**Abstract**

Salt marshes gather a high diversity of prokaryotes across their environmental gradients. Most of this diversity and the factors determining their community assemblage are unknown. We massively sequenced a portion of the 16S gene to characterize the diversity of prokaryotes in soils from a salt marsh in Río Piedras, Southern Spain. We sampled in the four seasons, and in five plots dominated by a different halophyte (*Spartina maritima*, *S. densiflora*, *Salicornia ramosissima*, *Arthrocaulon macrostachyum* and *Atriplex portulacoides*) growing under different environmental conditions and representing different stages in the marsh ecological succession. Soil was sampled in their rhizosphere and adjacent bulk soil. We report the effects of different factors explaining prokaryotic beta diversity in the marsh: zonation (50%), seasonality (14%), and halophyte rhizosphere (7%). Proteobacteria and *Bacteroidota* were the most abundant phyla. Firmicutes had a peak in winter and *Desulfobacterota* with other bacteria involved in sulfur cycling were abundant in the low marsh plots from *S. maritima*. Alpha diversity was highest in spring and decreased in winter. We detected a marked phylogenetic turnover between seasons and in rhizospheric soil respect to adjacent bulk soil for most pairwise comparisons. The effect of halophyte on its rhizosphere was species-specific, being *S. maritima* the species with more differentiated taxa between rhizosphere versus surrounding bulk soil. Our work highlights how the complex interaction between marsh zonation, seasonality and rhizosphere, onsets processes structuring bacterial community assemblage in salt marsh soils.

Keywords: bacteria, metabarcoding, Río Piedras, salt marsh, halophytes, microbial ecology

# Introduction

The tidal regime in salt marshes determine strong environmental gradients, and their fast dynamics permit to study coastal chronosequences. Accordingly, salt marshes are a suitable system to investigate species zonation and ecological succession. Related research has been mainly focused on halophytes (Castillo et al., 2021; Castillo et al., 2008; Garbutt, de Groot, Smit, & Pétillon, 2017; Isacch et al., 2006; Pomeroy & Wiegert, 2012). While soil microbiome in salt marshes constitute a valuable ecocline to assess these ecological processes and describe biodiversity, the works on this system are limited due to methodological difficulties to study the microbiological compartment (Hugenholtz et al., 1997; Poretsky et al. 2014).

The advent of sequencing technologies is boosting the description of the large-unknown microbial diversity through the sequencing of environmental samples (Delgado-Baquerizo et al., 2018; Thompson et al., 2017). In salt marshes, a diverse phylogenetic community of bacteria and archaea has started to be described (Camacho-Sanchez et al., 2020; Du et al., 2020; Wang et al., 2017; Wang et al. 2016). Furthermore, the study of microbial diversity from salt marshes contributes to build up ecological theory through the description of the complex mechanisms proposed for their assemblage (Dini-Andreote et al., 2014; Horner-Devine et al., 2004; Tripathi et al., 2018).

The interaction between abiotic factors, environmental temporality and the halophyte community are considered the main drivers of bacterial community heterogeneity across salt marshes. Soil pH has been identified as one of the most influential factors constraining phylogenetic diversity of bacteria at a global scale (Delgado-Baquerizo et al., 2018), and also in salt marsh ecosystems, followed by salinity (Camacho-Sanchez et al., 2020; Dini-Andreote et al., 2014; Hollister et al., 2010; Tripathi et al., 2018; Zhao et al., 2018). Additionally, some of the variation in the bacterial community has been attributed to temporality with day or season (Camacho-Sanchez et al., 2020; Dini-Andreote et al., 2014; Mesa et al. 2016). At last, the ecological effect of the halophyte community is debated. Some studies have found little (Wang et al., 2016; Zhang et al., 2020) or no effect (Camacho-Sanchez et al., 2020; Horner-Devine et al., 2004) of halophyte on their rhizosphere microbiome, while others suggest specific associations (Bagwell et al., 2001; Burke et al., 2002; Cui et al., 2017; Cunha et al., 2005; Ribeiro et al., 2013; Wang et al., 2007). For instance, plant-growth promotion activity (Mateos-Naranjo et al., 2020; Paredes-Páliz et al., 2018), or aeration of the rhizosphere by the plant and the exudation of organic compounds could be playing key roles in species-specific microbiome in salt marshes (Bagwell et al., 2001; Cunha et al., 2005). In fact, it has been suggested that bacteria in marsh soil could enhance ecological succession by ameliorating environmental stress and facilitating plant establishment under suboptimal conditions (Wang et al., 2017).

