monitored in sediment samples (520 ds m⁻¹). Regarding the concentration of the ions quantified, Cl⁻ was the most abundant ion as expected (174112.5 mg/l in brines samples and 0.0343mg/l in sediment), while Mg⁺² and Ca⁺² concentrations were as follows: 23.4, 14.02 mg/l in brine and sediment respectively, for Ca⁺² 46.7 and 2.15 mg/l in brine and sediment, respectively, in the case of Mg⁺². All parameters have a significant value *p*< 0.05 between both samples (Table 1).

3.2. Metagenomic analysis

3.2.1. Quality Control

The NGS Illumina sequencer was used to generate 250000 read pairs from brine samples and 50000 read pairs from sediment samples using the metagenome sequencing libraries. FASTQC v0.11.4 software was used to perform sequence quality control, with a high number of sequences passing with 100 % accuracy (Figure 2). And metagenomic data was deposit under EBI Metagenomic database (www.ebi.ac.uk) with an accession number ERS11432032 and ERS11432033 for brine and sediment sample respectively.

3.2.2. Taxonomic analysis and phylogenetic diversity of brine and sediment samples

Metagenomic analysis was carried out to analyze the diversity of the microbial community in the studied samples, and the results identified species from the three Domains and the comparative analysis of both sample datasets at the Domain level. 68.98% of the reads were from Domain Archaea in brine samples compared with the sediment samples (accounting for 0.518% of the overall). In comparison, Domain bacteria accounted for 99.38% of the reads in sediment whilst in brine samples were 30.05%. Eukaryotes abundance was lower in both samples. Finally, viruses at 0.689% were detected only in the brine samples (Figure 3A). At the phylum level, Euryarchaeota was the most abundant in brine (75.9%) compared with the sediment samples (0.53%), followed by Bacteroides at 13.51% and 0.131%, and proteobacteria at 6.13% and 0.37% in brine and sediment, respectively. On the other hand, the phylum Firmicutes represents 98.57% of the overall in sediment, while 0.52% in brine. Remarkably, the brine sample contained a higher abundance than the sediment at the phylum level (Figure 3B). 16 phyla were recorded in brine samples only as the following: candidatus Bipolaricaulota 0.09%, Candidatus Marinimicrobia 0.067%, Candidatus Thermoplasmatota 0.088%, Candidatus Aenigmarchaeota 0.077%, Candidatus Nanohaloarchaeota 0.008%, and Candidatus Bathyarchaeota 0.076% (Figure 3C). At family level, *Halobacteriaceae* represents 10.17% and 0.11% in brine and sediment, respectively. A high abundance of *Haloferacaceae* at 26.68% followed by *Nanohaloarchaea* at 7.02%, *Halorubraceae* at 6.9%, and *Natrialbaceae* at 2.1% was detected with the brine samples only beside a minor abundance of other families (Figure 3D). At the level of genus, *Bacillus* dominated the sediment samples (61.08%) followed by *Mesobacillus*, but both genera were not detected in the brine samples. On the other hand, *Haloquadratum* accounted for 18.38% in brine, and it was not detected in sediment samples (Figure 3E). At the species level, relevant differences were recorded when comparing archaea and bacteria in sediment and brine samples (Bacterial species identified in sediment sample were *mesobacillus persicus* 25.1%, *metabacillus litoralis* 10.04%, *cytobacillus oceanisediminis* 9.7%, *mesobacillus selenatarsenatis* 4.8%, whilst archaeal species in brine samples were: *Haloquadratum walsbyi* 25.7% *salinibacter ruber* 7.08% *Halorubellus sp.*JP.LI 3.1%.

Finally, Methanonatronarchaeia was recorded at a minor abundance of 0.27% in the brine samples only. The summary of the phylogenetic diversity of brine and sediment samples was illustrated using a snake make chart (Figure 3F) and radial tree chart (Figure 3G).

3.2.3. Functional analysis

The metagenome-predicted functions classified using EggNog, EC, and SEED databases in MEGAN software. InterPro matches to our studied samples yielded the functional protein of the microbial community, the highest protein value was Transcriptional regulatory protein WalR-like 1.5% in the sediment sample while .9% in brine sample. On the other hand Universal stress protein, A family was detected only in brine sample at 1.3% (Table 2S1). Eggnog functional inference categorized the genus into information Storage and Processing 1209.2083 and 3164, including (translation, ribosome structure and biogenesis beside transcriptions, replication, recombination and repair) followed by cellular Processes, Signaling 1428.9884 and 3793 including (defense mechanism, intracellular trafficking, secretion, vesicular transport, signal transduction mechanism, post-translational modification, protein turnover, chaperons, cell motility, cell wall, cell membrane, envelope biogenesis, cell cycle control, cell division and chromosome partitioning) while metabolism 2470.2212 and 8358 in brine and sediment, respectively including(lipid transport and metabolism, inorganic acid transport and metabolism, carbohydrate transport, nucleic acid transport, amino acid transport, secondary metabolites biosynthesis, transport and catabolism, energy production and conversation (Figure 4A). The comparative analysis of SEED functional genes in both samples showed that the Cell Envelope was 1.152111% and 0.703584%, cellular processes 3.883574% and 12.573462%, DNA processing 9.869749% and 8.169853%, Energy 14.761424% and 13.450873%, Experimental Subsystems 0.508849% and 0.008277%, membrane transport 5.952573% and 5.827332%, Metabolism 37.779652% and 36.387714%, protein processing 15.567898 % and 11.0173%, regulation and cell signaling 0.2688265% and 0.877411%, RNA processing 5.765355% and 4.097343%, and stress response, defense, virulence 3.864372% and 6.32398% in brine and sediment, respectively (Figure 4B). Furthermore, in the comparative analysis of EC datasets for functional process in the brine and sediment samples, the results showed similarity between the two samples for functional process and were categorized as follows: Oxidoreductases 9.2%, Transferases 14.9%, Hydrolases 11%, Translocases 2.2%, and Isomerases 2.7%, but differentiation

