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Mediterranean seasonality and the halophyte *Arthrocnemum macrostachyum* determine the bacterial community in salt marsh soils in Southwest Spain

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ABSTRACT

Perennial halophytes dominate coastal ecosystems, but little is known about how the biotic and abiotic factors alter their microbial community. This study compares the bacterial diversity associated with the rhizosphere, with respect to adjacent soil, for the halophyte *Arthrocnemum macrostachyum* in high salt marshes in Southern Spain, in dry and wet seasons (July and October, respectively) using metagenomics coupled with High-Throughput Sequencing. The read-based analysis revealed that seven phyla (*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Chloroflexi*, *Firmicutes* and *Acidobacteria*) accounted for the 90% of the diversity and 94% of the abundance and that, seasonal and spatial variations in soil salinity were the main determinants of the bacterial community diversity in these marshes, compared to the apparent no effect of the plant. However, at *A. macrostachyum* flowering stage, the bacterial community associated with the rhizosphere was comparatively more diverse than that inhabiting adjacent bulk soil, suggesting a more intensive plant-microbe interaction during this period.

1. Introduction

The rhizosphere bacterial community may have important interactions with plants, mainly involved in nutrient mineralization or competition for nutrients (Kaye and Hart, 1997; Van Der Heijden et al., 2008). It can also provide protection against pathogenic fungi (Heydari and Pessarakli, 2010), promote root growth or improve tolerance to toxic compounds (Morgan et al., 2005). Besides, in soils under harsh abiotic conditions such as high salinity, these microorganisms can ameliorate salt deleterious effects by phytohormone stimulation or nutrient mobilization (Mayak et al., 2004; Egamberdieva et al., 2008; Cassán et al., 2009; Sgroy et al., 2009; Yuan et al., 2016). These so called plant stress homeostasis-regulating bacteria (PSHB) can therefore have important applications for phytoremediation (Oliveira et al., 2014) or crop growth in saline soils (Mayak et al., 2004; Egamberdieva et al., 2008; Sáenz-Mata et al., 2016; Etesami and Beattie, 2018). In turn, plants can produce exudates and favor given groups of microorganisms in their rhizosphere (Morgan et al., 2005; Haichar et al., 2008). In extreme environments, plants can change the microclimatic conditions under their canopy, modifying moisture and soil nutrients (Pugnaire et al., 2011; Jing et al., 2019), which also affects the associated microbial communities (Wardle et al., 2004; Hortal et al., 2013; Iwaoka et al., 2018). In addition to the effect of the plant, the physicochemical properties of the soil can modulate the microbial assemblages (Mora-Ruíz et al., 2016; Lopez et al., 2019). The relative strengths of the effects of plant (biotic factors) or soil (abiotic factors) in these assemblages is highly variable in different systems (Zak et al., 2003; Singh et al., 2009; Dean et al., 2014; Matthews et al., 2019).

Salt marshes are biodiverse coastal ecosystems with tidal influence from sea water which are well represented in Southwestern Spain, along the Gulf of Cádiz. One of the dominant halophytes in these marshes is *Arthrocnemum macrostachyum* (Moric) C. Koch (Rubio-Casal et al., 2001), a C3 shrub from the family *Amaranthaceae* (Valdés et al., 1987). It is a primary colonizer of the salt pans, having a broad optimum of soil salinity from 171 to 510 mM NaCl (Redondo-Gómez et al., 2010). The family *Amaranthaceae*, has been traditionally described to interact little with soil microorganisms (Gerdemann, 1968; Hirrel et al., 1978), so that, the potential of mutualistic interactions of beneficial soil microorganism with *A. macrostachyum* have been, for a long time, overlooked. However, recent studies already unveiled that *A. macrostachyum* can establish associations with endophytic fungi and bacteria (Maciá-Vicente et al., 2008; Sonjak et al., 2009; Becerra et al., 2016)

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and, that, in common with other halophytes, its rhizosphere can have a distinctive bacteria community (Oliveira et al., 2014; Mora-Ruíz et al., 2016; Yamamoto et al., 2018), from which several bacteria species have recently been described (Camacho et al., 2016a, 2016b, 2017; Lucena et al., 2017). All this points to the possible existence of physiological mechanisms in these halophytes that could enable them to establish mutualism with their microbiome for surviving in harsh environments with soil contaminants (Oliveira et al., 2014; Navarro-Torre et al., 2016). Supporting this idea, several endophytic bacteria have been identified in *A. macrostachyum* with a potential role in tolerance to high salinity (Mora-Ruíz et al., 2016; Navarro-Torre et al., 2017, 2018; Mora-Ruíz et al., 2018).