Salt marshes in Southern Spain span across a large territory in the Gulf of Cádiz and encompass important protected saltmarshes areas. They represent a suitable area to study the relative importance of halophytic vegetation as driver of bacterial assemblages in a spatial and temporal context, given the Mediterranean seasonality and the well described plant community chronosequence and particular environmental characteristics associated. Ecological zonation and succession have been extensively studied across decades in specific locations across these marshes (i.e. Odiel marshes: (Castellanos et al., 1994; Castillo et al., 2021). Although initial studies have described a rich community of halotolerant prokaryotes in their sediments (Vera-Gargallo et al., 2019), little is known about the effect of salt marsh ecological succession stage on their diversity and community assemblage. While most studies focus on a single explanatory variable, it is little known how different environmental variables interplay to generate the observed diversity. Unveiling this prokaryote diversity can have valuable implications to evaluate ecological theories on community assemblage or highlight the role of microbiome in nutrient cycling and understand their implications in ecological succession of salt marshes. The goal of this study was to characterize the alpha and beta diversity of prokaryotes in salt marshes from Río Piedras, Southern Spain. We evaluated their seasonal variations and structure associated with the halophyte community corresponding to different stages in the ecological succession by sequencing a partial region from the 16S rRNA gene. We hypothesize the bacterial community will be mostly determined by marsh zonation, being further modulated by the presence of specific halophytes and the changing environmental conditions across seasons.

# Material and methods

## Study site

The study was carried out in the salt marshes of Río Piedras, on the Atlantic coast of the Iberian Peninsula (Huelva, SW Spain) at 37.211 N 7.174 W (Figure 1). The Mediterranean climate in this region is characterized by a strong seasonality, with dry and hot summers, and mild wet winters. These salt marshes constitute a natural mesotidal enclave of fluvial-tidal modeling and coastal feeding, covering about 2,595 ha. It presents a high ecological value, being protected as a ‘Natural Area’ since 1989 (Law 2/1989, of July 18; BOJA N. 60, of 07/27/1989). It has a mean tidal range of 2.10 m. Relative to Spanish hydrographic zero, mean high water neap is +2.44 m; mean high water +2.91 m; mean high water springs +3.37 m; and highest astronomical tides +3.71 m (Instituto Hidrográfico de la Marina, Spain).

Specifically, the study was carried out in a gently sloping silty-clay plain with tidal influence of approximately 1.2 ha at the southwest area of the Piedras Marshes. This area is colonized by dominant perennial halophytic species patches, heterogeneously distributed along the river channel, and accompanied by other annual halophytic species. The principal halophytes are distributed throughout the tidal range as follows: the halophyte *Spartina maritima* (Curtis) Fernald (Bortolus et al., 2019) is a pioneer due to its high tolerance to flooded soils with low redox, forming patches of tillers in low marsh areas. These patches accumulate sediments, forming tussocks that accrue to higher elevations, making them less exposed to inundation. The subsequent increase in redox potential facilitates the colonization of a series of shrubby species, being the perennial halophytic shrub *Atriplex portulacoides* (L.) Aellen a dominant species of salt marsh areas at a midpoint in the succession (Caçador et al., 2013). Despite its dominance, several middle saltmarsh areas have been extensively invaded in the last decades by the halophytic cordgrass *S. densiflora* Brongn., which has demonstrated potential to compete with this native middle salt marsh dominant species, and develops monospecific communities in the middle marsh and even in the least tidal influenced upper marsh areas (Mateos-Naranjo & Redondo-Gómez, 2016). The greater topographic elevation in the tidal range, where tidal inundations are occasional and combined with seasonal hypersalinity, is characterized by shrub formations of the perennial halophyte *Arthrocaulon macrostachyum* (Moric.) Piirainen & G. Kadereit (Redondo-Gómez et al., 2010; VV.AA, 2009) accompanied by other annual halophytic species. Among those, the annual halophyte *Salicornia ramosissima* J. Woods creates compact formations during spring and summer. This species tolerates a wide range of salinity and some immersion (Davy, Bishop, & Costa, 2001; Pérez-Romero et al., 2019).