appeared in Lyases at 4.1% and 3.7% and Ligases at 6.4% and 4.8% for brine and sediment, respectively (Figure 4C).

3.2.4. The correlation of physicochemical parameters and microbial community

To understand the effect of environmental parameters on microbial location and abundance of microbial communities, analysis on the correlations between parameters was explored. While a strong negative connection was found between EC and pH and salinity, Cl^- , Mg^{2+} , and Ca^{2+} , strong positive correlations were found between salinity, Ca^{2+} , and Cl^- (Figure 5A.) Various bacterial and Archaeal species were shown to have both positive and negative correlations, as demonstrated in (Figure 5B).

4. Discussion

Sinai region is a location in which three continents interact. The extreme climatic changes that this region has experienced over time make it a biologically significant environment. Despite the importance of halophilic microorganisms to the salt works' biological system, studies on Egyptian hypersaline ecosystems, particularly the North Sinai solar saltern, are scarce. This work is the first research of a natural solar saltern closed to the EL-Bardawil lagoon. The recorded physicochemical parameters showed Ph values ranging from 6 to 7.5 (brine and sediment) which are slightly lower than those from other hypersaline ecosystems Elevi et al. (2004). Regarding salinity, brine salinity was higher than in sediment, like the results of other studies madkour et al. (2012); Rasooli et al. (2016), Elevi et al.(2004). In comparison with Hazelton et al. (2016) and Kargas et al. (2018), the relation between physico-chemical parameters showed Cl^- was the dominant anion in this investigation. Because the concentration of NaCl defines the diversity and structure of microbiomes in these environments, it has been found that a North Sinai solar saltern with an intermediate salinity contains a greater diversity of microorganisms than a saltern with

a higher salt concentration Fernandez et al. (2013) and Wang et al. (2011). This finding suggested that this habitat's physical, chemical, and biological characteristics are unlike any other El-bana et al. (2002). So, the salinity- induced pH change is one of the most important ecological factors affecting microbial populations, especially in coastal areas (SenGupta, 1999). Based on a comparison with a prior study by Maturrano et al. (2006), we found that the average community composition of archaea and bacteria was more heavily represented in the brine and sediment samples, respectively. Another study by Ma et al. (2010) found that the diversity of the halophilic world shrank with increasing salt concentration, and they found that salinities above 30‰ were dominated by archaea OTUS with a low percentage of bacteria OTUS. In the Archaeal family *Halobacteriaceae*, life in hypersaline environments (Ventosa, 2004) and most bacteria have been recorded in numerous hypersaline habitats Vavourakis et al. (2016) and Jacob et al. (2017). The phylum level of the structure in our investigation is equal, but there are significant variances at the genus level that are linked to regional ecological variables Fernandez et al. (2013). And according to our research, the predominant prokaryotic groups were related to Eukaryote, Nanoarcheota, and Firmicutes. This differs from the findings of Cyclic et al. (2020), who found that Phyla Euryarchaeota, Bacteroidetes, Proteobacteria, and Firmicutes members were found in the distribution of archaeal and bacterial communities in the Karak salt mines. On the other hand, most of the bacteria we observed belonged to the genus *Bacillus*, which is aerobic and typically found in habitats of intermediate salinity and high altitude mamani et al. (2011) and menas et al. (2019). Our findings contrast with those of a previous study on solar saltern, which found bacteria from the phylum Proteobacteria and a low percentage of firmicutes Ali et al. (2021) and Xiao-Ran et al. (2018). We also found that *Haloquadratum*, one of the most abundant archaeal genera, had a similar effect (Welsh, 2000; Oren, 2012) and Dillon et al. (2013),

