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Metagenomic insights into the prokaryotic communities of heavy metal-contaminated hypersaline soils

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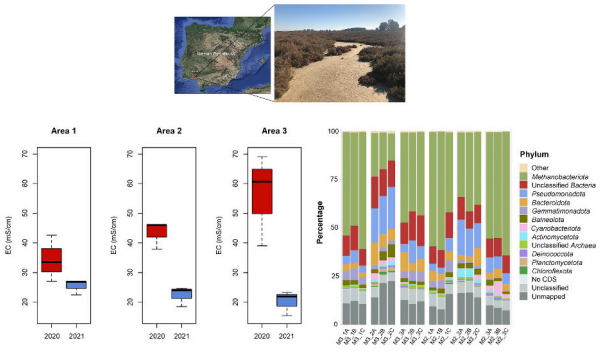
HIGHLIGHTS

- High salts and heavy metals concentrations define these soils as extreme environments.
- *Archaea* and *Bacteria* are equally distributed, but *Bacteria* members are more diverse.
- Copper and zinc transporters are main functions within the prokaryotic community.
- *Methanobacteriota* (haloarchaea) is relevant in the metabolism of arsenic and osmolytes.

GRAPHICAL ABSTRACT



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ABSTRACT

Saline soils and their microbial communities have recently been studied in response to ongoing desertification of agricultural soils caused by anthropogenic impacts and climate change. Here we describe the prokaryotic microbiota of hypersaline soils in the Odiel Saltmarshes Natural Area of Southwest Spain. This region has been strongly affected by mining and industrial activity and feature high levels of certain heavy metals. We sequenced 18 shotgun metagenomes through Illumina NovaSeq from samples obtained from three different areas in 2020 and 2021. Taxogenomic analyses demonstrate that these soils harbored equal proportions of archaea and bacteria, with *Methanobacteriota*, *Pseudomonadota*, *Bacteroidota*, *Gemmatimonadota*, and *Balneolota* as most abundant phyla. Functions related to the transport of heavy metal outside the cytoplasm are among the most relevant features of the community (*i.e.*, ZntA and CopA enzymes). They seem to be indispensable to avoid the increase of zinc and copper concentration inside the cell. Besides, the archaeal phylum *Methanobacteriota* is the main arsenic detoxifier within the microbiota although arsenic related genes are widely distributed in the community. Regarding the osmoregulation strategies, "salt-out" mechanism was identified in part of the bacterial population, whereas "salt-in" mechanism was present in both domains, *Bacteria* and *Archaea*. *De novo* biosynthesis of two of the most universal compatible solutes was detected, with predominance of glycine betaine biosynthesis (*betAB*

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genes) over ectoine (*ectABC* genes). Furthermore, *doeABCD* gene cluster related to the use of ectoine as carbon and energy source was solely identified in *Pseudomonadota* and *Methanobacteriota*.

1. Introduction

Aquatic hypersaline environments have traditionally been studied more extensively than their terrestrial equivalents. A large number of studies have been carried out on aquatic hypersaline habitats, including saline lakes and salterns, such as the Santa Pola and the Isla Cristina salterns (Oren, 2008; Ventosa et al., 2014, 2015). However, hypersaline soil systems are currently attracting attention, due both to their more variable and largely unexplored biodiversity, as well as the trend of increasing salinity and desertification of agricultural soils (Li et al., 2021; Das and Dhal, 2022; Ramos-Tapia et al., 2022).

Soils of the Odiel Saltmarshes Natural Area present high salinity and heavy metal concentrations due to nonregulated industry and mining activity in the past (Luque et al., 1998; Morillo et al., 2002; Pérez-López et al., 2023). They constitute an interesting environmental model for the study of the microbial diversity harboring adaptation mechanisms. These two extreme conditions are commonly caused by human activity, such as saline soils from Egypt (Rady et al., 2023), salterns from India (Pereira et al., 2013), and saline-alkaline paddy soils in China (Liu et al., 2023), although it is also present in nature, such as Mono and Searles Lakes in the western USA (Kulp et al., 2006). Preliminary metagenomic studies of the prokaryotic community of hypersaline soils from the Odiel Saltmarshes Natural Area have been reported, although limited to a single metagenome sequenced by pyrosequencing technology at rather low coverage (Vera-Gargallo and Ventosa, 2018; Vera-Gargallo et al., 2019). These investigations revealed that these soils harbor a more diverse prokaryotic community than in aquatic saline environments and identified *Methanobacteriota* (previously, "*Euryarchaeota*"), *Pseudomonadota* (previously, "*Proteobacteria*"), *Balneolota* (previously, "*Balneolaeota*"), *Bacteroidota* (previously, "*Bacteroidetes*"), and *Rhodothermota* (previously, "*Rhodothermaeota*") as major community members.

The purpose of the present work is to provide an in-depth analysis of the prokaryotic diversity inhabiting soils with extreme salt and heavy metal concentrations, particularly those located at the Odiel Saltmarshes Natural Area (Huelva, Southwest Spain). Furthermore, we aim to dig into their adaptative mechanisms, focusing on: (a) heavy metal transporters and metabolism of main contaminants (arsenic, cadmium, copper, and zinc); (b) identification of "salt-in" and "salt-out" strategies in *Archaea* and *Bacteria* domains and the transport and/or *de novo* biosynthesis of universal compatible solutes (glycine betaine and ectoine).

2. Materials and methods

2.1. Sampling sites and physico-chemical features

The Odiel Saltmarshes Natural Area, declared a UNESCO biosphere reserve in 1983, is located in Huelva, Andalucía, Southwest of Spain, adjacent to the Odiel and Tinto rivers. The area has suffered from mining activity for years and acid mining drainages have contaminated both rivers and the surrounding fields, with low pH and high concentrations of heavy metals, such as aluminum, arsenic, cadmium, chromium, cobalt, copper, iron, lead, and nickel in its water and estuary. This state has been aggravated by nonregulated emissions from chemical and mining industries in the past (Luque et al., 1998; Morillo et al., 2002; Pérez-López et al., 2023).

Three sampling sites (1, 2 and 3) were selected in order to obtain a representative subset of the prokaryotic community of the soils from the Odiel Saltmarshes Natural Area (Fig. S1; Table 1). Lack of vegetation, visible salt crust, and distance between samples were the main criteria for their selection. Area 1 had been previously studied following a metagenomic approach (Vera-Gargallo and Ventosa, 2018; Vera-Gargallo et al., 2019). Three different subareas separated by 2–7 m, designated as A, B, and C, were selected within each sampling site. These subareas were handled as biological replicates to assess reproducibility of the results within the same sampling. The samples were collected in July 2020 (called M2) and June 2021 (named M3) in order to observe potential temporal variations. The surface layer was discarded, and the subsurface stratum (2–4 cm) was mixed with a sterile spatula. Approximately 300 g of homogenized soil were collected and kept in sterile Whirl-Pak bags during transport to the laboratory, where they were subsequently stored at 4 °C until DNA extraction.

The temperature of soil and air was measured in the field with a Checktemp 2 thermometer (Electron Microscopy Sciences). After diluting the soil 1:5 with Q-grade water, pH and electrical conductivity (EC) were measured with a pHmeter CRISON BASIC 20 and a conductometer CRISON 35+, corrected for temperature, respectively. Physico-chemical properties of the 18 soil samples were determined by Innoagral Laboratories (Brenes, Sevilla, Spain): aluminum, calcium, phosphorus, potassium, sodium, sulfur, chloride, copper, iron, manganese, and zinc concentrations were measured by atomic absorption spectroscopy; arsenic, cadmium, and nickel, by Inductively Coupled Plasma–Mass Spectrometry (ICP-MS); cobalt and lead concentrations, by Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES); nitrate, nitrite, and sulfate concentrations, by ultraviolet-visible spectroscopy; carbon concentration by infrared combustion system; nitrogen concentration by Kjeldahl method; and clay, sand, silt, and texture, by Bouyoucos method. The multivariate analysis of the parameters above mentioned was carried out using 'vegan' v. 2.5-7 package for R (Oksanen et al., 2020).

2.2. Total prokaryotic DNA extraction and sequencing

Total environmental DNA was extracted from the 18 samples collected during this study (Table 1) using the commercial FastDNA™ SPIN Kit for Soil (MP Biomedicals) and the high-speed benchtop homogenizer FastPrep®-24™ (MP Biomedicals), following the manufacturer's recommendations. DNA concentration and quality were measured with Qubit™ 4 dsDNA HS assay kit (Thermo Fisher Scientific) and NanoDrop™ One (Thermo Fisher Scientific), respectively. Sequence libraries were constructed from the prokaryotic DNA using the

Table 1
Information about the two samplings carried out in the soils from Odiel Saltmarshes Natural Area (Southwest Spain).

Sampling	Date	Area	Samples	Coordinates
M2	July 2020	1	M2_1A	37°12′26.6″N 6°57′52.5″W
			M2_1B	
			M2_1C	
		2	M2_2A	37°12′28.4″N 6°57′27.9″W
			M2_2B	
			M2_2C	
		3	M2_3A	37°13′18.0″N 6°57′44.8″W
			M2_3B	
			M2_3C	
M3	June 2021	1	M3_1A	37°12′26.6″N 6°57′52.5″W
			M3_1B	
			M3_1C	
		2	M3_2A	37°12′28.4″N 6°57′27.9″W
			M3_2B	
			M3_2C	
		3	M3_3A	37°13′18.0″N 6°57′44.8″W
			M3_3B	
			M3_3C	

Novogene NGS DNA library prep set (catalog no. PT004) and the genomic DNA was sequenced on the Illumina NovaSeq 6000 platform following a 2 × 150-bp paired-end strategy, by Novogene Europe (Cambridge, United Kingdom).