## Sampling protocol

For sample collection, permanent monospecific plots of the perennial species *A. macrostachyum*, *A. portulacoides, S. densiflora*, *S. maritima* and the annual *S. ramosissima* with at least 50 m2 were established, considering a minimum separation between them of 20 m. In each plot, we collected soil samples considering two different factors: rhizosphere/bulk sediment, and season throughout a year (spring, May 2017; summer, July 2017; autumn, October 2017; winter, January 2018) for all perennial species. The annual *S. ramosissima*, was only collected in spring and summer, coinciding with its appearance in the field. A total of 36 soil samples were collected (Table 1). In each plot, we randomly selected three healthy and completely developed adult plants from the center of the species patch. Soil samples from the rhizosphere were collected by shaking its roots. Bulk soil samples (15 cm depth) were collected in un-vegetated sites in three points and at least one meter from the vegetated patches. Each set of three point samples with the same conditions were pooled in a sterile 100 ml plastic bottle (Panelli et al., 2017), preserved at 4 °C and immediately transported to the laboratory. A fraction of each sample was dried in a hot air oven at 60 °C during 48 h and homogenized (Camacho-Sanchez et al., 2020).

## DNA extraction and sequencing

DNA was extracted from 0.25 g of oven-dried soil with PowerSoil™ DNA Isolation Kit following manufacturer's instructions. Library preparation and sequencing was externalized to AllGenetics & Biology SL ([www.allgenetics.eu](http://www.allgenetics.eu); La Coruña, Spain). For library preparation, a fragment of the bacterial 16S rRNA gene which includes the hypervariable region V3-V4 was amplified using the primers Bakt\_341F (5’ CCT ACG GGN GGC WGC AG 3’) and Bakt\_805R (5’ GAC TAC HVG GGT ATC TAA TCC 3’) (Herlemann et al., 2011), with Illumina sequencing primer attached to their 5’ ends. PCRs were carried out in a final volume of 25 μL, containing 2.5 μL of template DNA, 0.5 μM of the primers, 12.5 μL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure water up to 25 μL. The reaction mixture was incubated with an initial denaturation at 95 ºC for 5 min, followed by 25 cycles of 95 ºC for 30 s, 50 ºC for 30 s, 72 ºC for 30 s, and a final extension step at 72 ºC for 10 minutes. The index sequences were attached in a second PCR round with identical conditions but only 5 cycles and at 60 ºC of annealing temperature (Meyer & Kircher, 2010). A negative control with no DNA was included during library preparation (BPCR). The libraries were run on a 2 % agarose gel stained with GreenSafe (NZYTech), and library size verified under UV light. Libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), following manufacturer instructions. Then, they were pooled in equimolar amounts according to the quantification data provided by the Qubit dsDNA HS Assay (Thermo Fisher Scientific). The pool was sequenced in a MiSeq PE300 run (Illumina).

## Determination of 16S rRNA sequence variants

Unique variants, or amplicon sequence variants (ASVs), of the 16S reads were determined with *DADA2* (Callahan et al., 2016). Briefly, raw FASTQ files were quality filtered and trimmed. ASV determination was carried on forward and reverse reads with *dada* function, followed by merging paired-end reads and ending with removal of chimeric sequences (details in *github.com/csmiguel/marsh\_metabarcoding*).

We used the function *IdTaxa* (Murali et al., 2018) from the *DECIPHER* 2.18.1 package (Wright, 2016), to assign taxonomy of the ASVs using as reference the Silva 138 prokaryotic small subunit rRNA taxonomic training set for organismal classification (Quast et al., 2013). All analyses were carried in R4.0.5 (R Core Team, 2021).