playing a crucial part in the formation of polyhydroxyalkonates and the Halocin protein that shields tissues from dehydration and chemical stress. As Zenke et al. (2015) reported, Like Jacob et al. (2017), we found a small percentage of eukaryotes in the present investigation. The previous study on solar saltern by culture method, was detected a few species from Archaea and bacteria in comparison with the results from insight metagenomic analysis Elshafey et al. (2022). From metagenomic functional analysis the creation of antimicrobial proteins to stop the expansion of other populations, thickening of the cell wall, an increase in pigmentation, the production of suitable solutes, solute transport systems, it was vital to investigate the functionality of microbes in habitats with intermediate salinities in order to comprehend how these processes might be used by microbes in these circumstances. Additionally, compared to previously examined saltern datasets, more contigs from the brine and sediment databases had annotations for SEED subsystems Overbeek et al. (2005). Most of the genes involved in membrane transport, cellular processing, and DNA processing were overrepresented in soil databases due to the frequently low nutrient concentrations in soil habitats Torsvik et al. (2008). Previously discovered that there was a rise in the gene classed as pathogenicity, virulence, and defense in sediment databases compared to brine. According to Abrudan et al. (2015), it has been hypothesized that nutritional limitation encourages the production of antibiotics and, subsequently their resistance mechanisms. Since haloarchaea do not contain any known pathogens, databases that contain more bacteria are likely to contain more virulent genes. Many of the genes were associated with central carbohydrate metabolism. According to the percentage of SEED subsystems annotated in contigs from the brine and sediment samples of the solar saltern, the overabundance of these amino acids in the brine samples included RNA processing and modification, protein biosynthesis, folate, pterins, isoprenoids, lysine, threonine, methionine, and cysteine. The findings refuted

the hypothesis that the stress response genes SEED category would be one of the most common in hypersaline environments. For example, Vera-Gargallo Ventosa (2018) and Hagagy et al. (2021) suggest that not all the genes implicated in the basic mechanisms of osmoadaptation (such as trehalose production and ion transport) are present in this SEED subsystem, which may contribute to this predicament. Furthermore, it is important to note that halophiles can create bioactive compounds that have a wide range of impressive biological properties, such as strong antibacterial, antifungal, and antiviral effects. Furthermore, it has been observed that they include antioxidant, anticancer, and immunomodulatory characteristics Naughton et al. (2019). In contrast, halophiles have attracted considerable attention owing to their potential as a valuable source of bioactive chemicals that exhibit promise for numerous applications in the field of biotechnology. Commercial fructans, which are frequently derived from chicory root (known as inulin-type fructans) and agave plants, are widely acknowledged as functional substances that have diverse applications in the pharmaceutical, food, and cosmetic sectors according to Bhanja et al. (2022). The present investigation showed the presence of Haloarchaea, specifically *Haloarcula* sp., which is like the findings of Aragón-León et al. (2023). These haloarchaea have demonstrated the capability to manufacture fructans, which are polysaccharides based on fructose and play important roles in the fields of biotechnology and cosmetics production.

5. Conclusion

The objective of this study was to investigate and assess the richness and functional diversity of halophilic microorganisms in a significant geographic area in Egypt known for its high salinity levels. To the best of our current understanding, this study represents the initial investigation utilizing metagenomic and bioinformatical analysis to examine an ecosystem situated in the North Sinai, Egypt. In the present study, we investigated the relationship between microbial communities, salinity, and pH in brine and sediments. The

analysis revealed that the main prokaryotic groups exhibited associations with Eukaryotes, Nanoarcheota, and Firmicutes. Certain genera discovered in this study exhibit significant involvement in global biogeochemical cycles and hold considerable significance in the fields of industry and biotechnology. This understanding has the potential to be applied in the development of more sustainable management strategies for solar systems. Furthermore, the findings of this study may result in the creation of novel industrial products, improved solar saltern management techniques, and new bioremediation methods for contaminated environments. The presented discoveries in the metagenomic study of solar salterns have potential applications in various domains, including the manufacturing of bioplastics, the creation of novel antibiotics, and the remediation of environmental pollutants. Metagenomic investigation of solar salterns is making a significant contribution to our complete understanding of microbial diversity and the environmental functions of microorganisms. This knowledge is necessary for creating sustainable solutions to a variety of environmental problems.

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Tables

Table1. Physico-Chemical characterization for samples

Samples	Cl (mg/l)	pH	EC	Salinity (%)	Ca mg/L	Mg mg/L
Brine	174112.5 ± 10 a	6.06 ± 0.1 b	13840 ± 7 a	29 ± 1 a	23.45 ± 2.5 a	46.73 ± 1.9 a
Sediment	0.0343 ± 0.001 b	7.48 ± 0.3 a	520 ± 3 b	12 ± 0.7 b	14.0294 ± 2 b	2.1542 ± 0.12 b
p value	0.00	0.001	0.00	0.00	0.007	0.00