Raw sequences were filtered prior to assembly with 'PRINSEQ' v. 0.20.3 (Schmieder and Edwards, 2011). 'N' strings at the terminal positions were trimmed. Reads with entropy <70 and length <60 bp were removed, as recommended, and singletons were discarded. Data were processed following 'SqueezeMeta' v. 1.6.0 pipeline (Tamames and Puente-Sánchez, 2019) with 'sequential' option, meaning that each dataset was analyzed individually. 'MEGAHIT' v. 1.2.9 (Li et al., 2015, 2016) was used to assemble reads into contigs. 'Barrnap' v. 0.9 (Seemann, 2017) performed annotation of rRNAs, which were masked with 'N' in the contigs to avoid their prediction as proteins. 'Prodigal' v. 2.6.3 (Hyatt et al., 2010) predicted the open reading frames (ORFs), which were searched against the GenBank nr (Clark et al., 2016) and the KEGG (lastest version publicly available) (Kanehisa and Goto, 2000) databases by 'DIAMOND' v. 2.0.14.152 (Buchfink et al., 2021) for taxonomy and functional annotation, respectively. 'Bowtie2' v. 4.8.2 (Langmead and Salzberg, 2012) was used to calculate coverage. All these tools are implemented in the automated pipeline 'SqueezeMeta'. The G+C content of assembled contigs from the 18 metagenomic datasets was determined using the 'geecee' tool from 'EMBOSS' package v. 6.5.7.0 (Rice et al., 2000). The isoelectric point profile of the annotated protein sequences was calculated by 'iep' tool also from 'EMBOSS' package v. 6.5.7.0 (Rice et al., 2000).

Initial analysis of the output tables generated by 'SqueezeMeta' was performed in R environment with the package 'SQMtools' v. 0.8.0 (Puente-Sánchez et al., 2020). Gene abundances were normalized to Features Per Million (FPM), a metric conceptually similar to Transcripts Per Million (TPM) that represent the times a given feature would be found after sampling one million features from a population, and which controls for both feature length and library size (Puente-Sánchez et al., 2020). The following R packages were used for plot generation: 'ggplot2' v. 3.3.3 (Wickham, 2009), 'ggtext' v. 0.1.2, and 'paletteer' v. 1.4.0 (Hvitfeldt, 2021). The R packages 'reshape2' v. 1.4.4 (Wickham, 2007) 'scales' v. 1.1.1 (Wickham and Seidel, 2020) allowed the data edition and 'vegan' v. 2.5-7 (Oksanen et al., 2020) was employed to calculate the rarefaction curve and the Shannon diversity index.

3. Results and discussion

3.1. Extreme concentration of salt and multiple heavy metal contaminants

Saline soils are not well defined in the literature. Richards (1954) proposed that soils with electrical conductivity (EC) >4 mS cm^{-1} at 25 °C should be considered saline. The World Reference Base for Soil Resources sets the threshold in EC 15 mS cm^{-1}, or 8 mS cm^{-1} when pH exceeds 8.5. The United States Department of Agriculture (USDA) has more recently raised this threshold to 30 mS cm^{-1} (Abrol et al., 1988; IUSS Working Group WRB, 2007). The EC of the 18 collected samples exceeded at least two of the aforementioned thresholds, with values ranging from 15.4 to 69.2 mS cm^{-1} (Fig. 1a; Table S1). The soils from 2020 showed a higher median (42.6 mS cm^{-1}) than those from 2021, with 27.1 mS cm^{-1} as minimum value and 69.2 mS cm^{-1} as maximum value for the former. Soils from 2021 had an EC median of 23.3 mS cm^{-1}, with a minimum value of 15.4 mS cm^{-1} and a maximum of 27.0 mS cm^{-1} (Fig. 1a; Table S1). These variations can be explained by hot temperatures the days prior to collection in 2020. Nevertheless, salinity was consistent with a previous study of the microbial community of the Odiel Saltmarshes Natural Area (Vera-Gargallo et al., 2019). The pH in soils sampled in area 1 and area 2 were similar across both timepoints, but slightly higher in area 1 (pH 8.2–8.5) compared to area 2 (pH 7.4–8.4). Soils from area 3 were more acidic in 2020, with pH 6.0–6.4, compared to 2021, showing a range of pH 8.3–8.5.

To determine the contribution of the individual physico-chemical parameters to the variation in environmental state observed between the samples, we evaluated their normalized values using a principal component analysis. The two first principal components (PC1 and PC2) captured 32.7 % of the variation present in the 18 samples, which indicates that the correlations between the physico-chemical variables were not strong. The graphical representation of this analysis (Fig. 1b) showed that samples from area 1 were highly similar to each other. On the other hand, samples from area 2 and 3 were clearly separated by sampling date along PC2. This axis, that represents 25.7 % of relative variance, mostly captured the variation in electrical conductivity (EC), soil and air temperature, and pH.

The Odiel River has been affected by contamination from industrial and mining activities in the area. Previous studies have revealed a high concentration of arsenic, cadmium, copper, lead, and zinc in water and sediments of the river (Sainz et al., 2002, 2004). Although the contamination of the saline soils of the Odiel Saltmarshes Natural Area

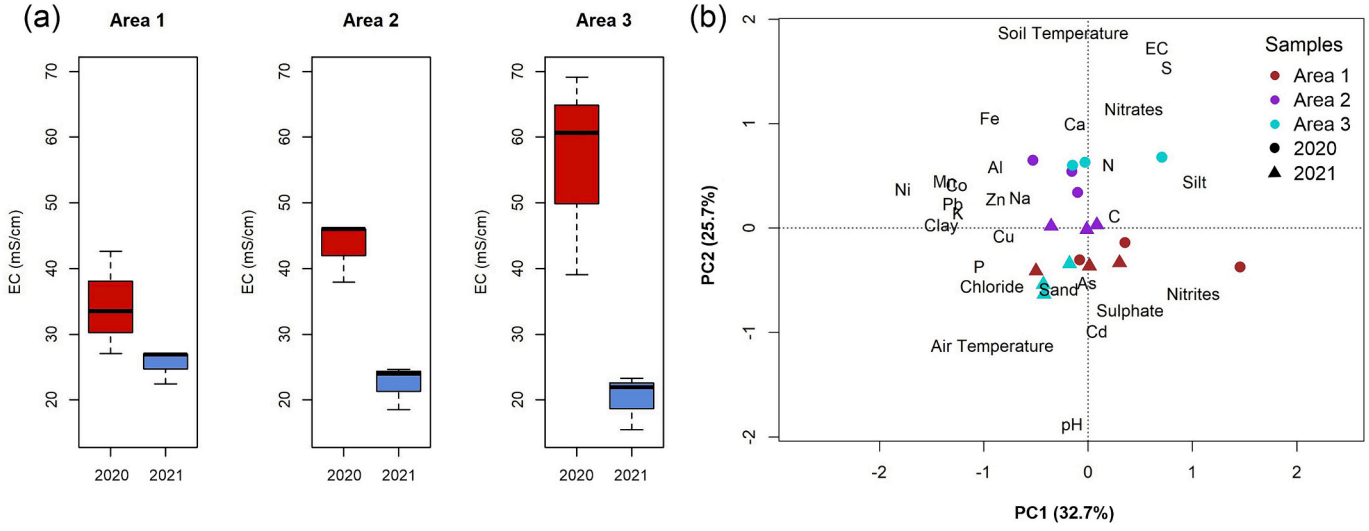

Fig. 1. (a) Boxplot of the measured electroconductivity (EC) of the soil samples after a 1:5 dilution, clustered by area (1, 2, and 3) and sampling date (M2, year 2020; M3, year 2021). (b) Principal component analysis (PCA) of all normalized physicochemical parameters measured for the 18 collected samples. Relative inertia of each component is indicated on the axes.

has not been studied in detail, the Government of the region of Andalucía considers the following reference criteria for non-contaminated soils (mg kg^{-1}): arsenic, 2–5; cadmium, 0.4–0.8; copper, 17–100; lead, 10–50; and zinc, 10–70 (Consejería de Medio Ambiente, 1999). According to these criteria, the 18 collected samples were contaminated with arsenic and zinc. None of the samples had copper concentrations above 100 mg kg^{-1}; however, 12 of them were very close to this limit, with values exceeding 90 mg kg^{-1}. Cadmium concentration was within the range for non-contaminated soils (Table S1). The variation in heavy metal concentrations between the samples did not allow to differentiate them (Fig. 1b).

Therefore, the extreme values obtained for salinity and heavy metal concentrations turn the soils of Odiel Saltmarshes Natural Area into an excellent model ecosystem for the study of the prokaryotic diversity on soils with poly-extreme conditions, with relevance on terrestrial habitats affected by desertification processes or intensive agriculture practices.

3.2. General features of the assembled metagenomic datasets

The metagenome sequencing yielded 87,797,025–53,595,850 paired-end sequences of 2 × 150 bp and a mean Phred quality score (Q) of 36. After trimming, minimum length of the reads was between 60 and 101 bp (Table S2). Filtered reads from the 18 metagenomic datasets were assembled into 2,662,817–1,197,119 contigs, with a total size of 1.10 Gb for the smaller metagenome and 2.07 Gb for the largest one. Contig mapping recruited between 92.66 % and 77.39 % of the total filtered reads. Further parameters related to the assembly and annotation of the 18 metagenomic datasets are detailed in Table S2.

The G + C content profile of the assembled contigs in the 18 metagenomic datasets shared a similar distribution, spotting a high peak at around 70 mol% and another lower peak at around 45 mol% (Fig. S2a). Halophilic microorganisms generally exhibit a G + C content above 60 mol%, which is thought to be a protection mechanism against thymidine dimerization by UV radiation, particularly relevant in hypersaline systems (Salwan and Sharma, 2022). This corresponds to the large peak at about 70 mol%, which comprises a high proportion of the contigs (Fig. S2a).

The isoelectric point profiles determined for the 18 metagenomic databases were very similar. They revealed a peak at pH 4 (Fig. S2b), which is usually related to the adaptation of halophilic microorganisms to high salt concentration. Similarly, the amino acid frequencies of the metagenomic datasets were analogous between the 18 metagenomic datasets. The major amino acids were apolar alanine (A) and leucine (L), while polar cysteine (C) and aromatic tryptophan (T) were the least abundant molecules constituting the proteins of the population under study (Fig. 3C).