There are no studies taking account the spatio-temporal changes that occurs in the microbial communities of halophytes, and about how the plant influences them. Our goals were (1) to study the relative effects of abiotic (seasonal and spatial changes in soil conditions) versus biotic factors (the effect of the halophyte *A. macrostachyum*) in structuring bacterial diversity in the marsh soil, and (2) to identify bacterial strains with potential interactions with *A. macrostachyum*.

2. Materials and methods

2.1. Study site and sample collection

Soil from high salt marshes in Lebrija, Seville, Spain (36.907 N 6.207 W) was sampled considering three different variables: plot location in the marsh, rhizosphere/bulk soil and season. The landscape is crossed by channels to allow water drainage and soil desalination for agriculture, so that two sampling plots were defined: high salt marsh and slope of a drainage channel (referred to as "channel" hereafter) (Fig. 1). In each plot, soil from the rhizosphere of *A. macrostachyum* and from adjacent bulk soil in two seasons (July, dry season; October, wet season) were sampled. Soil from the rhizosphere of *A. macrostachyum* was sampled by shaking few roots. Plants were marked so that the samples from October were taken from the same plants than those from July. Samples from bulk soil were collected at ~15 cm depth.

In total, 8 unique combinations of conditions were considered (3 variables, each with 2 levels) (Table 1). For each unique condition, 4 soil replicates were sampled in different points within the same plot and pooled in sterile 100 ml plastic bottles (Panelli et al., 2017). Samples were preserved at 4 °C and transported to the laboratory where they were manually disaggregated with a mortar and homogenized. Following the kit manufacturer's instructions, for a more efficient DNA extraction, a fraction of each sample was dried in a hot air oven at 60 °C during 48 h, while the remaining one was stored at −80 °C for further analysis. Dried samples were crushed and sifted through a sieve of a mesh size of 3 mm.

The salinity (electrical conductivity, EC) from the homogenized dried soil samples was measured in the lab. Samples were saturated with distilled water, adding 2× volume of water. The EC was measured after 30 min with an electrical conductivity meter (handylab LF11, SCHOTT Instruments, Germany). The salinity was highest in soil from the desalination channel in July (12–44 mS/cm) while it was around 10 times lower in the high salt marsh in July (1.1–3.3 mS/cm) (Table 1).

Table 1
Samples (in parenthesis) and their salinity (mS/cm) in rhizosphere (R) and bulk soil (B) (sampled at 15 cm depth).

	July	October
High salt marsh	R: 1.1[a] (Jul3) B: 1.4[a] (Jul4)	R: 3.2 (Oct3) B: 3.3 (Oct4)
Desalination channel	R: 22.0 (Jul1) B: 44.0 (Jul2)	R: 12.0 (Oct1) B: 12.8 (Oct2)

[a] A salt crust was observed on the surface of this soil.

2.2. DNA extraction and sequencing

DNA was extracted from 0.25 g of oven-dried soil with PowerSoil™ DNA Isolation Kit following manufacturer's instructions. For each sample, the products of 3 independent DNA extractions were pooled together. DNA concentrations from the extracts ranged from 13 to 36 ng/µl in volumes of 100 µl per DNA extraction.

We targeted V3 and V4 variable regions of the 16S rRNA gene using primers F341/R785 (Klindworth et al., 2013) with P5/P7 Illumina tails attached to their 5′ end (Table 2). This region was amplified in a 1st PCR with 1.5 U of Velocity (Bioline), a high-fidelity DNA Taq polymerase (Brandariz-Fontes et al., 2015), in PCR volumes of 25 µl, using ~30 ng of template, 1× of Hi-Fi Reaction Buffer, 1 mM of dNTPs and 0.2 µM of each primer. The PCR program started with the activation of the enzyme at 98 °C for 2 min, followed by 25 cycles of 98 °C for 30 s, 55 °C for 30 s and 72 °C for 15 s, with a final extension at 72 °C for 10 min. Two randomly chosen samples were replicated in independent indexing PCRs to control for potential methodological biases. We included a negative control. The PCR products were purified with FavorPrep GEL/PCR Purification Kit (FavorGen) and eluted in 40 µl of TE. Illumina adapters and sample-unique barcodes were ligated to the amplicons in a 2nd PCR in reaction volumes of 25 µl using 4 µl of purified template, 1 U of Velocity, 1× of Hi-Fi Reaction Buffer, 1 mM of dNTPs and 1.2–1.6 µM of each indexing oligonucleotide. We ran the PCRs for 10–12 cycles using the same conditions as in the 1st PCR. The products from the 2nd PCR were purified again with FavorPrep GEL/PCR Purification Kit and total DNA was quantified in Nanodrop Spectrophotometer ND-1000. These concentrations were used to pool samples in equimolarity. Libraries were further cleaned with SPRI beads (Rohland and Reich, 2012) and sequenced in Illumina MiSeq PE 300 at

Fig. 1. Study area. Soil was sampled from high salt marshes dominated by *Arthrocnemum macrostachyum* (left) and the channel (right) in Southern Spain.