ASVs with assigned taxonomy and metadata (sample characteristics) were stored in a *phyloseq* object in R using the *phyloseq* 1.34.0 package (McMurdie & Holmes, 2013). ASVs with no phylum, or assigned to Chloroplast or Mitochondria were discarded. Three ASVs present in the blank (Supplementary Material SM1) were filtered from all samples using the *decontam* 1.10.0 (Davis et al., 2018) package in R. After inspecting the distribution of prevalence and abundance of ASVs across samples and replicas, we applied a filter to remove low-abundant ASVs. We only retained ASVs meeting three conditions: (1) product of their non-zero abundances across samples above 185, (2) present in 3 or more samples, and (3) with an average proportion of reads across samples above 5e-5. Replicas and the blank were removed from the *phyloseq* object for downstream analysis.

Sequences corresponding to each ASV were aligned using *DECIPHER::AlignSeqs* and written into PHYLIP format for tree inference. A maximum likelihood tree was reconstructed with RAxML 8.2.9 (Stamatakis, 2014).

## Alpha diversity

Alpha diversity was computed over rarefied counts of ASV abundances using *rarefy\_even\_depth* and *estimate\_richness* functions from *phyloseq* in R. Bootstrapped values of Shannon H’ diversity were estimated by resampling a fraction of the counts. We assessed the effects of season (*lme4::lmer*(*Shannon ~ season + (1|species\_plot)*) and species plot (*lme4::lmer(Shannon ~ species\_plot \* rhizosphere\_or\_bulk + (1|season)*) on Shannon H’ using linear models with mixed effects with *lme4::lmer* (Bates et al., 2015) in R. The significance of the effects from the independent variables were tested with Wald tests using *sjPlot* (Lüdecke, 2018). We evaluated the homogeneity of variance of Shannon H’ for each plot and soil type across seasons with a Levene’s test in R using *car::leveneTest* function.

## Beta diversity

Beta diversity was explored with a Multidimensional Scaling (MDS) and a hierarchical clustering from weighted UniFrac distances (Hamady et al., 2010) on the log-transformed ASV counts using *phyloseq*. The variance of this distance matrix was partitioned amongst the different explanatory variables (season, rhizosphere, species plot), and 9999 permutations were run to assess their statistical significance using the function *vegan::adonis* (Oksanen et al., 2020).

Differential abundances of taxa between levels from each variable (rhizosphere, season, species plot) were estimated with *DESeq2* (Love et al., 2014) in R. ASVs were agglomerated by family, as a good trade-off to retain taxonomic groups with shared functional diversity with the lesser sacrifice in success of taxonomic assignment: 58% of ASVs are successfully assigned to a family. The resulting families with differential abundances (Benjamini and Hochberg corrected *p-values* < 0.05) were explored with a clustered heatmap using *pheatmap* 1.0.12 (Kolde, 2019), and Venn diagrams.

## Phylogenetic filtering of diversity

The environmental conditions can impose a filter on the taxonomic groups that stablish in a location. This deterministic process can be assessed by comparing the phylogenetic distance between taxa in each community. Multiple studies have contributed to develop the mathematical methods to study phylogenetic turnover in microbial communities (Stegen et al., 2012; Stegen et al., 2013; Stegen et al., 2015). The metric β-mean nearest taxon distance (βMNTD) quantifies the phylogenetic distance between each ASV in one community and its closest relative in a second community. Lower βMNTD than expected indicates phylogenetic clustering which can be interpreted as selection by constraining environmental conditions. This metric can be compared against permutated values of βMNTD by permutating labels (ASVs) and recalculating βMNTD. The significancy of this effect can be approached by computing the β-nearest taxon index (βNTI), which is the number of standard deviations (from permutations) that has the difference between the observed βMNTD and the average permutated βMNTD. Absolute βNTI values above 2 are indicative of selection. We used these metrics to evaluate phylogenetic filtering on the prokaryotic community in the rhizosphere from its surrounding bulk soil and in bulk soil across successive seasons. We used *picante::comdistnt* (Kembel et al., 2010) to compute βMNTD and custom R script to run 999 permutations and pairwise contrasts.