3.3. Similar spatio-temporal prokaryotic diversity among the hypersaline soils of the Odiel Saltmarshes Natural Area

3.3.1. A dominant archaeal but multiple bacterial phyla as main inhabitants

The 18 metagenomic datasets from the hypersaline soils of the Odiel Saltmarshes Natural Area shared a similar taxonomic profile at the phylum level. More than 100 phyla were detected in each sample, where M2_2A and M3_3B harbored the highest number of phyla (183) and M2_3C the lowest number (137). Despite the high richness, the samples were dominated by the phyla *Methanobacteriota* (previously, "*Euryarchaeota*") within the domain *Archaea*, *Pseudomonadota*, *Bacteroidota*, *Gemmatimonadota*, and *Balneolota* (formerly, "*Proteobacteria*", "*Bacteroidetes*", "*Gemmatimonadetes*", and "*Balneolaeota*", respectively) within domain *Bacteria*. On the other hand, *Cyanobacteriota*, *Actinomycetota*, *Deinococcota*, *Planctomycetota*, and *Chloroflexota* (formerly, "*Cyanobacteria*", "*Actinobacteria*", "*Deinococcus-Thermus*", "*Planctomycetes*", and "*Chloroflexi*", respectively) had overall lower and highly variable contributions. Although archaeal diversity was mainly restricted to a single phylum, there were typically equal proportions of archaeal and bacterial reads, with the exception of samples M3_2A, M3_2B, and M3_2C, where *Methanobacteriota* relative abundance was lowered to around 25 % (Fig. 2a). Approximately, 10 % of the reads assigned to the domain *Bacteria* in each sample could not be confidently attributed to any known phylum. <2 and 0.4 % of the sequences were assigned to eukaryotes and viruses, respectively. Additionally, around 20 % of sequences from each sample could not be classified or were not assembled into contigs.

Even if the most prevalent phyla were the same for each of the 18 samples, the relative proportions differed slightly depending on the area and year. Samples M3_2A, M3_2B, and M3_2C (area 2, year 2021) had a decrease in the *Archaea* and a concomitant increase in the bacterial phylum *Pseudomonadota*. The opposite phenomenon occurred for samples M3_1C (area 1, year 2021), M2_1A, and M2_1B (area 1, year 2020), where the relative abundance of *Pseudomonadota* decreased and the relative abundance of *Methanobacteriota* increased (Fig. 2a). These results are generally similar to those found in the pioneering metagenomic shotgun studies of the microbial population of a hypersaline soil from Odiel Saltmarshes, conducted by pyrosequencing technology (Vera-Gargallo and Ventosa, 2018; Vera-Gargallo et al., 2019). In these previous studies, the ratio of *Bacteria* and *Archaea* was also balanced, but the proportion of *Balneolota* and *Bacteroidota* was higher, while *Gemmatimonadota* and *Pseudomonadota* displayed a lower community contribution. These discrepancies could be explained by the complexity of the microbial diversity in this environment and the limited sequencing depth with 454 pyrosequencing method.

At the phylum level, the results agree with other diversity studies performed in geographically distant hypersaline terrestrial systems. *Pseudomonadota*, *Bacteroidota,* and *Gemmatimonadota* are generally the most abundant phyla (de León-Lorenzana et al., 2017; Xie et al., 2017; Rath et al., 2019; Fariq et al., 2021; Li et al., 2021; Ramos-Tapia et al., 2022; Zeng et al., 2022), sometimes accompanied by *Balneolota*, *Actinomycetota*, *Acidobacteriota*, *Chloroflexota*, or *Bacillota* (de León-Lorenzana et al., 2017; Vavourakis et al., 2018; Vera-Gargallo and Ventosa, 2018; Rath et al., 2019; Fariq et al., 2021; Li et al., 2021; Ramos-Tapia et al., 2022; Zeng et al., 2022).

The abundance of *Methanobacteriota* is typically correlated to the electrical conductivity (EC) (Xie et al., 2017; Zeng et al., 2022) with highly saline soils harboring a larger proportion of *Archaea* than those with lower salinity (Rath et al., 2019; Li et al., 2021). Our results partially agree with this pattern, as samples M3_2A, M3_2B, and M3_2C presented the lowest proportion of haloarchaea and their EC values ranged from 22.5 to 18.5 mS cm^{-1}, which is below the 46.1–37.9 mS cm^{-1} range for the corresponding samples collected a year earlier (Table S1) where the proportion of *Archaea* was considerably higher. Nevertheless, samples from area 1 and area 3 collected in 2021 (M3_1A, M3_1B, and M3_1C; M3_3A, M3_3B, and M3_3C, respectively) also exhibited lower EC than their 2020 counterparts (M2_1A, M2_1B, and M2_1C; M2_3A, M2_3B, and M2_3C, respectively), but the relative abundance of archaea was higher in 2021 in comparison to 2020. Therefore, salinity cannot be regarded as the only factor that constrain and control archaeal phylum *Methanobacteriota* proliferation in the environment, as also evidenced by Pandit et al. (2015). On the other hand, our data agree with earlier reports that *Gammaproteobacteria* is the most predominant class of the phylum *Pseudomonadota* in saline habitats (Vavourakis et al., 2018; Fariq et al., 2021), suggesting that members of this phylum are better adapted to stressful osmotic conditions (de León-Lorenzana et al., 2017; Xie et al., 2017).

Considering the bimodal distribution of the global G + C content of the samples (Fig. S2a), we analyzed this parameter for the seven main phyla. Typically, low G + C content is much less common among halophiles, although some examples can be found, such as the archaeal species *Haloquadratum walsbyi*, with 47.9 mol% G + C content (Salwan and Sharma, 2022). However, *H. walsbyi* favors aquatic environments (Vera-Gargallo and Ventosa, 2018), such as the Santa Pola salterns (Fernández et al., 2014a, 2014b) and the hypersaline Lake Urmia (Kheiri

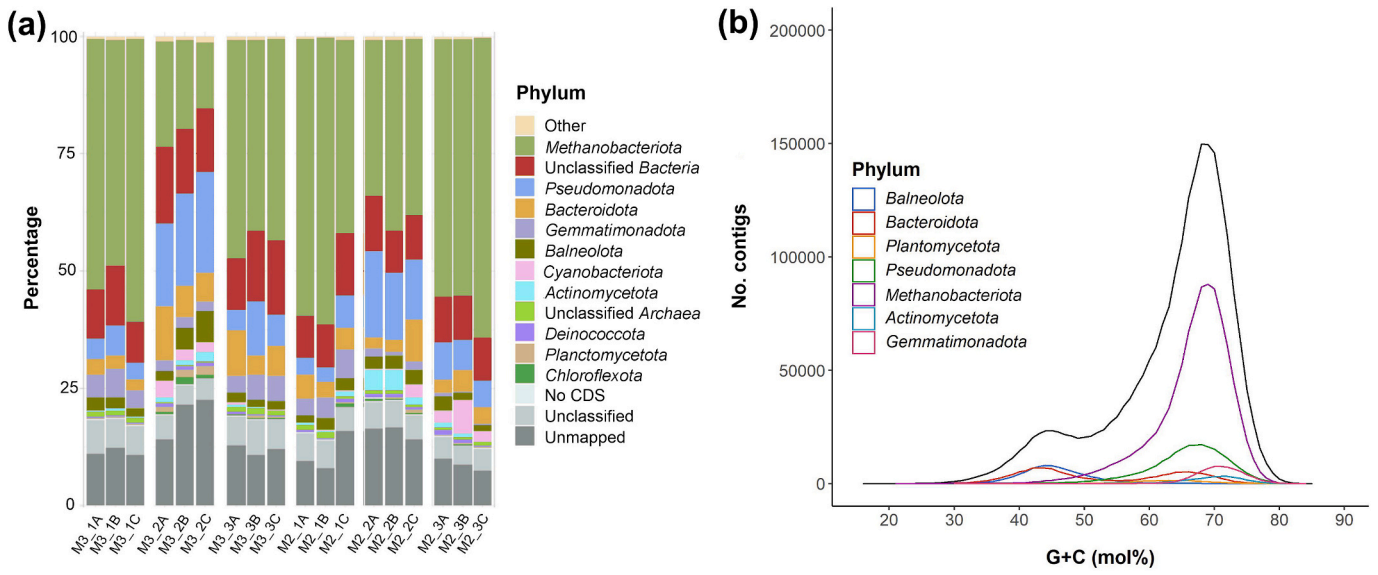

Fig. 2. (a) Relative abundance (%) of the phyla detected in the hypersaline soils of the Odiel Saltmarshes Natural Area based on contig taxonomic assignment. (b) G + C content distribution (%) of the global M2_1C sample (black) and of the seven most abundant phyla (color key displayed on the plot). Given that the 18 samples showed similar G + C profiles (Fig. 3A), sample M2_1C was selected as a representative due to its balanced proportion of the main phyla contributing to the G + C content peaks.

et al., 2023), among others. Otherwise, members of the phylum *Balneolota*, which preferably grow in soil habitats, have been determined as a relevant group in the biota of soils from Odiel Saltmarshes Natural Area (Vera-Gargallo and Ventosa, 2018; Galisteo et al., 2023b) and also exhibit a G + C content below 50 mol% (Munoz et al., 2016) or even below 40 mol% in the case of *Balneola*, the type genus of the family *Balneolaceae* (Urios et al., 2006, 2008). Hence, it is highly likely that species of the phylum *Balneolota* are partly responsible for the G + C content peak at 45 mol% (Fig. S2a). The plot revealed that the lower peak at 40–50 mol% was mainly constituted by representatives of the phyla *Balneolota* and *Bacteroidota*, while the latter phylum was also represented in the second peak along with *Methanobacteriota*, *Pseudomonadota*, and other minor phyla (Fig. 2b). However, the sequences assigned to the phylum *Balneolota* consistently featured low G + C content, similar to cultured members of this taxonomic group (40.8 to 49.0 mol%) (Galisteo et al., 2023b). Aliperti et al. (2023) correlate G + C content with r/K selection, where low G + C content genomes are linked to specialist in stable environments with scarce resource availability and high-cost homeostasis (K-strategists). This theory agrees with the preference of the members of the phylum *Balneolota* to inhabit hypersaline terrestrial environments (Kimbrel et al., 2018; Vavourakis et al., 2018; Vera-Gargallo and Ventosa, 2018; Vera-Gargallo et al., 2019).