Table 2
Primers used for library preparation.

16s primers with Illumina tails (1st PCR) [**tail**-PRIMER]:	
Forward	**acactctttccctacacgacgctcttccgatct**CCTACGGGNGGCWGCAG
Reverse	**gtgactggagttcagacgtgtgctcttccgatct**GACTACHVGGGTATCTAATCC
Indexing oligos (2nd PCR):	
P5	AATGATACGGCGACCACCGAGATCTACAC-Index-ACACTCTTTCCCTACACGACGCTCTT
P7	CAAGCAGAAGACGGCATACGAGAT-Index-GTGACTGGAGTTCAGACGTGTGCTCTTCCG

the Genetics Resources Core Facility at John Hopkins University.

2.3. Sequence processing

Paired reads which had the expected flanking 16S primers in the forward (R1) and reverse (R2) reads with CUTADAPT 1.8.3 (Martin, 2011) were filtered in paired-end mode. Details on the bioinformatics workflow are available at https://github.com/csmiguel/metagenomics_halophytes. Then, Amplicon Sequence Variants (ASV) were determined with DADA2 v1.8 (Callahan et al., 2016a) following the strategy described by Callahan et al. (2016b), in R 3.4.3 (R Core Team, 2017). This strategy aims to identify all unique real amplicon sequence variants (ASVs) in the dataset. The aforementioned approach allows interpretations on microbial diversity which are more independent from the data, increasing the reproducibility while yielding an increased resolution in alpha diversity (Amir et al., 2017; Callahan et al., 2017). After inspecting the quality plots for R1 and R2 reads, the reads were truncated to a length of 275 nucleotides (nt) (R1) and 180 nt (R2), and low-quality reads were trimmed. Then, the independent error matrices for R1 and R2 reads from a subset of 10,000 random reads from each sample were determined. These error matrices learnt from the data and the de-replicated sequences were used to determine unique sequence variants with the DADA2 algorithm for R1 and R2 independently (Callahan et al., 2016a). After that, R1 and R2 ASVs were merged with *dada2::mergePairs* and bimeras were removed with *dada2::removeBimeraDenovo*. A taxonomy was assigned to each ASV using the SILVA v128 database to the lowest possible taxonomic rank, down to species level (Quast et al., 2013).

Phyloseq 1.22.3 (McMurdie and Holmes, 2013) was used for subsequent metacommunity analysis in R. ASVs with no Phylum, or assigned to Chloroplast or Mitochondria were discarded. After inspecting the distribution of their prevalence and abundance across samples and replicas, a filter to remove low-abundant ASVs was applied. Consequently, all variants for which the product of their abundances across samples was below 100, $\prod_{Abundance_j>0}^{1\leq j\leq n} Abundance_j < 100$ were removed. The negative control contained very few reads from some of the most frequent variants across all samples and was removed for downstream analysis.

A phylogenetic tree from ASVs with PASTA (Mirarab et al., 2015) an iterative aligner-tree builder was reconstructed and added to the *phyloseq* object.

2.4. Diversity analysis

We estimated alpha diversity as the total observed variants and the Shannon Index, H′, for each sample with *phyloseq::estimate_richness* in R. This alpha diversity showed no correlation with sequencing depth. Tables with relative per-sample abundance of ASVs for each taxonomic level were produced (S1).

We compared the bacterial communities (beta diversity) between samples with a Multidimensional Scaling (MDS) of the data using weighted UniFrac distances (Hamady et al., 2010) with *phyloseq::ordinate*, in R, on a log-transformed matrix of abundances.

2.5. Differential abundances

To determine ASVs with differential abundances between conditions (season, sampling site and rhizosphere/bulk soil) we used DESeq2, an R package initially developed for studying differential RNA expression (Love et al., 2014), but that can also be used in environmental metagenomics to estimate differences in OTU abundances (McMurdie and Holmes, 2014). DESeq2 uses a generalized linear model where counts are modeled using a Negative Binomial distribution, which are function of the fitted mean, adjusted by size factor and ASV-specific dispersions. This method has been shown to outperform others in determining true differentially abundant taxa (McMurdie and Holmes, 2014). *p*-values were adjusted with the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

3. Results

3.1. Sequencing results

After sequencing, a total of 1,979,453 raw paired-end reads were generated. They were deposited in the NCBI in the project SRP133164. After all cleaning and filtering steps, a total of 460,583 sequences in 1944 ASVs were successfully assigned to Bacteria (S1). ASVs were deposited in GenBank under accession number KDPU01000000. The average number of sequences per sample were 57,573 (minimum 40,834; maximum, 95,359; standard deviation, sd = 28,809).

3.2. Alpha and beta diversity

The number of observed ASVs ranged from 228 to 876 (mean ± *sd*: 660 ± 346). ASV classification against the Silva v128 database revealed 98.8% of the variants were assigned to class, 88.4% to order, 79.7% to family and 46.2% to genus.