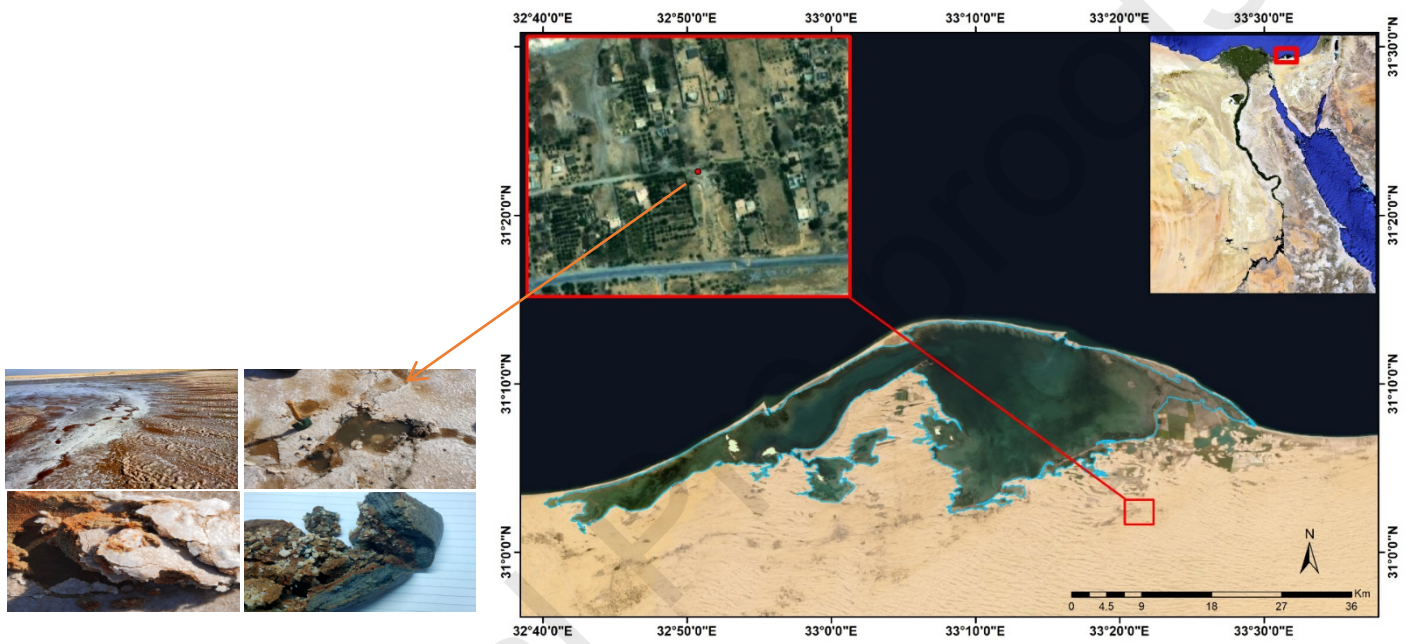


Figure 1. Location map of El-Rawda solar saltern, North Sinai, Egypt