3.3.2. *Not-yet described bacterial families dominated the studied saline soils*

Between 27 and 15 % of the contigs of the 18 metagenomes could be annotated to lower taxonomic level where most prokaryotic sequences remained unclassified at family rank (Fig. S3a). Still, representative halophilic archaeal families were detected in significant proportions, *i.e.*, *Halorubraceae*, *Haloferacaceae*, *Haloarculaceae*, and *Halobacteriaceae*. Bacterial sequences could only be annotated at phylum and class level, except for the family *Balneolaceae*. This family is one of the two that constitute the phylum *Balneolota* and one of its representatives, *Fodinibius salsisoli*, has recently been isolated and described as a new species from the saline soils of the Odiel Saltmarshes Natural Area (Galisteo et al., 2023b). The lack of family identification for *Gemmatimonadota* (Fig. S3a) is expected, as this is still an unexplored taxon with only a few species described to date (Parte et al., 2020). On the other hand, *Pseudomonadota* is a broad group that contains genera with a large number of known halophilic species, such as *Marinobacter* (62 species with validly published names) or *Halomonas* (78 validly published species names) (Parte et al., 2020). However, their respective families were not abundant in our datasets, nor was any other family within the phylum *Pseudomonadota* (Fig. S3a). The lack of proper resolution at lower taxonomic levels suggests that many of the prokaryotes inhabiting the hypersaline soils of the Odiel Saltmarshes Natural Area have not yet been isolated in pure culture or described taxonomically. To ensure that the samples represented the major biodiversity present in the soils under scrutiny, we estimated rarefaction curves based on the number of reads identified at family level. The 18 metagenomic databases reached a plateau at lower sample size than the current libraries (Fig. S3b), indicating sufficient sequencing depth.

Alpha diversity at the family level based on the Shannon index enabled us to explore the differences in diversity between areas and over years. Area 2C increased its diversity from 3.36 in 2020 to 3.54 in 2021, and it was the sample with highest alpha diversity in the 18 metagenomic samples (Fig. S3c). Within this area, approximately 25 % of the reads were assigned to families with lower abundances in M3 (year 2021), showing a clear decline of unclassified representatives from the class *Halobacteria* with respect to the previous year (M2, year 2020) (Fig. S3a). The diversity in areas 1A and 2A was stable during both years, with a minimal decrease in 2021. For areas 1B and 2B, the diversity increased slightly in 2021, especially in area 1B (Fig. S3c), and for areas 1C and 3C there was a considerable decrease in alpha diversity between years (3.20 for M2_1C; 2.78 for M3_1C, and 3.28 for M2_3C; 2.96 for M3_3C; respectively). Lastly, area 3 (3A, 3B, and 3C) had a decrease in alpha diversity from year 2020 (sampling M2) to 2021 (sampling M3), with convergent Shannon values for 3A, 3B, and 3C in the second year (Fig. S3c).

The main taxonomic groups from the hypersaline soils of the Odiel Saltmarshes Natural Area were strikingly different than those found in hypersaline aquatic ecosystems. In hypersaline salterns, the prokaryotic community is much less diverse and mostly dominated by *Pseudomonadota* and *Methanobacteriota* (Benlloch et al., 2002; Ghai et al., 2011; Fernández et al., 2014a, 2014b). Even if these two phyla were also abundant in the soils under scrutiny here, they present a higher proportion of rare population than in the aquatic hypersaline environments. At lower taxonomic levels, *Salinibacter ruber* (Antón et al., 2002) is

considered one of the main inhabitants of the crystallizer ponds from Spanish salterns, such as Santa Pola (Alicante) and Isla Cristina (Huelva), with a relative abundance of up to 9 % (Ghai et al., 2011; Fernández et al., 2014a, 2014b), and almost 20 % in hypersaline lake Urmia (Iran) (Kheiri et al., 2023). Likewise, one of the predominant bacteria inhabiting intermediate salinity ponds of salterns is *Spiribacter salinus*, with up to 15 % of relative abundance in Santa Pola saltern (León et al., 2014). These genera were detected with a relative abundance lower than 2 and 0.1 %, respectively, in the hypersaline soils of the Odiel Saltmarshes either, suggesting that they are both specifically adapted to aquatic saline habitats.

Concerning the domain *Archaea*, aquatic and terrestrial hypersaline environments were more similar. Our study revealed that the families *Halorubraceae*, *Haloferacaceae*, *Haloarculaceae*, and *Halobacteriaceae*, within the phylum *Methanobacteriota*, were among the most abundant community representatives in the hypersaline soils from the Odiel Saltmarshes Natural Area (Fig. S3a). These families also include the most abundant genera detected in the abovementioned salterns and in hypersaline lakes, such as the Dead Sea (Bodaker et al., 2010; Jacob et al., 2017; Rhodes et al., 2012), the Great Salt Lake (Tazi et al., 2014), the lake Tyrrel (Narasingarao et al., 2012; Podell et al., 2013), and the lake Urmia (Kheiri et al., 2023). Still, the archaeon *Haloquadratum walsbyi* (family *Haloferacaceae*), which constitutes one of the main archaeal populations of Santa Pola salterns (79 % of relative abundance) (Ghai et al., 2011), the Great Salt Lake (Tazi et al., 2014), and the hypersaline lake Urmia (Kheiri et al., 2023), was not well represented in the hypersaline soils from the Odiel Saltmarshes Natural Area, with a relative abundance lower than 0.02 %. The absence of this haloarchaeon in our hypersaline soils could be linked to the salt concentration, close to saturation in the aforementioned aquatic environments, specifically 37 to 27 % (*w/v*) NaCl (Ghai et al., 2011; Asem et al., 2014; Tazi et al., 2014; Jookar Kashi et al., 2021), which is much higher than that measured in the soils of the present study. "*Candidatus* Nanohaloarchaeum" was detected in the 18 metagenomic samples, but this archaeal lineage did not exhibit the same high representation as observed in the hypersaline lake Tyrrel (Narasingarao et al., 2012; Podell et al., 2013), with a relative abundance lower than 1 % in our soils.

3.4. Heavy metals transporters among the most widely distributed functions in the community

Besides the study of the taxonomic diversity, we carried out a detailed functional analysis of the metagenomes focused on survival strategies to cope with extreme concentration of heavy metal and salts. Between 3,917,390 (for M2_1B metagenome) and 1,916,882 (for M3_3C dataset) ORFs were predicted from contigs and translated to proteins. Afterwards, KEGG Orthology identifiers (KO numbers) were assigned to the identified proteins, yielding from 1,924,101 (M2_1B) to 902,706 (M3_3C) annotated proteins (Table S2) that were further manually inspected.

To detect relevant functions in the prokaryotic community under study, we selected the 20 most abundant KO numbers according to FPM. There were two functions with considerably higher abundance in samples M3_2A, M3_2B, and M3_2C: ABC-2 type transport system permease protein (K01992) and the eukaryotic-like serine/threonine-protein kinase (K12132) (Fig. S4). These samples had the lowest relative proportion of *Methanobacteriota* and the highest relative abundance of *Pseudomonadota* (Fig. 2). Thus, the differences in the functional profile of the samples are likely a consequence of the variable contributions from these two taxonomic groups. The majority of the 20 top abundant KO in the 18 samples belonged to unnamed proteins or with generic functions such as UDP-glucose 4-epimerase and acetyl-CoA synthetase (Fig. S4). However, our results revealed the strong presence of two functions related to resistance to high concentrations of heavy metals, particularly a Cu^{+} exporting ATPase (K17686) and a Cd^{2+}/Zn^{2+} exporting ATPase (K01534) (Fig. S4). This emphasizes the importance of resistance strategies to cope with enhanced heavy metal exposure in this particular extreme environment. These transporters and other manually identified functions related to heavy metal tolerance will be discussed in the next section.

3.4.1. Heavy metal tolerance strategies developed by the prokaryotes from the Odiel Saltmarshes Natural Area

3.4.1.1. Arsenic extrusion and metabolism are widely present in haloarchaea and other predominant phyla. The Odiel River and its sediments are well known for their heavy metal contamination. The samples collected during this study showed concentrations above the recommended limit for several heavy metals (Consejería de Medio Ambiente, 1999), particularly with regards to arsenic, lead, and zinc (Table S1). The metals arsenic and lead, along with silver, cadmium, aluminum, mercury, and gold, do not have a biological role and their accumulation is toxic to most microorganisms (Nies, 1999; Hobman and Crossman, 2015). Among the most predominant phyla with species detected in different heavy metal contaminated environments are *Pseudomonadota*, *Chloroflexota*, *Acidobacteriota*, *Actinomycetota*, *Bacteroidota*, *Gemmatimonadota*, *Nitrospirota*, *Desulfobacterota*, *Myxococcota*, and *Bacillota* (Hao et al., 2021). These groups constituted a minor proportion in the soil metagenomes of the present study, except for *Pseudomonadota*, *Chloroflexota*, and *Actinomycetota*, which were abundant in our samples. Additionally, members of the phyla *Bacillota* and *Balneolota*, previously isolated from the Odiel Saltmarshes Natural Area hypersaline soils, carried genes for heavy metal resistance in their genome sequences (Galisteo et al., 2023a, 2023b).