We detected 26 bacterial phyla. Seven of them (*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Chloroflexi*, *Firmicutes* and *Acidobacteria*) accounted for the 90% of the diversity (32.5, 20.4, 14.7, 8.4, 7.3, 3.6 and 4.5%, respectively), and 94% of the abundance (32.4, 28.6, 12.3, 7.4, 5.7, 5.0 and 2.8, respectively) (Figs. 2 and 3). Overall, *Proteobacteria* were most abundant in the channel in July, while *Actinobacteria* were most abundant in the channel in October (Fig. 2). There were little changes in the most dominant phyla associated with the rhizosphere of *A. macrostrachuym* (Fig. 2), except between the samples collected in the channel in July (jul1 and jul2). However, these most dominant phyla did seem to change between sampling site (channel vs open soil) and season (July vs October) (Fig. 2).

Across all samples, alpha diversity was 2–4 times lower in the soil from the channel in July. Under these conditions, alpha diversity in the rhizosphere (jul1) was almost two times greater than in bulk soil (jul2) (observed ASVs, 397 versus 228; Shannon H′, 5.4 versus 5.0) (Fig. 4). For the other samples, alpha diversity varied little between conditions (average ASVs, 775; range 600–876).

The MDS based on weighted UniFrac distances concentrated 74.6% of the variance of beta diversity in the first 2 dimensions (Fig. 5). Dimension 1 (44.7%) separated all samples from the wet season plus a rhizosphere sample from the channel in July (jul1), from the rest (jul2–4). There was no evident separation of the samples from the rhizosphere with respect to bulk soil for the other conditions in the subsequent dimensions 3 to 5 (98.5% of accumulated eigenvalues). All the samples from October were very similar (little beta diversity), while the beta diversity for the samples from July was very high (Fig. 5).

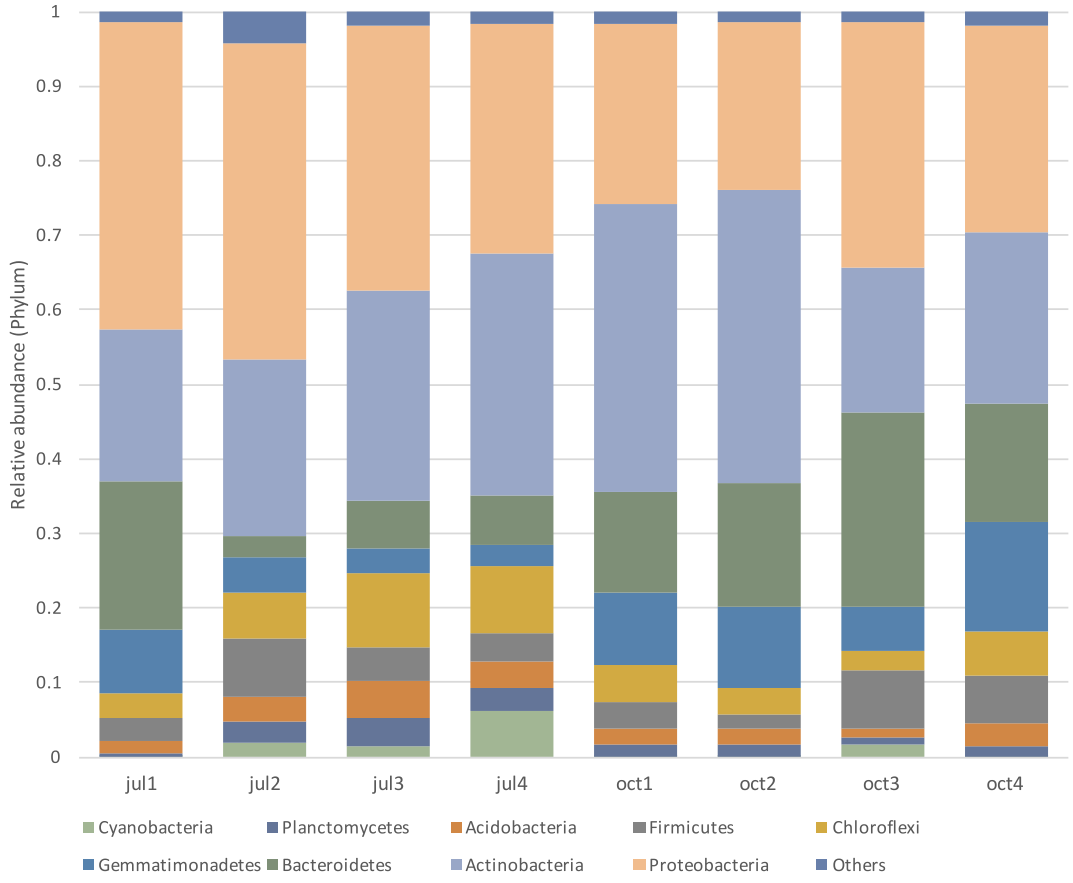

Fig. 2. Relative abundance of the 9 most abundant bacterial phyla (98% of the variants) for each sample. The legend of the samples includes *jul* and *oct* for dry (jul) and wet (oct) seasons, rhizosphere (1–3) and bulk soil (2–4) and location of the sample (1–2 for channel, 3–4 for high salt marsh).