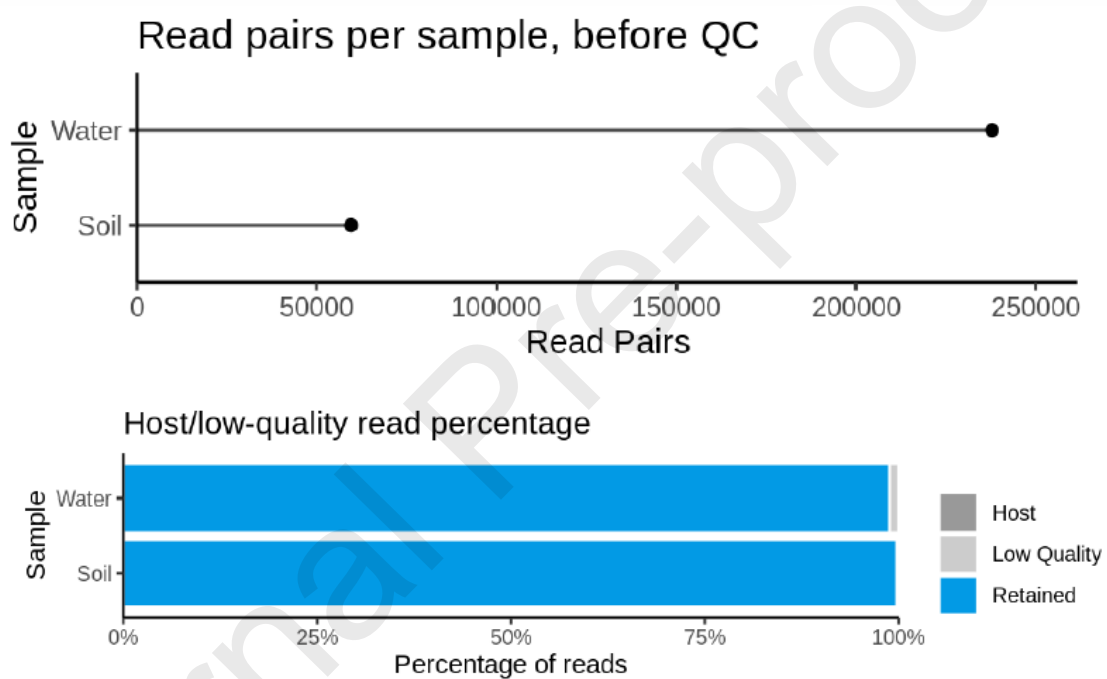
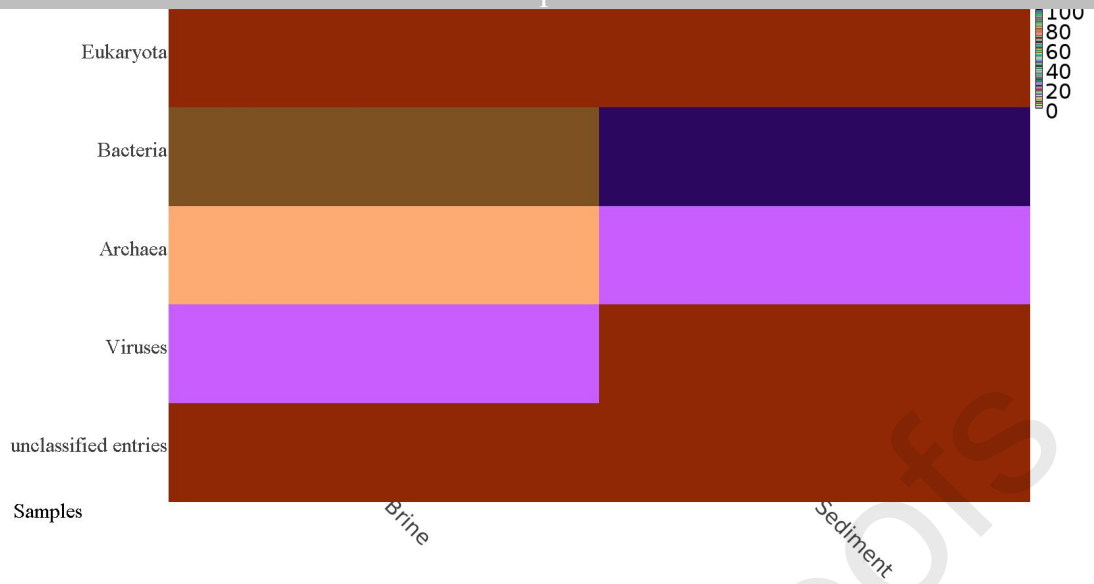
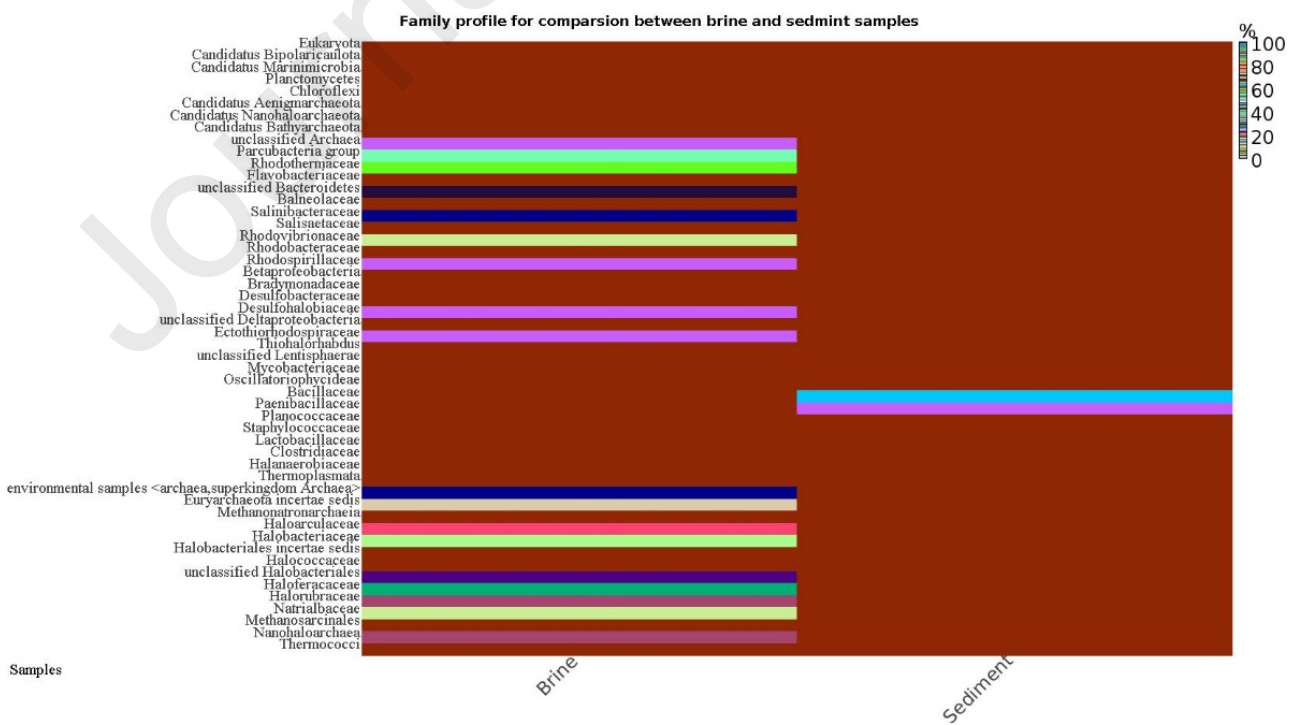