In nature, arsenic is mostly found as trivalent species [As(III), arsenite, AsO_2^-], which strongly binds to sulfhydryl groups, and pentavalent species [As(V), arsenate, AsO_4^{3-}], which mimics PO_4^{3-} for uptake. Generally, As(III) is more soluble and 60 times more toxic than pentavalent species. Arsenic detoxification is widely distributed across prokaryotes and more complex eukaryotic organisms, such as humans (Andres and Bertin, 2016; Voica et al., 2016; Ben Fekih et al., 2018; Chen and Rosen, 2020), as this metalloid may have been continuously present since the early emergence of life the Earth (Chen et al., 2020). The *ars* operon provides an ancient active mechanism for arsenic resistance (Nies, 1999). ArsC (K03741) transforms As(V) to As(III), which is then extruded from the cytoplasm by the energy-dependent proteins ArsA (K01551) and/or ArsB (K03893). The transcription of the operon is regulated by ArsR (K03892), which dissociates from its DNA binding site when interacting with arsenite (Ben Fekih et al., 2018; Chauhan et al., 2019; Islam et al., 2022). All these functions were identified in our soil metagenomes, with *arsR* as the most abundant gene and *arsB* being the least abundant (Fig. 3a). Arc3 transporter (K03325), also known as ACR3 or ArsY, has an As(III) efflux function, and its combination with ArsA has been shown to be more efficient for As(III) extrusion (Castillo and Saier, 2010). The predominance of *arc3* over *arsB* (Fig. 3a) in our samples has also been reported for other arsenic-contaminated soils (Cai et al., 2009), implying that Acr3 is a more efficient arsenic transporter than ArsB in conditions where concentrations of this metal is high. The *ars* operon was present in the ten most abundant phyla in the metagenomic datasets, with the exception of the phylum *Deinococcota*, which only harbored the *arsC* gene (Fig. 3b). Regarding the different distribution of ArsB and Acr3, the latter was present along with ArsA in the majority of the identified phyla. However, ArsB was almost absent from the phylum *Actinomycetota*, being only detected in sample M3_2B. In the case of haloarchaea, ArsA was the predominant arsenic transporter in all metagenomes and, once again, the Acr3 transporter overcomed ArsB (Fig. 3b).

The biotransformation of inorganic arsenic to organoarsenical species by the enzyme S-adenosylmethionine methyl transferase, ArsM (K07755), is another strategy to avoid the fatal consequences of the

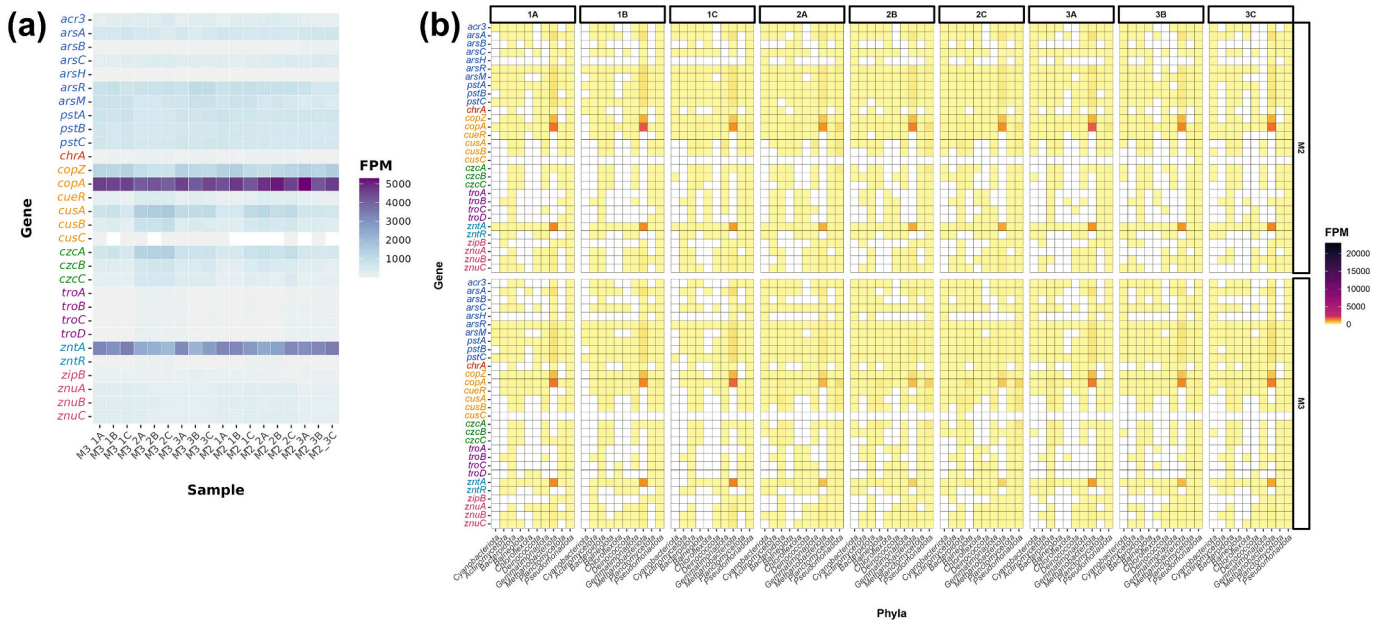

Fig. 3. Abundance (expressed as Features Per Million, FPM) of genes related to arsenic (dark blue), chromium (red), copper (yellow), cadmium, zinc and copper (green), manganese import system (purple), zinc efflux (light blue), and zinc uptake (pink), annotated against the KEGG database in the 18 metagenomic databases under study (a) and for the most predominant phyla (b). Respective KO numbers are described in the main text.

accumulation of this heavy metal. This gene was first identified in the haloarchaeon *Halobacterium* sp. (Wang et al., 2004; Qin et al., 2006) and is typically located in the *ars* operon with other arsenic resistance related genes and the arsenic regulator *arsR* (Ben Fekih et al., 2018), which is widely distributed in prokaryotes according to the results of the genomic database search performed by Zhu et al. (2014). This gene, *arsM*, is also abundant in the most dominant phyla in soil environments (Dunivin et al., 2019; Keren et al., 2022). However, the first methylated product from ArsM activity, known as methylarsenites [MA(III)] (Dheeman et al., 2014), is extremely toxic in environments where anoxic and reducing conditions prevail (Ben Fekih et al., 2018; Chen and Rosen, 2020). Hence, ArsM may have an inhibitory role and thus mediate ecological competition in complex communities, forcing other populations to invest in strategies against MA(III) (Chen and Rosen, 2020). The most widely distributed mechanism for such detoxification is oxidation to MA(V) by the NADPH-dependent MA(III) oxidase, ArsH (K11811), encoded by *arsH* (Chen et al., 2015; Páez-Espino et al., 2015). However, the *arsH* gene was not abundant in our soil metagenomes (Fig. 3a) and scarcely detected in five of the ten most abundant phyla, that is, *Bacteroidota*, *Balneolota*, *Cyanobacteriota*, *Methanobacteriota*, and *Pseudomonadota*. In contrast, the *arsM* gene was found in the top ten phyla in this study (Fig. 3b), as well as in the most dominant taxa in soil environments such as *Acidobacteriota*, *Deltaproteobacteria*, *Gemmatimonadota*, *Nitrospirota*, *Actinomycetota*, *Methanobacteriales*, *Chloroflexota,* and *Cyanobacteriota*, among others (Dunivin et al., 2019; Keren et al., 2022).

The annotation of these proteins for transport and methylation of arsenite in the metagenomes could indicate that the prokaryotic population from the environment under study harbor mechanisms to coexist with this compound. The soils were sampled at 2–4 cm depth from the surface. Lack of oxygen is not a concern in such surface soils and therefore MA(III) could be spontaneously oxidized to the less toxic MA (V) (Le et al., 2000). Given that a high proportion of the *arsM* gene annotated from the metagenomic datasets under study belonged to the phylum *Methanobacteriota*, its members may play an important role in the methylation of inorganic arsenic in this environment.

The Pst phosphate-specific transport system, encoded by *pstABC* genes, was present in the 18 samples (Fig. 3a) and in the 10 most abundant phyla of this environment (Fig. 3b). Considering that the surrounded arsenic concentration values surpassed the contamination limits (Table S1), this phosphate transporter may play a significant role in the unspecified uptake of As(V) instead of PO_4^{3-} (Kabiraj et al., 2022; Corrales et al., 2024). It entails that at least one mechanism for arsenic tolerance should be present for the cell survival. As(V) could be reduced to As(III) by ArsC and later be excreted by ArsA, Acr3 and, to a lesser extent, by ArsB transporter. Only sequences affiliated to *Deinococcota* did not harbored arsenite transporters (*arsAB*, *acr3*). In this case, methylation of As(III) by ArsM is favored. The gene coding for this methyltransferase, *arsM*, was present in the 10 most abundant phyla, where the phylum *Methanobacteriota* gathered most of the annotated *arsM* genes in the studied soils.

In summary, we hypothesize that arsenic may be carried inside the prokaryotic cell by a phosphate transport system if an extremely high concentration of this heavy metal is present in the environment. Functions related to arsenic extrusion and metabolism were widely distributed in the major taxonomic groups found in the Odiel Saltmarshes Natural Area and they have also been detected in the genome sequence of strains isolated from this habitat (Galisteo et al., 2023a). Thus, our data suggest that the prokaryotic community, and in particular the haloarchaea, could play a role in the detoxification of soils.

3.4.1.2. Zinc, cadmium, and copper tolerance are achieved by efflux and chelation strategies. In addition to arsenic, zinc is an abundant contaminant in the hypersaline soils of the Odiel Saltmarshes Natural Area (Table S1). One of the main transporters detected among the most abundant KO numbers in the 18 metagenomic databases was ZntA (K01534) (Fig. S4; Fig. 3a). This protein is a P-type ATPase transporter enabling efflux of zinc, lead, and cadmium (Beard et al., 1997; Rensing et al., 1997, 1998), where the latter is the main trigger (Brown et al., 2003), and it is regulated by ZntR (K13628) (Brocklehurst et al., 1999; Brown et al., 2003) The concentrations of lead and cadmium in our samples did not exceed the limits to consider the soils contaminated, but zinc concentrations were clearly above such limits (Table S1). ZntA was detected in all the predominant phyla (Fig. 3a) and was especially prevalent in the phyla *Methanobacteriota* and *Balneolota* (Fig. 3b). Thus, ZntA may play a significant role extruding zinc and avoiding its accumulation inside the prokaryotic cells from this habitat. On the contrary

zntR gene was less represented than *zntA* in the samples (Fig. 3a), or even missing from some major phyla (Fig. 3b). We hypothesize that ZntR may not be the main regulator of *zntA* in these individuals, or that its sequence may not be conserved enough as to be found in databases, and thus, the protein could not be annotated. In line with this, *zntAR* genes have previously been found in the genome sequence of isolates from these saline soils (Galisteo et al., 2023a, 2023b).