## Prediction of potential functions

The software FAPROTAX with its curated database was used to determine metabolic functional groups in the bacteria community between conditions. This database is optimized to be used with the SILVA taxonomy and it includes more than 80 metabolic functions over 4600 taxa (Louca et al., 2016).

# Results

A total of 1,790,778 paired-end sequences were generated for the 36 samples, averaging 49,744 reads per sample (minimum = 25,032; maximum = 74,339; standard deviation, *sd* = 11,429) (Table 1).

## Alpha diversity

Taken altogether, 4,898 ASVs were identified, ranging from 634 to 2,625 (*sd* = 419) per sample (Table 1). All filtered variants had a phylum assigned. The proportion of ASVs successfully assigned to a given taxonomic rank decreased from 96% matching a class, 69% to an order, 58% to a family and 38% to a genus (OTU table in SM2). Rarefaction curves showed an increase of the ASVs with greater sequencing depth, up to about 10,000 reads, after which the slope gradually decreased for most samples (SM3). A total of 31 bacteria phyla (plus eight candidate phyla) were present in the data, with *Proteobacteria*, *Bacteroidota* and *Desulfobacterota*, accounting for around 75% of the abundance in most samples (Figure 2). The dominance of certain phyla changed between sampling conditions (i.e. season and zonation). Noticeably, *Firmicutes* had a peak in winter, *Desulfobacterota* were most abundant in the low marsh plots of *S. maritima*, and *Campilobacterota* were more abundant in non-rhizospheric sediments. The diversity within archaea was restricted to 26 ASVs (0.12 % of overall relative abundance). All archaea corresponded to halophilic taxa from multiple genera in the order *Halobactorales*, phylum *Holobacterota*.

Shannon diversity varied across seasons (*p* = 0.01). It was greatest in spring (*H’* = 6.39) and decreased in the following seasons to a minimum in winter (*H’* = 5.84) (Figure 3; SM4). For *S. maritima*, Shannon H’ was 0.75 points greater in the rhizosphere compared to bulk soil (*pwald-test* = 0.02), but this pattern was not consistent for other species (*pwald-test* = 0.23-.93). Variance of alpha diversity was unequal across seasons depending on the species (Levene’s test: *F9* = 3.0, *p* = 0.01), with a more stable diversity in the plots of *S. maritima* (*sdS.maritima* = 0.11; sdother\_halophytes = 0.15-0.79).

## Beta diversity

In the ordination analysis, Dimensions 1 and 2 explained 44 and 21% of the variance, respectively (Figure 4; SM5). Beta diversity was low in the plots of *S. maritima* while samples from *A. macrostachyum* were very dissimilar between seasons. Dimension 1 explained much of the variation attributed to zonation (species plots), while Dimension 2 to rhizospheric versus bulk soil communities. The dendrogram from hierarchical clustering backed this effect of species the clustering (SM6). Permutation tests to assess the partition of the variance among explanatory conditions showed that halophyte plots (zonation) explained most of the beta diversity (*R2* = 0.50; *p* < 0.001), whereas rhizosphere (*R2* = 0.07; *p* < 0.001) and season (*R2* = 0.14; *p* < 0.001) explained less of the variation, and only when controlling for species (Table 2).

The phylogenetic turnover in pairwise samples using the βMNTD yielded in most cases lower values of observed βMNTD versus permuted βMNTD, which indicates selection on the bacterial community in the rhizosphere of all the halophytes studied in at least one of the seasons (SM7). For *Spartina maritima*, the pressure of this selection was consistent across all seasons, whereas for the other species the effect varied between seasons. Regarding the selection across successive seasons, pairwise contrasts of bulk soil across successive seasons also revealed lower values of observed βMNTD versus permuted βMNTD. This indicates phylogenetic filtering at all stages of the succession, although this effect was little or even non-significant in the low marsh *Spartina maritima* plots (SM7).