Figure 2. Quality control of read pairs per samples sediment and brine



A

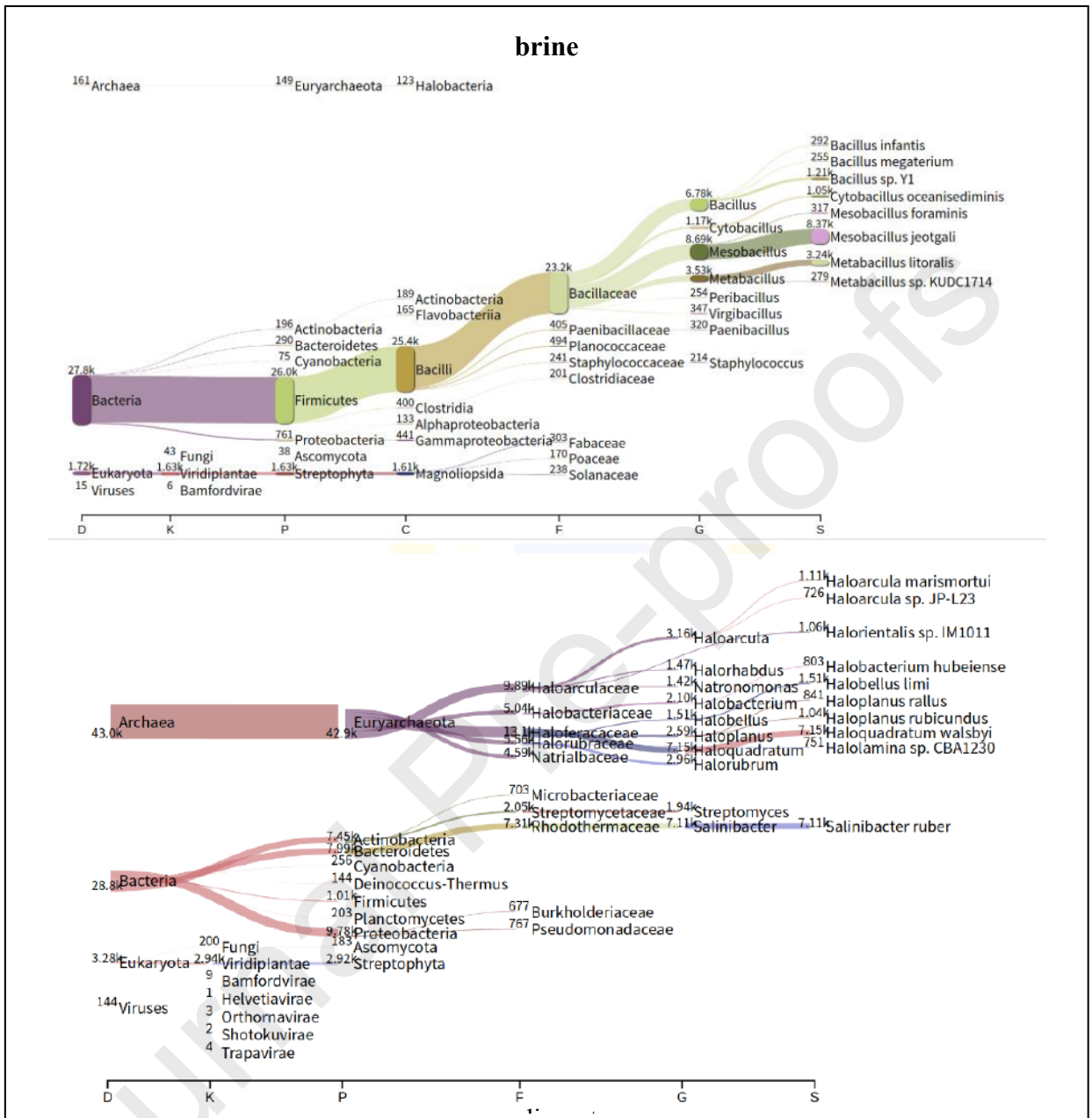
B