In contrast, functions related to the uptake of zinc from the environment such as *zipB* (K16267), which also transports cadmium (Lin et al., 2010), and *znuABC* (Patzer and Hantke, 1998; Ducret et al., 2022), were considerably less abundant in the metagenomic datasets (Fig. 3a). Furthermore, *zipB* was completely missing from some of the main phyla, *i.e.*, *Cyanobacteriota*, *Balneolota*, *Chloroflexota*, and *Deinococcota*, and *znuABC* cluster was incomplete in *Cyanobacteriota* and *Balneolota* (Fig. 3b). Thus, the low proportion of these genes implies a lack of need to acquire this metal by active mechanisms as it is passively obtained from the surroundings.

Another efficient strategy for extrusion of cadmium, zinc, and copper from the cytoplasm is the CzcCBA efflux system (K15725, K15726, K15727) (Diels et al., 1995; Legatzki et al., 2003), which was identified in some of the most abundant phyla dwelling in the studied soils (Fig. 3b). Although, copper was not detected at values above the contamination limits in any of the 18 samples (Table S1), CzcCBA was not the only transporter related to copper found in the samples. Cu^{+} exporting ATPase (CopA; K17686) constituted one of the most abundant functions in the community (Fig. S4). Besides, it was the most prevalent among the heavy metal-related functions (Fig. 3a) and it was annotated for all the major phyla. Its absence in *Cyanobacteriota* from the samples M2_1A, M2_1B, and M2_1C (Fig. 3b) may be related to a lower relative abundance of this phylum in area 1, particularly in 2020 (sampling M2) (Fig. 2a). Moreover, this transporter was also found in the newly described genus and species recently isolated from the hypersaline soils of the Odiel Saltmarshes Natural Area (Galisteo et al., 2023a). CopA has been identified as the main copper exporter in some species, such *as Streptococcus suis,* and its deletion has been revealed to decrease copper tolerance (Yang et al., 2023).

Although not directly related to copper mobilization, recent studies have demonstrated that when the manganese import system TroABCD (K11707, K11710, K11708, K11709) is missing from the organism, its tolerance to copper increases even when *copA* is not present (Yang et al., 2023). For our metagenomes, the presence of this system was scarce (Fig. 3a), and entirely missing in some taxa, such as *Cyanobacteriota*, *Methanobacteriota*, and *Balneolota* (Fig. 3b). Therefore, the activity of CopA and the absence of TroABCD could confer an active and passive protection, respectively, against intracellular copper poisoning along with other copper-related systems such as the copper/silver efflux system encoded by *cusABC* genes (K07787, K07796, K07798) (Bagai et al., 2007). The *cusA* and *cusB* genes were annotated mostly for *Balneolota*, *Gemmatimonadota*, *Planctomycetota*, and *Pseudomonadota*, but not for *Methanobacteriota*, *Actinomycetota*, *Chloroflexota*, and *Deinococcota* (Fig. 3b), which means that they were limited to certain taxonomic groups. On the other hand, the *cusC* gene was almost nonexistent in the metagenomes (Fig. 3a), and it was only annotated for sequences related to *Bacteroidota* and *Pseudomonadota* (Fig. 3b).

The last function related to copper that was evaluated in the present work was the copper efflux regulator coded by *cueR* gene (K19591) (Thaden et al., 2010). This transcriptional regulator was detected in all samples (Fig. 3a), although never associated to *Methanobacteriota* (Fig. 3b). The response of CueR to copper increases when the copper chaperone encoded by *copZ* is deleted (Stoyanov et al., 2003). CopZ was poorly represented in the samples (Fig. 3a), with the exception of haloarchaea. In this group, CopZ was relatively abundant and also lacked *cusABC* and *cueR* (Fig. 3b). Therefore, we could conclude that *Methanobacteriota* may be using the chelation strategy enabled by CopZ coupled to copper export by CopA. On the other hand, the bacterial phyla are potentially extruding copper outside the cell using CopA, CusABC, and CzcCBA.

These *in silico* results are partially in agreement with *in vitro* studies in prokaryotes previously isolated from this habitat (Nieto et al., 1989a, 1989b). These pioneering investigations revealed that 90 % of the isolated archaeal strains were tolerant to copper, 82 % to chromium, 78 % to lead, and 33 and 30 % to arsenate and cadmium, respectively (Nieto et al., 1989b). Our data displayed a high abundance of copper (*copA*) and chromium (*chrA*) efflux. However, our results suggest that the arsenic detoxification is focused on As(III) (arsenite) rather than on As (V) (arsenate), either by efflux (*acr3*, *arsA*) or methylation systems (*arsM*) (Fig. 3b). On the other hand, these previous studies tested 250 moderately halophilic bacteria and all of them were sensitive to zinc, but the response to cadmium, copper, arsenate, and chromium was mixed, highlighting two *Halomonas* strains as the most tolerant microorganisms to cadmium. Only six strains were tolerant to copper, but all of them were tolerant to lead (Nieto et al., 1989a). This apparently differential results are probably related to cultivation bias, as *in vitro* analysis considers easy-growing bacteria, such as members of the genera *Halomonas*, *Chromohalobacter*, and *Marinobacter*.

The study presented here was based on the analysis of metagenomic DNA, which allows the detection and functional classification of undescribed taxa. Therefore, the results of Nieto et al. (1989a, 1989b) encompass a reduced landscape of the total microbial population and, thus, they are not fully representative, although they agree with the tolerance strategies unveiled by metagenomic analyses. Another potential heavy metal strategy would be the formation of biofilms, as this has been found to be prevalent in soils with high copper pollution (Shaw et al., 2020; Zhu et al., 2022) and to protect against Hg^{2+} and As^{3+} toxicity (Nocelli et al., 2016). In any case, the microorganisms from our soils could be an interesting source for bioremediation processes, due to a widespread ability to grow in hypersaline soils contaminated with high concentrations of multiple heavy metals.

To sum up, the distribution of zinc, cadmium, and copper tolerance genes in the most predominant phyla from the soils of the Odiel Saltmarshes Natural Area, we observed an almost complete absence of zinc uptake genes (*zipB*, *znuABC*) but widespread occurrence of zinc efflux mechanisms (*zntA*), enhanced by shared efflux systems, such as CzcCBA (cadmium, zinc, and copper). Likewise, copper extrusion was favored by copper related proteins, particularly CopA and CusABC. In addition, the absence or scarcity of the manganese efflux system (TroABC) favors copper tolerance by increasing the activity of CueR regulator. Overall, our study suggests that both prokaryotic domains use different strategies, with a preference for copper transport in bacteria and a copper chelation in haloarchaea (Fig. 3b).

3.4.2. Proteome profile as an indicator of the osmoregulation strategy

The survival of prokaryotes under hypersaline conditions is accomplished by means of two different strategies known as “salt-in” and “salt-out”. The “salt-in” mechanism avoids salinity-induced denaturation of proteins in the cytoplasm by acidifying the cell proteome. This strategy is characteristic for haloarchaea (Oren, 2013) and extremely halophilic bacteria (Antón et al., 2002). The “salt-out” mechanism is more prevalent in prokaryotes and allows microorganisms to thrive under a wide range of salt concentrations (Oren, 2008). Some of the microorganisms that present “salt-out” adaptation also have an acidic proteome just like the “salt-in” microorganisms (Elevi Bardavid and Oren, 2012; Oren, 2013), but their main strategy consists of cytoplasmic accumulation of osmolytes to cope with high osmotic pressure for prolonged periods of time (Hoffmann and Bremer, 2016, 2017), which facilitate accumulation of water in the cell cytoplasm, thereby maintaining biochemical processes (Gregory and Boyd, 2021). In this study, we focus on the two more universal osmolytes: glycine betaine and ectoine, together with its 5-hydroxylated derivative (hydroxyectoine) (Hosseiniyan Khatibi et al., 2019).

As previously indicated, the isoelectric profiles of the 18 metagenomes were very similar, with the highest peak at pH 4, representing

between 30 and 50 % of the predicted proteome (Fig. S2b). The proportion of proteins in this peak was lower than for the extremely halophilic archaeon *Haloarcula vallismortis* DSM 3756^T (GCF_900106715.1), a "salt-in" microorganism used as a reference. Around 70 % of the proteome of this haloarchaea presents an isoelectric point at pH 4, whereas the relative abundance of protein above pH 6 was almost nonexistent. However, our metagenomes display a second much lower peak at pH 10 (approximately, 5–10 % of the global proteome) which matches the pattern for the "salt-out" reference species *Spiribacter salinus* M19-40^T (GCF_000319575.2) (Fig. S2b). Hence, according to the isoelectric point predictions for annotated proteins, our metagenomes seem to feature a mix of both strategies. Considering the balanced distribution of archaea and bacteria in these soils (Fig. 2a), we split the predicted proteins by domain, and the result showed a clear concentration of archaeal proteins at around pH 4, while bacteria displayed an isoelectric point across a wider pH range (Fig. 4a). We conclude that haloarchaea of the hypersaline soils of the Odiel Saltmarshes Natural Area use the "salt-in" strategy to adapt their metabolism to high salt concentrations, while the bacterial community uses both "salt-in" and "salt-out" strategies, as evidenced from the wider predicted isoelectric point within their proteomes.

Regarding amino acid frequency in the predicted proteomes, alanine (A) was the most abundant amino acid (Fig. S2c). Previous metagenomic studies have revealed that the proportion of this amino acid increases with salinity (Ghai et al., 2011; Rhodes et al., 2012; Fernández et al., 2014b). Leucine was the second most abundant amino acid in the 18 metagenomes (Fig. S2c), and also this amino acid appears to be less significant in metagenomes of non-saline environments (Rhodes et al., 2012). Hence the predicted amino acid profiles with alanine and leucine as major components are in line with a microbiome adapted to hypersaline environments.