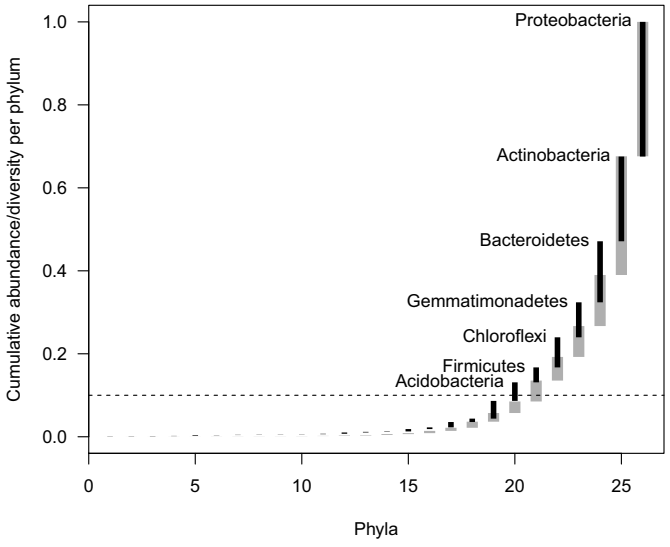

Fig. 3. Ranked cumulative abundance of bacterial phyla (grey bars) and their corresponding relative observed diversity (number of ASVs, black bars), for all samples together.

3.3. Differentially abundant taxa between conditions

The analysis of differentially abundant bacteria between conditions revealed 163 ASVs significantly (adjusted p-value < 0.05) associated with one or more of the environmental variables (Fig. 6). Most of these ASVs (147) were associated to a given season: 10 to July (dry) and 137 to October (wet). Sampling sites, either channel or high salt marsh, had 24 differential ASVs correlated: 8 to the channels and 16 to the high salt marsh. However, the rhizosphere had no taxa associated while only one ASV (*Cystobacter* sp) was linked to non-rhizosphere soil in October (Fig. 6).

Among the 147 variants associated to a season, all variants from 7 phyla were exclusively associated with October: *Bacteriodetes* (37), *Gemmatimonadetes* (29), *Acidobacteria* (3), *Planctomycetes* (3), *Saccharibacteria* (2), *Verrucomicrobia* (1) and *Firmicutes* (1) (Fig. 6). Only variants in *Cyanobacteria* (2) were exclusively associated with July. The remaining 3 phyla (*Actinobacteria*, 27; *Proteobacteria*, 38; *Chloroflexi* 3) had representatives with abundances correlated to either month (Fig. 6). Many of the *Actinobacteria* were associated to the community in October, 13 of which belonged to the orders *Acidimicrobiales* and *Nitriliruptorales*. Only 5 variants in this phylum were assigned to a genus: *Actinopolyspora*, *Mycobacterium*, *Kocuria*, *Longispora* and *Euzebya* (Fig. 6). One variant from the Class *Thermoleophilia* and another one from the Genus *Rubrobacter* were the only Actinobacteria significantly correlated with July. All the highlighted 37 variants in *Bacteriodetes* belonged to orders *Cytophagales*, *Flavobacteriales* and *Sphingobacteriales*, and Orders II and III. Many of these (22) were assigned to genus: *Rubrivirga*, *Catalinimonas*, *Cesiribacter*, *Fulvivirga*, *Nafulsella*, *Tunicatimonas*, *Owenweeksia*, *Salinimicrobium* and *Lewinella* (Fig. 6). Variants from the phyla *Gemmatimonadetes*, *Chloroflexi*, *Saccharibacteria* and *Verrucomicrobia*, were not assigned to any genus. In *Proteobacteria*, 6 out of 38 variants were more abundant in July, with two recognizable genera *Skermanella* (1) and *Sphingomonas* (3). However, most of the genera were correlated to October: *Tepidisphaera*, *Planctomyces*, *Rhodomicrobium*, *Tepidamorphus, Maribius*, *Palleronia*, *Paracoccus*, *Rhodovulum*, *Altererythrobacter*, *Croceicoccus*, *Porphyrobacter*, *Sphingomonas*, *Cystobacter*, *Haliangium*, *Cellvibrio*, *Halomonas*, *Methylohalomonas* and *Lysobacter* (Fig. 6). We identified other 2 genera, *Planococcus* and