## Differential abundances

ASVs were aggregated into the 229 families identified from Bacteria and Archaea for the comparison of differential abundances between conditions. Around ⅔ of the families (n = 148) changed their abundances associated with a specific halophyte plot (Table 3). Particularly, the plots from low marsh *S. maritima* were the most differentiated with respect to the others. Multiple families from *Desulfobacterota* were very abundant in *S. maritima* compared with the other halophytes plots, while many from *Actinobacterota* and *Proteobacteria* were less frequent (SM8 and SM9.A).

The rhizosphere exerted a strong effect on the number of families with differential distribution in the plots of *S. maritima* (n = 40), *A. portulacoides* (n = 28) and *S. densiflora* (n = 18), but it had no effect on *A. macrostachyum* (n = 0) nor *S. ramosissima* (n = 0) (Table 3; SM8 and SM9.B). However, the taxa associated with the rhizosphere were species-specific (Figure 5).

Finally, there was a seasonal effect on families' abundances. Thereby, warmer seasons had 17-20 families overrepresented compared to winter. Warmer seasons differed little between them, and only two families differed at most (Table 3). Particularly, some groups from *Firmicutes* and *Actinobacteriota* were more abundant in winter, whereas multiple groups of *Bacteroidota* and *Proteobacteria* were more abundant in the warmer seasons (SM8 and SM9.C).

## Prediction of functions

In the FAPROTAX analysis, 616 ASVs were assigned to at least one group. Metabolic functions related to sulfur cycling were especially prevalent in soils from low marsh compared with the rest of locations (SM10).

# Discussion

We studied diversity of bacteria in marsh soils from Southern Spain, and show environmental factors and halophytic vegetation interact to structure communities. Thus, the halophyte patches characteristic to the different stages in the chronosequence were strong determinants of bacteria diversity: 50% of beta diversity was explained by this factor, while rhizosphere and season accounted for the 7% and 14%, respectively (Table 2).

Communities from soils in the initial stages in the succession, associated to *S. maritima*, were highly differentiated compared to those from later stages (Table 3; SM9.A). The mechanisms causing this structure across the chronosequence are debated, and could be related to changes in salinity, pH, redox potential, deterministic/stochastic processes or phylogenetic filtering (Dini-Andreote et al., 2014; Hollister et al., 2010; Tripathi et al., 2018; Wang et al., 2016; Zhang et al., 2020; Zhao et al., 2018). Given the functional groups we found across patches, redox potential seems crucial in bacterial assemblages. Remarkably, we report an excess of *Desulfobacterota* in the lower parts of the marsh. Many of these strictly anaerobic bacteria reduce sulfur compounds to sulphide (Waite et al., 2020), which coincides with a well-described multiplication of sulphide concentrations in these low marshes (Castillo et al., 2000). This high sulfur cycling activity in the low marsh was also detected in the FAPROTAX analysis (SM10). The inundation of the low marsh reduces oxygen availability in the soil, with a consequent decrease in redox potential in these marshes (Castellanos et al., 1994; Castillo et al., 2021). Redox potential together with pH limit nutrients bioavailability in the soil and which compounds can be used as electron acceptors for metabolic pathways of microorganisms, imposing phylogenetic constrains on microbial diversity (Husson, 2013; Tokarz & Urban, 2015; Zheng et al., 2017). At the other end, the high seasonal salinity and dryness, and higher redox potential also seemed to affect the community associated to the high marsh patches of *A. macrostachyum* (Camacho-Sanchez et al., 2020). Therein, we report high abundance of the halophilic archaea *Halobacterota*. These generally neutrophilic or alkaliphilic extremophiles have also been described in salty habitats in the nearby Odiel saltmarshes (Vera-Gargallo & Ventosa, 2018). Most of the bacterial taxa associated to this high marsh were aerobic mesophilic or thermophilic, and rather alkalophilic, many of which also described from marine or arid habitats, such as *Entotheonellaceae*, *Blastocatellaceae*, *Geodermatophilaceae*, *Idiomarina* or *Solirubrobacteracea* (Whitman, 2015) (SM9.A).