3.4.2.1. Ion and osmolyte transport for short- and long-term salt stress. The first barrier of defense against sudden increase in salt stress is uptake of K^+ into the cytoplasm by KtrAB potassium importers (K03498, K03499) to balance the osmotic pressure in the cell (Holtmann et al., 2003; Hoffmann and Bremer, 2016), followed by efflux of sodium ions mediated by Mnh (K05565, K05566, K05567, K05568, K05569, K05570, K05571), and NhaA (K03313) sodium antiporters (Ito et al., 1999; Ohyama et al., 1994; Blanco-Rivero et al., 2005; Patiño-Ruiz et al., 2022). KtrAB is the only sodium-dependent potassium importer, emphasizing its role in cellular osmoprotection (Nakamura et al., 1998; Tholema et al., 1999). All 18 metagenomes featured genes for potassium uptake and sodium extrusion by the aforementioned transporters. Samples M3_2A, M3_2B, and M3_2C (area 2, year 2021) had a lower abundance of potassium importers than the other analyzed metagenomes (Fig. 4b), possibly due to lower abundance of *Methanobacteriota* compared to the rest of the samples (Fig. 2a). As stated above, we hypothesize that members of the phylum *Methanobacteriota* present a "salt-in" strategy according to their acidic proteome (Fig. 4a), and the literature also classified them as such (Oren, 2013). KtrAB potassium importers were accordingly the most abundant archaeal osmoprotective function in the present study (Fig. 5a).

Other organisms cope with long-term salt stress by accumulating osmolytes in the cytoplasm. The *opuABC* genes encode the Opu uptake system (K05845, K05846, K05847), an ABC transporter known to import a diverse range of osmolytes into the cytoplasm with particularly high affinity for choline (Hoffmann and Bremer, 2017; Teichmann et al., 2018). Other transporters such as OpuD (K05020) is a betaine-choline-carnitine-transporter (BCCT) with a preference for glycine betaine (Kappes et al., 1996) analogous to ProVWX transporters (K02000, K02001, K02002) (Gregory and Boyd, 2021). Unlike KtrAB, OpuABC was slightly more prevalent in samples M3_2A, M3_2B, and M3_2C (area

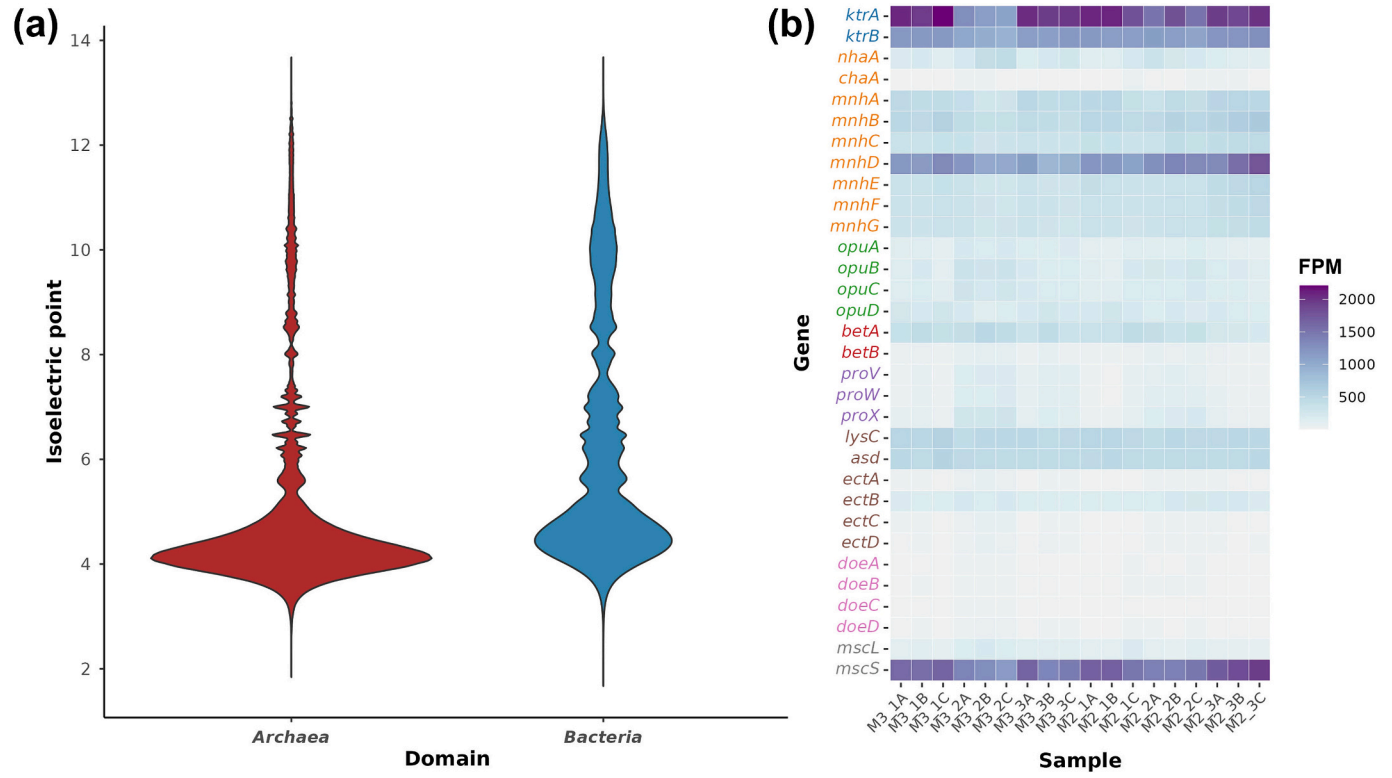

Fig. 4. (a) Distribution of the isoelectric point of the translated CDS sequences assigned to the domains *Archaea* or *Bacteria* of the 18 metagenomic samples. (b) Normalized abundance of genes related to osmotic stress survival strategies annotated against the KEGG database in the 18 metagenomic databases under study. Potassium uptake (dark blue), sodium extrusion (orange), osmoprotectant transporters (green), glycine betaine biosynthesis (red), glycine betaine transporters (purple), ectoine biosynthesis (brown), ectoine degradation (pink), and ions/osmolytes extrusion channels (gray). Respective KO numbers are described in the main text.

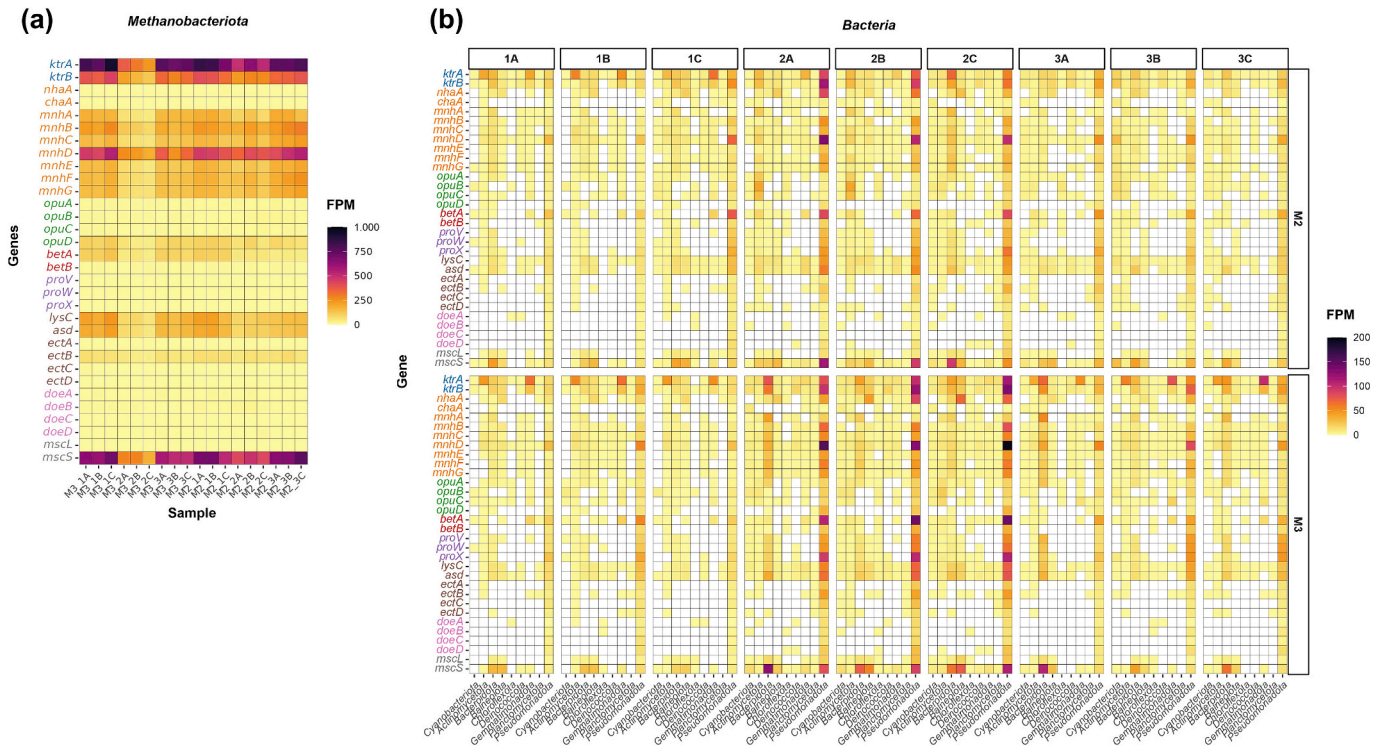

Fig. 5. Normalized abundance of genes related to osmotic stress survival strategies annotated against the KEGG database in the 18 metagenomic databases under study for the phylum *Methanobacteriota* (a) and for the nine most predominant bacterial phyla (b). Potassium uptake (dark blue), sodium extrusion (orange), osmoprotectant transporters (green), glycine betaine biosynthesis (red), glycine betaine transporters (purple), ectoine biosynthesis (brown), ectoine degradation (pink), and ions/osmolytes extrusion channels (gray). Respective KO numbers are described in the main text.

2, year 2021), which correspond to the datasets where the relative abundance of archaea was lower (Fig. 4b). Still, Opu transporters were present in *Methanobacteriota* contigs, with higher representation of OpuD (Fig. 5a) compared to OpuABC (Hoffmann and Bremer, 2017). In contrast, *opuD* was missing from five of the most abundant bacterial phyla in our metagenomes, *i.e.*, *Cyanobacteriota*, *Balneolota*, *Chloroflexota*, *Deinococcota*, and *Gemmatimonadota* (Fig. 5b). Our finding suggests that bacteria rely on ABC transporters rather than BCCT transporters to assimilate osmoprotectants, or that bacteria use choline over glycine betaine as compatible solute. However, the ProVWX transporter, with affinity for glycine betaine, was present in the five bacterial phyla that missed the *opuD* gene. Therefore, it is possible that some bacteria rely on only a single glycine betaine uptake mechanism. This does not mean that the OpuD and ProVWX are mutually exclusive, as they were both detected in the phyla *Actinomycetota*, *Bacteroidota*, *Pseudomonadota*, and *Methanobacteriota*.