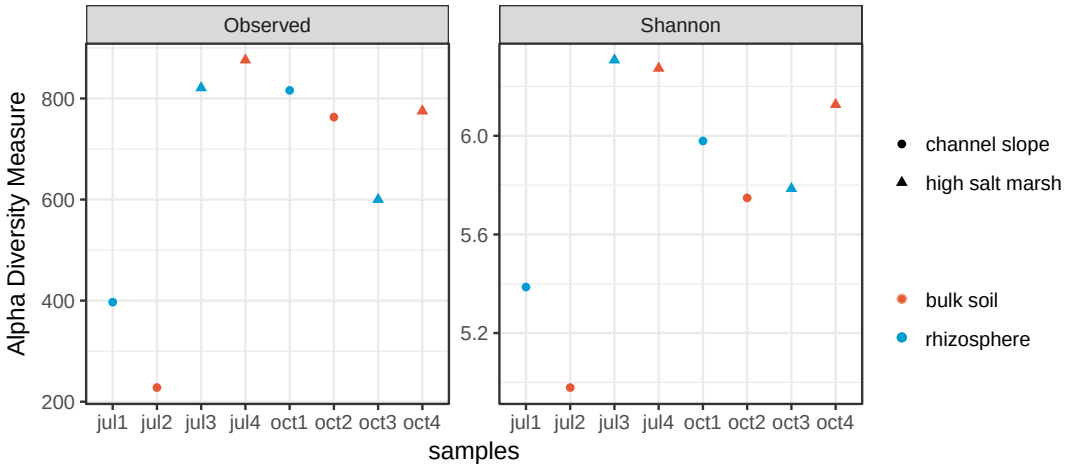

Fig. 4. Alpha diversity based on ASVs. Observed (left panel) and Shannon Index (right panel) for all samples.

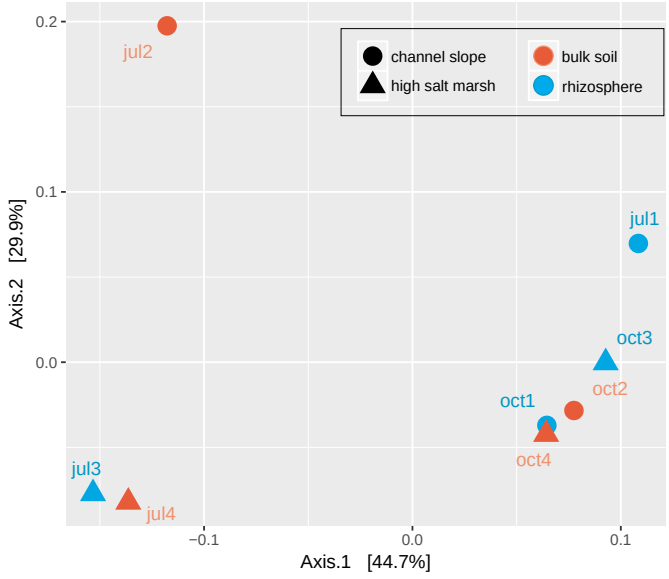

Fig. 5. Beta diversity. MDS based on weighted UniFrac distances for all samples for Dimensions 1 (44.7% of the variance) and 2 (29.9% of the variance).

Blastocatella, from phyla *Firmicutes* and *Acidobacteria*, respectively, that were more abundant in October.

We found twice the number of variants associated with the high salt marsh (16) compared to the channel (8) (Fig. 6). They belonged to 5 phyla: *Proteobacteria* (12), *Actinobacteria* (5), *Bacteroidetes* (3), *Cyanobacteria* (2) and *Firmicutes* (2). There was no clear association between these phyla in either sites. A total of 17 variants were successfully assigned to 15 genera: 4 in the channel, *Halomonas*, *Longispora*, *Marmoricola* and *Salinimicrobium*, and 10 in the bulk soil, *Archangium*, *Corallococcus*, *Methylibium*, *Microcoleus*, *Nonomuraea*, *Paenibacillus*, *Paracoccus*, *Planococcus*, *Skermanella* and *Spirulina* (Fig. 6). In Proteobacteria, all the 4 significant variants in Order *Myxococcales* were linked to the high salt marsh.

4. Discussion

We characterized bacterial communities from high salt marshes in southern Spain associated with the rhizosphere of the halophyte *A. macrostachyum* in comparison with surrounding bulk soil, in wet and dry seasons and for two different sites (high salt pans and water channel). Our bioinformatics workflow using DADA2 allowed us to identify 1944 ASVs from 26 phyla, averaging 660 ± 346 ASVs per sample. Determination of ASVs with DADA2 has been shown to outperform OTU clustering methods implemented in popular QIIME environment to determine more accurate estimates of real variants and overall diversity estimates for metagenomics analysis (Callahan et al., 2016a).