Species profile for comparison between brine and sediment samples



F



G

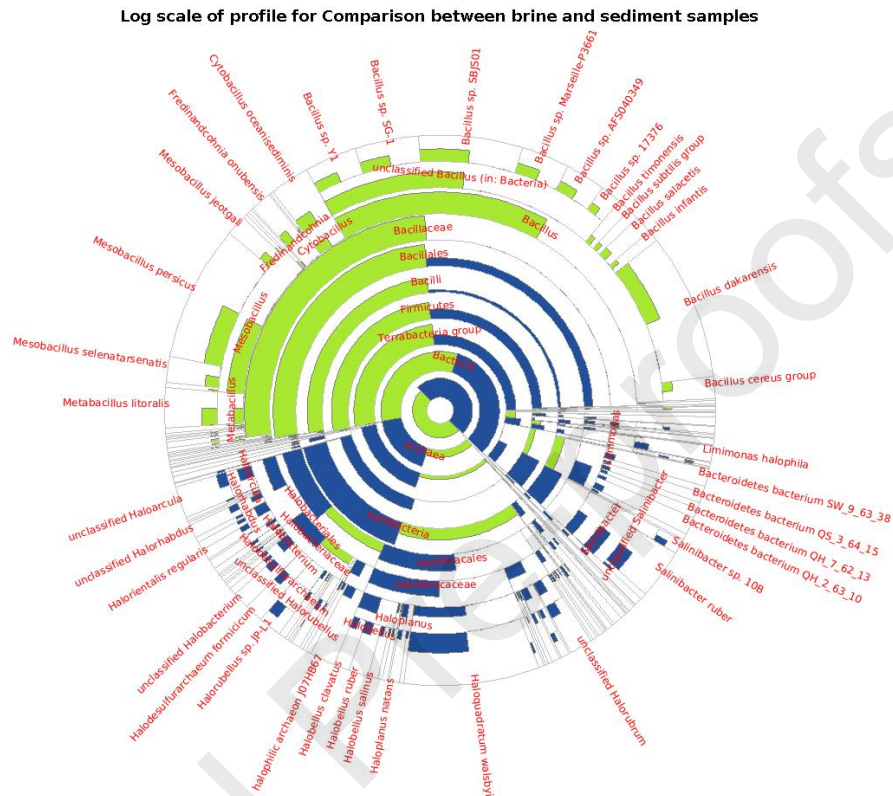
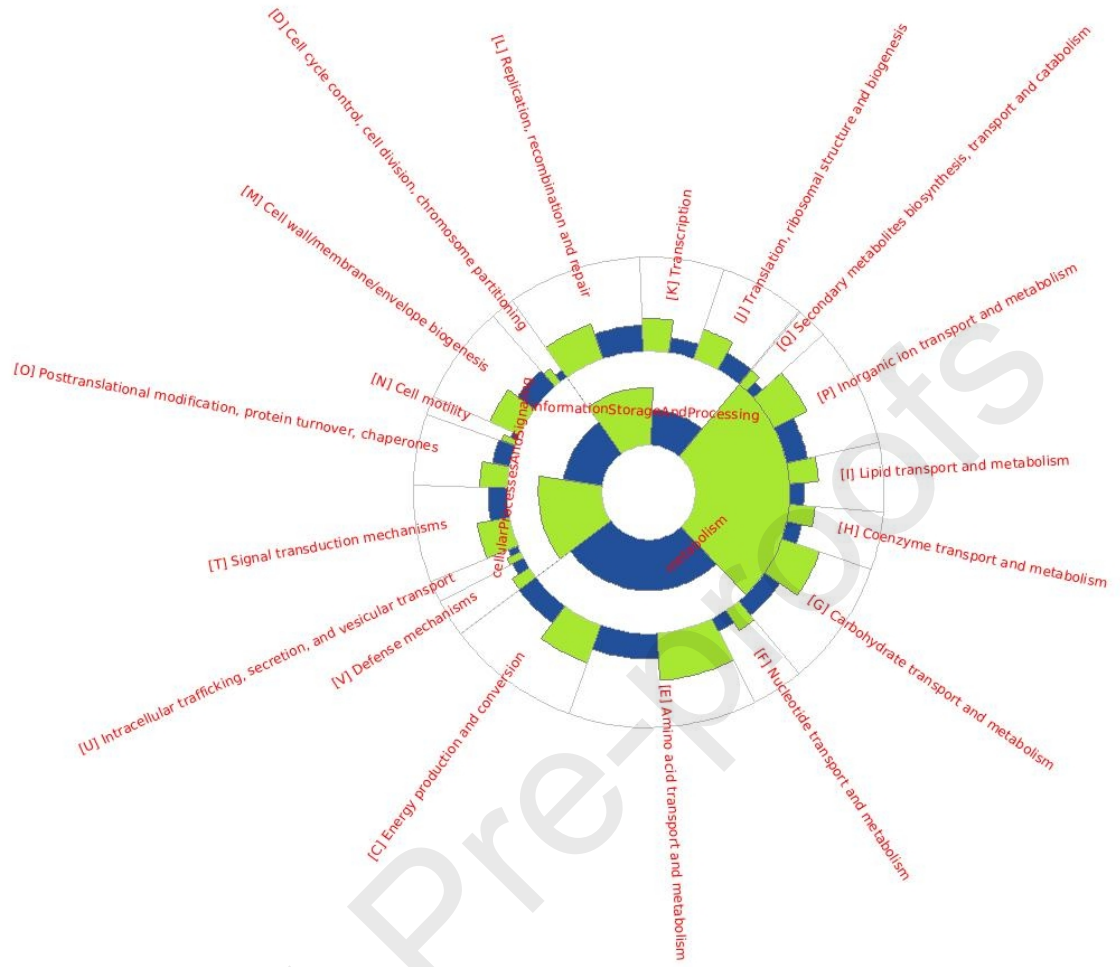