The effect of the rhizosphere on its surrounding bacterial community was species-specific: while *S. maritima*, *S. densiflora*, and *A. portulacoides*, showed multiple taxa positive or negatively associated to their rhizosphere, *S. ramosissima* or the high marsh species *A. macrostachyum* did not alter their rhizospheric microbiome (Table 3), as previously described (Camacho-Sanchez et al., 2020). Species-specific interactions are spread across halophytes (Bagwell et al., 2001; Burke et al., 2002; Cunha et al., 2005; Ribeiro et al., 2013; Wang et al., 2007), although the causes of these associations have not been investigated.

Our data revealed the low marsh *S. maritima* exerted the strongest effect on its rhizospheric bacteria compared to the other four halophytes studied (Table 4; SM9.A). This is cohesive with the reported selection associated to the rhizosphere of all halophytes, being *S. maritima* the only species filtering the surrounding bacterial community in the four seasons studied. At the same time, the bulk soil in this low marsh experienced the lesser overall phylogenetic turnover across successive seasons (SM7), probably because of the homogenizing high inundation regime. The low-marsh *S. maritima* tolerates the largest inundation regimes, even beyond its congeneric and sympatric *S. densiflora* (Castillo et al., 2000). Its rhizospheric community was more diverse in term of alpha diversity (Figure 3) and in taxa related to sulfur cycling: increased abundances of sulfur reducers *Desulfovibrionaceae* and *Thermodesulfovibrionaceae*, and nitrogen fixators from *Xanthobacteraceae*, and lesser abundance of the sulfur oxidizers *Sulfurovaceae* and *Sulfurimonadaceae* compared to adjacent bulk soil (SM9.A) (Waite et al., 2020; Whitman, 2015). Other studies report *S. maritima* to boost bacterial-mediated nitrogen fixation and sulfur reduction in its rhizosphere through sugary exudates (Nielsen et al., 2001). This increased sulfur-reducing activity rather indicates an accelerated sulfur oxidation-reduction cycling (Zheng et al., 2017), which was also supported by the metabolic function analysis (SM10). Research on its congeneric marsh ecophysiological model, *S. alterniflora*, also describe *Desulfobacteriaceae* intimate related to their period of vegetative growth (Rooney-Varga et al., 1997), increased activity of sulfide oxidation in their roots (Lee et al., 1999), and transport of oxygen through the aerenchyma to the roots (Teal & Kanwisher, 1966), which may promote bacteria oxidative processes. This increased intensity of bacterial interactions with the plant rhizosphere could be related to the harsh redox conditions in the low marsh and supports a potential role of the rhizospheric bacteria in such tolerance to low redox potential. Indeed, redox potential decrease to minimum of around -300 mV in the areas of *S. maritima* in low marsh (Castellanos et al., 1994; Castillo et al., 2021). Plants under such conditions suffer from physiological stress (Husson, 2013) and sulfide toxicity (Maricle et al., 2006), and they could interact with their rhizosphere microbiome to ameliorate pH and redox potential around their roots (Husson, 2013).

We found little seasonal variation in bacterial communities in sampling plots from low marsh compared with those from high marsh (Figure 4). This pattern could be explained by the continuous influx of nutrients and bacteria from the sea to homogenize the bacterial assemblages in low marsh (Wang et al. 2016), even though under these conditions some differential abundances associated to the seasonal vegetative states of the plant are expected to be detected (Rooney-Varga et al., 1997). The changes in salinity and humidity characteristic of Mediterranean seasonality, with dry summers and wet mild winters, could be partially responsible for the variation observed in the halophyte plots less exposed to inundation, as in *A. macrostachyum* (Camacho-Sanchez et al., 2020). Indeed, some of the overrepresented taxa in the drier seasons corresponded to aerobic halophilic bacteria such as *Pseudoalteromonadaceae* or *Saccharospirillaceae*.