4.1. Alpha diversity

We report an alpha diversity that is in the lower range of that found for a broad world survey by the Earth Microbiome Project for saline sediments and other kinds of soils (Thompson et al., 2017). These differences could be associated to methodological issues related to the OTU versus ASVs approach to determine taxonomic units (Callahan et al., 2016a). The most abundant and diverse phyla were: *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Chloroflexi*, *Firmicutes* and *Acidobacteria* (Figs. 2 and 3; S1). These phyla are also among the most abundant in other kinds of soils (Rodríguez-Echeverría et al., 2013; Yang et al., 2017), including saline soils (Yang et al., 2016; Tian and Zhang, 2017). Phyla *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* have been described as dominant in other halophytes (Shi et al., 2015; Mukhtar et al., 2017; Tian and Zhang, 2017; Yamamoto et al., 2018; Furtado et al., 2019). Thus, the bacterial species belonging to these phyla could play key roles in these plants. As an example, members of phylum *Actinobacteria* are well known by their capacity to degrade organic compounds and to produce bioactive metabolites (Ningthoujam et al., 2009; Buée et al., 2009) and could be playing an important role as plant growth promoting bacteria under these harsh environments. In the same way, phylum *Planctomycetes* has been described in halophytes as *Glaux maritima* and *Salicornia europeae* (Yamamoto et al., 2018), *Salsola stocksii* (Mukhtar et al., 2017) and *A. macrostachyum* (Mora-Ruíz et al., 2016). In accordance with this last author, we found this phylum abundant in high salt marshes in July. Also, and in agreement with Mirete et al. (2015) and Mora-Ruíz et al. (2016), we found phylum *Firmicutes* as one of the most abundant in the rhizosphere of *A. macrostachyum*. This phylum has also been described inhabiting the endophyllosphere of this plant (Mora-Ruíz et al., 2015; Navarro-Torre et al., 2017) and has been proposed to improve seed germination in *A. macrostachyum* under saline conditions by the presence of enzymatic activities able to break the seed cell wall (Navarro-Torre et al., 2017). In accordance, Szymańska et al. (2016) described a high proportion of variants from phylum *Firmicutes* in *Aster tripolium* grown in higher salinities than ours (55 mS/cm) and proposed it could be a consequence of the higher tolerance of spore-producing bacteria under unfavorable conditions. The domination of Gram-positive bacteria in the rhizosphere of plants growing in saline areas has been confirmed by other authors (Upadhyay et al., 2011; Damodaran et al., 2013).