Figure 3. Comparison Taxonomic profile between brine and sediment samples (A). domain profile comparison ;(B). family profile comparison;(C). phylum profile comparison;(D). Genus profile comparison;(E). Species profile comparison; (F).Snake make chart On the x-axis are the taxonomic levels: D, domain; P, phylum; C, class; O, order; F, family; G, genus; S, species. Numbers correspond to the assigned;(G). Radical tree for comparison.

EGGNOG profile for Comparison between brine and sediment samples

A



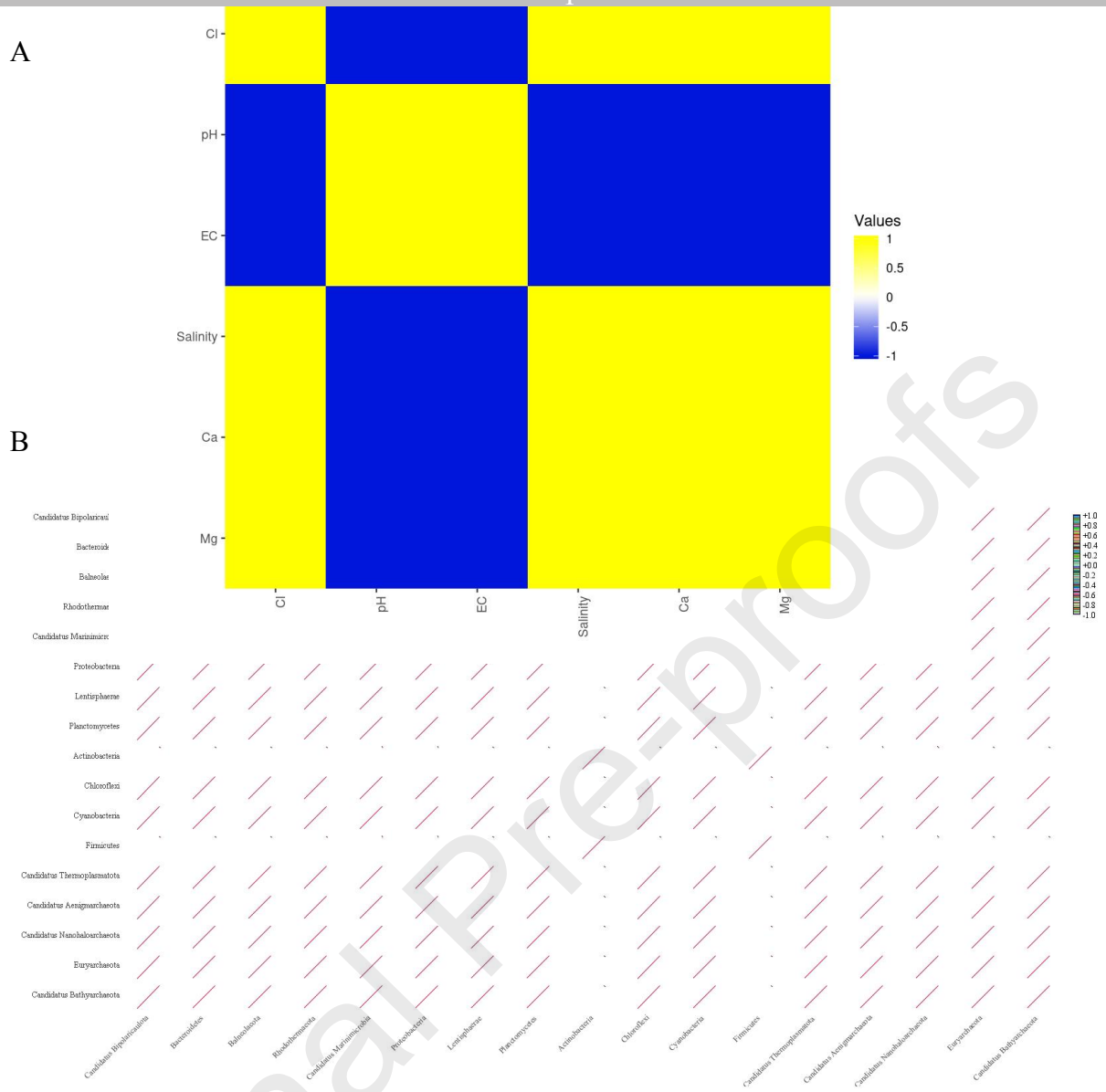


Figure. 5 the correlation comparison between brine and sediment (A); physico-chemical parameter; (B) .Microbial community

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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