# Conclusions

Bacterial community assemblage in salt marsh offers a rich field to study ecological assembly rules and described a major unknown biodiversity. Previous research had only focused on a single cause (either, pH, halophytes, chronosequence, salinity) to explain bacterial diversity in salt marshes. This study provides a novel overview of the relative importance of key factors governing bacterial diversity in a salt marsh from southern Spain: ecological stages in the succession were the main factor driving diversity, being further modulated by halophytes and seasonality. This better understanding of the bacterial diversity and structuring factors in saltmarsh soils permits to assess the validity of ecological theory (mostly developed in plants ecology) to the microbiological compartment, and further identifies functional groups which can have important ecological roles in the environment related to nutrient cycling in coastal systems or ecological succession, for instance. Thus, many of the bacterial taxa associated to low marsh (early chronosequence) were associated to the dominant halophyte *S. maritima*, suggesting an important role in the onset of ecological succession in salt marshes. However future manipulative experimental studies should be necessary to determine the relative importance of specific environmental variables along the chronosequence on structuring bacterial diversity.

# Data availability

Genetic data was deposited in GenBank under the BioProject PRJNA765018. Raw sequences were deposited in the GenBank Sequence Read Archive with accessions SRA21540851-SRA21540891, and assigned to BioSamples SAMN21540851-SAMN21540891. ASVs were deposited in the nucleotide database with accession KFLF00000000. Code to replicate the analyses and outputs is available in the GitHub repository github.com/csmiguel/marsh\_metabarcoding. A stable release is available at Zenodo doi:xxx.

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# Supplementary Information

Supplementary Material SM1: table with ASVs present in the blank and removed for downstream analysis.

Supplementary Material SM2: OTU table. Table with filtered ASV counts per sample.

Supplementary Material SM3: rarefaction curves.

Supplementary Material SM4: results from linear mixed models evaluating the effect of season and halophytes (zonation and rhizosphere) on alpha diversity.

Supplementary Material SM5: Beta diversity. Dimensions 3 and 4 from a MDS using weighted UniFrac distances.

Supplementary Material SM6: dendrogram clustering samples according to their weighted UniFrac distances.

Supplementary Material SM7: Effect of the rhizosphere (A) and season (B) on selection of community assembly on the marsh soils studied. In the x-axis we have represented the difference between the observed β-mean nearest taxon distance (βMNTD) in pairwise comparisons and the averaged permuted βMNTD. The dispersion bars correspond to the standard deviation of permuted βMNTD. Each data point has been colored red if βMNTD was significant.

Supplementary Material SM8: CSV with results from the DeSeq2 analysis revealing the log2-fold change in abundances of families for multiple contrasts and their associated p-values.

Supplementary Material SM9: families with significant differential relative abundances (adjusted p-values) for any of the conditions for each environmental factor: species plot (A), rhizosphere (B) and season (C).

Supplementary Material SM10: results from the FAPROTAX analysis describing the relative abundance of the metabolic functions associated to the bacterial community in each soil. The light/dark paired colors correspond to non-rhizospheric/rhizospheric soil in each halophyte plot.

# References

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# Tables

Table 1. Sample characteristics, sequencing depth (number of reads), and number of amplicon sequence variants (ASVs).

Table 2. PERMANOVA tests. Summary table from multiple test showing the partition of beta diversity (weighted UniFrac distances) among the factors evaluated: species plot, rhizosphere, and season (*Df*, degrees of freedom; *F*, Fisher’s F analogue; *R2*, effect size, sum of squares from factor divided by total sum of squares; *P(>F)*, probability of observed F being larger than permuted Fs; *strata*, groups within which to constrain permutations).

Table 3. Differential abundances between conditions. Number of families from a total of 229 that had different abundances (*p-adjusted* < 0.05 after results from DESeq2) between conditions for each contrast (in parenthesis for each factor). The total number is broken down into families with higher (+) or lower (-) abundances of level 1 respect level 2.

# Figures

Figure 1: sampling location (data from protected areas downloaded from the *Ministerio para la Transición Ecológica y el Reto Demográfico* and 0.5 m resolution 2013 orthophotos from the *Instituto de Estadística y Cartografía de Andalucía*).

Figure 2: relative abundance of phyla across conditions.

Figure 3: alpha diversity across conditions. Each point is a boxplot from the first to the third quartiles from 100 bootstrapped values.

Figure 4: beta diversity. Dimensions 1 and 2 from a MDS using weighted UniFrac distances.

Figure 5: Venn diagram showing shared families whose abundance were positively (top) or negatively (bottom) associated with the rhizosphere respect to bulk soil.