3.4.2.2. Haloarchaea as relevant producers of compatible solutes. Some prokaryotic microorganisms have the ability to not only take up osmolytes from the environment, but also to synthesize them *de novo*. Common pathways to achieve this are the biosynthesis routes for glycine betaine, ectoine, and its derivative 5-hydroxyectoine. Halophilic archaea and bacteria (Gregory and Boyd, 2021), and even eukaryotes (Czech and Bremer, 2018), transform choline into glycine betaine *via* aerobic oxidation mediated by BetA (K00108) and BetB (K00130) (Boch et al., 1994; Czech and Bremer, 2018). This widely distributed biosynthesis pathway was found in all our 18 metagenomes (Fig. 4b). Regarding their distribution in the ten most abundant phyla across the samples, both enzymes were annotated in contigs related to *Methanobacteriota* (Fig. 5a), *Actinomycetota*, and *Pseudomonadota* for all samples. These genes were also detected in *Cyanobacteriota* and *Bacteroidota* for most samples from area 2 in years 2020 and 2021 but not for the rest of the samples (Fig. 5b). The fact that only one of the genes could be detected in less abundant phyla could be due to paucity of data and incomplete genome coverage in the metagenomes.

De novo biosynthesis of ectoine consists of five steps and is specific to prokaryotic microorganisms (Czech and Bremer, 2018). Aspartate kinase (*lysC*; K00928), aspartate semialdehyde dehydrogenase (*asd*; K00133), diaminobutyrate-2-oxoglutarate transaminase (*ectB*; K00836), L-2,4-diaminobutyric acid acetyltransferase (*ectA*; K06718), and L-ectoine synthase (*ectC*; K06720) transform, subsequently, L-aspartate into ectoine (Ono et al., 1999), which can also be further conversed into 5-hydroxyectoine by means of the ectoine hydroxylase (*ectD*; K10674) (Bursy et al., 2007). These genes were less abundant in the metagenomic datasets as compared to *betA* and *betB* (Fig. 4b) and many of the more abundant phyla did not seem to carry the *ectABC* operon. Only *Methanobacteriota*, *Actinomycetota*, and *Pseudomonadota* harbored the three gene set (Fig. 5). The *lysC* and *asp* genes were not considered as exclusive for ectoine production because they also participate in the biosynthesis of threonine (Dong et al., 2012).

Ectoine and 5-hydroxyectoine are also a source of carbon and energy. Their catabolism is not yet fully understood, but it is known that the *doeBDAC* gene cluster (K15784, K15785, K15783, K15786) is involved. Specifically, accumulation of ectoine has been shown to increase when the *doeA* gene is deleted (Reshetnikov et al., 2020). Additionally, ectoine degradation proteins have been detected in some ectoine producers such as *Halomonas elongata* (Schwibbert et al., 2011) and *Methylotuvimicrobium alcaliphilum* (Reshetnikov et al., 2020). In our samples, *doeBDAC* genes were low in abundance, and most of them were missing from some of the predominant phyla. The two most abundant phyla in the samples (Fig. 2a), *Pseudomonadota* and *Methanobacteriota*, harbored both ectoine and 5-hydroxyectoine biosynthesis and degradation pathways. *Actinomycetota* and *Plactomycetota* also harbored the complete, or almost complete, ectoine biosynthesis pathway, but several ectoine degradation genes were missing (Fig. 5). Therefore, we can conclude that the metabolic use of ectoine and its derivatives as carbon and

energy sources was not widely distributed among the prokaryotes that inhabit the environment under study.

The main *de novo* producers of the osmolytes glycine betaine and ectoine in these saline soils seem to be representatives of *Pseudomonadota* and *Methanobacteriota*. Microorganisms from these groups were also ectoine degraders so they can catabolically use this osmolyte when the salinity decrease and, thereby, conserve energy. It should also be noted that we are only considering two of the most universal compatible solutes and other molecules such as sugars, polyols and their derivatives, or amino acids and their derivatives could also have osmoprotective activity (Hosseiniyan Khatibi et al., 2019). Besides, our analysis was focused on the ten most abundant phyla in the environment, thus, more rare community members could also play an important role as producers of compatible solutes, such as *Terrihalobacillus insolitus* and *Aquibacillus salsiterrae*, which were isolated from the hypersaline soils of Odiel Saltmarshes Natural Area and feature genes coding for glycine betaine and ectoine biosynthesis (Galisteo et al., 2023a).

The use of osmolytes for osmoregulation purposes is related to prokaryotes with "salt-out" strategy (Hoffmann and Bremer, 2016, 2017). However, we found a high abundance of proteins related to biosynthesis of osmolytes in haloarchaeal sequences. These results show that the role of compatible solutes in osmoregulation is also relevant in prokaryotes with "salt-in" mechanisms. The biosynthesis and uptake of compatible solutes have been uncovered before in *Halobacteriales* (Youssef et al., 2014), thus, our findings suggest that these osmoadaptation strategies may be more widely spread in archaea with "salt-in" strategies than previously thought.

Until now, we have only discussed about the stress associated to increasing salt concentration. However, osmotic stress can also arise when the salinity around the cell decreases. In that case, the cells must be ready to excrete the accumulated compatible solutes, and in this regard, mechanosensitive channels allow the release of both ions and osmolytes outside the cell (Booth and Blount, 2012; Booth, 2014). In the studied metagenomes, the small conductance mechanosensitive channel (MscS; K03442) appeared to be more abundant than the large conductance mechanosensitive channel (MscL; K03282) (Fig. 4b) and the same was true for each of the predominant taxa (Fig. 5). This is just a generic glimpse regarding the osmoprotectant strategies adopted by the prokaryotic community from the hypersaline soils of the Odiel Saltmarshes Natural Area. Further specific analyses should be performed including laboratory experiments.

4. Conclusions

The soils studied in this research, located at the Odiel Saltmarshes Natural Area (Huelva, Spain) are hypersaline with high content of arsenic, zinc, and other heavy metals. Cultivation-independent analysis of prokaryotic communities in these poly-extreme habitat revealed equal contributions from domains *Archaea* and *Bacteria*. However, the biodiversity was higher for bacteria, where *Pseudomonadota*, *Bacteroidota*, *Gemmatimonadota*, and *Balneolota* were abundant, along with a large proportion of sequences that remain unclassified even at phylum level. Archaea were mainly represented by *Methanobacteriota*, specifically by class *Halobacteria*, and most sequences were related to the following haloarchaeal families: *Halorubraceae*, *Haloferacaceae*, *Haloarculaceae*, and *Halobacteriaceae*. Although aquatic and terrestrial hypersaline habitat showed certain similarities and differences concerning their prokaryotic population, main inhabitants of aquatic hypersaline environments, particularly *Haloquadratum walsbyi* and "*Candidatus* Nanohaloarchaeum", only define a minor proportion in the terrestrial hypersaline environment under study.

The functional analysis also showed that the prokaryotic inhabitants of the studied hypersaline soils harbor diverse heavy metal tolerance mechanisms, which could enable survival at the very high metal concentrations measured in this habitat. Specially, CopA and ZntA were two of the most abundant global functions in the community. They are involved in the extrusion of copper and zinc outside the cytoplasm, along with CzcCBA, CusABC, CueR and/or CopZ. In addition, we found genes suggesting that the microbiota is likely to play a significant role in the transformation of toxic inorganic arsenic species, in particular, the conversion of arsenate into arsenite by ArsC. Arsenite is more easily expelled out of the cytoplasm mostly by ACR3 but also by ArsA and ArsB. Also, arsenite is biotransformed into organoarsenic species (less toxic) by ArsM. Although this enzyme is present in most of the main phyla, *Methanobacteriota* seems to be the key player for bioremediation in the analyzed soils.

Besides the heavy metal tolerance strategies, the prokaryotic community also encoded mechanisms to deal with salt stress. Haloarchaea exhibit "salt-in" strategies characterized by acidic proteomes with low isoelectric point (at around pH 4) and the presence of transmembrane ion transporters (KtrAB, MnhABCDEFG, NhaA) and mechanosensitive channels (MscL and MscS); however, compatible solutes may also be important as archaeal osmoprotectant. For bacterial population, "salt-in" and "salt-out" approaches were detected according to the broad distribution of the isoelectric point of the bacterial proteomes. Overall, *de novo* production of ectoine was less widespread than glycine betaine biosynthesis both in *Bacteria* and *Archaea* domains. The use of ectoine as a carbon and energy source was rather uncommon given that DoeABCD were exclusively identified in *Pseudomonadota* and *Methanobacteriota*.

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CRediT authorship contribution statement

Cristina Galisteo: Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Fernando Puente-Sánchez:** Writing – review & editing, Software, Resources, Methodology, Funding acquisition, Formal analysis, Data curation. **Rafael R. de la Haba:** Writing – review & editing, Resources, Investigation. **Stefan Bertilsson:** Writing – review & editing, Resources. **Cristina Sánchez-Porro:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Antonio Ventosa:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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.

Data availability

The GenBank accession numbers for the raw metagenomic sequences for the 18 samples are SRS20604429 (M2_1A), SRS20617295 (M2_1B), SRS20617297 (M2_1C), SRS20617301 (M2_2A), SRS20604431 (M2_2B), SRS20604430 (M2_2C), SRS20617303 (M2_3A), SRS20617302 (M2_3B), SRS20604432 (M2_3C), SRS20604428 (M3_1A), SRS20617304 (M3_1B), SRS20617300 (M3_1C), SRS20617299 (M3_2A), SRS20604433 (M3_2B), SRS20617296 (M3_2C), SRS20617298 (M3_3A), SRS20604749 (M3_3B) and SRS20604748 (M3_3C).

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