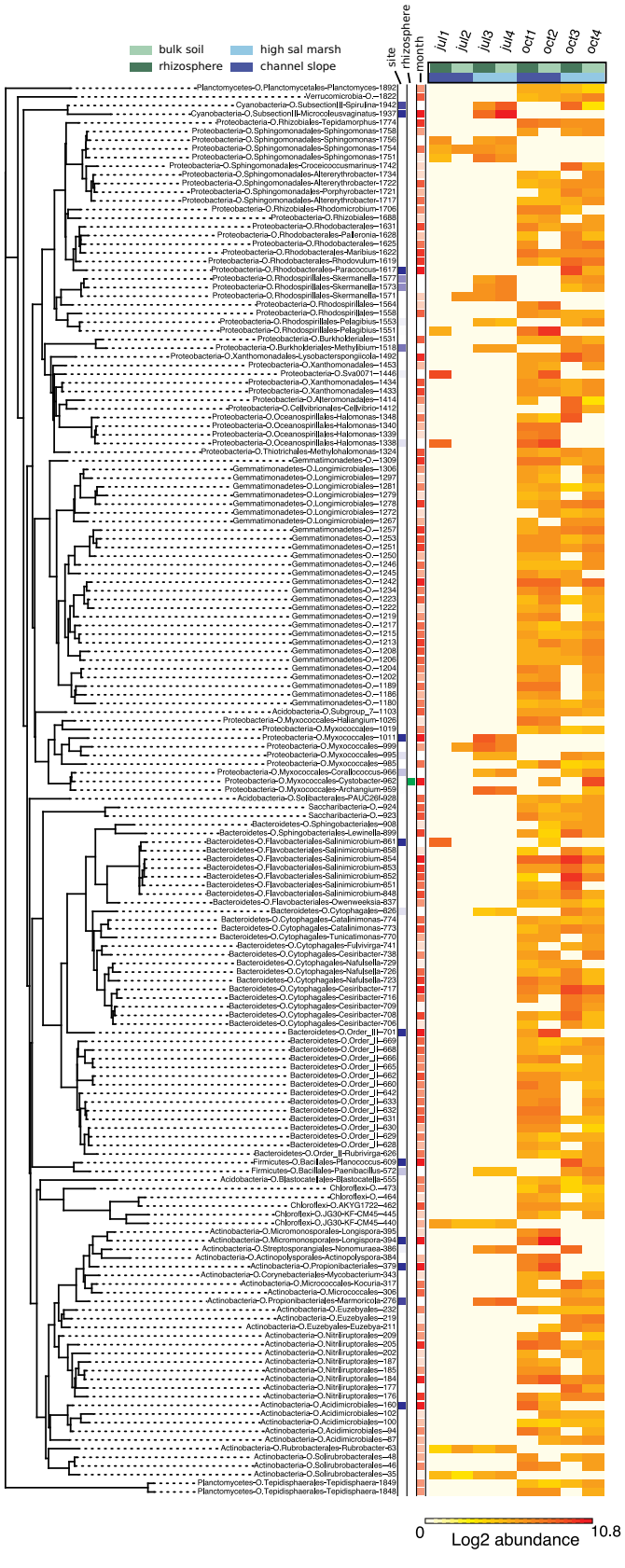

Fig. 6. Maximum Likelihood tree of the 163 variants which were significantly associated (adjusted *p*-value < 0.05) to any of the variables (site, rhizosphere and month). The opacity of the color squares is proportional to their *p*-values (more significant values correspond to darker colors). Normalized abundances (log2) for each sample are represented in the heatmap. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Across different studies there is no consensus on the association of alpha diversity with rhizosphere versus adjacent bulk soil. Indeed, the observed changes in the community could reflect a shift in their phylogenetic composition rather than a net increment in alpha diversity (Uroz et al., 2010; Hortal et al., 2013; Tian and Zhang, 2017; Yang et al., 2017; Iwaoka et al., 2018). In our case, alpha diversity from the rhizosphere of *A. macrostachyum* was similar to bulk soil across all conditions, except for the channel in July, in which alpha diversity in the rhizosphere was twice larger than in adjacent soil (Fig. 4). The alpha diversity found under these conditions (Channel/July) was the lowest found in this study. This correlates with the highest salinity of the channel in July, and it is in accordance with the taxa richness reduction found in saline environments (Hollister et al., 2010; Mora-Ruíz et al., 2016).

Much of the alpha diversity across all samples was associated to genera described in saline environments (e.g. *Halomonas*, *Roseimaritima*, *Maribius*), which highlights the importance of salinity in determining the microbial community (Mukhtar et al., 2018). Our results agree with Yang et al. (2016), who reported that species in the phylum *Verrucomicrobia* were in low abundance in saline soils; this phylum only appears in this study in autumn samples. In contrast, Jing et al. (2019) found this phylum at the highest salinities and proposed further investigation of the optimal environmental conditions for the growth of species in the Phylum *Verrucomicrobia*. Other phyla, as *Bacteroidetes* and *Clorobi*, seem to have a direct correlation with the salinity concentration, being less abundant at high salinities (Mirete et al., 2015). In agreement with these authors we found all their variants associated exclusively with October in this study.

4.2. Beta diversity

After exploring the beta diversity, none of the 5 first dimensions (98.5% of accumulated eigenvalues) separated bacterial communities in the rhizosphere from bulk soil, except for the samples in the channel in July in Dimension 1 (Fig. 5), in which the soil from the rhizosphere clustered with the samples from October. Apart from this, the first dimension (44.7% of the variance) seems to point to seasonal and site differences in soil salinity as a strong determinant for structuring the bacterial community. In fact, the low beta diversity for all samples in the wet season correlated with the little change in salinity between samples, whereas the high beta diversity in the dry season was associated with high changes between samples (Table 1; Fig. 5). This microbial diversity reduction in several halophytes in rainy season has been described by other authors (Rathore et al., 2017). The loss in beta diversity in October could be the consequence of the homogenization of the physical conditions in the soil, leading to the convergence of the bacterial communities between the sampling sites, regardless of the rhizosphere and site (channel/high salt marsh). However, in the samples from July, which correspond to the very dry summer conditions that characterize the Mediterranean climate, beta diversity was very high, indicating thus very different bacterial communities between nearby sampling sites. This higher beta diversity in July correlates with the larger differences in salinity between samples from this season. There was one exception to this pattern regarding the soil in the channel in July (Fig. 5), in which the rhizosphere of *A. macrostachyum* clustered with the samples from October. July coincides with the beginning of the fructification in this halophyte (Valdés et al., 1987). It is possible that the plant phenology could keep a relation with the microbial metacommunity in its rhizosphere when there is some humidity in the soil (closer to the water channel). This idea is supported by a study in this same plant from another region of Spain in the middle of its fructification period (August), which reported marked differences in the phylogenetic composition of the rhizosphere bacterial community versus bulk soil (Mirete et al., 2015). The activity in rhizosphere increases in the most active periods of the plant (Hanson et al., 2000) as during flowering (Fu et al., 2002). In such scenario, an interaction

between the plant phenology and the metacommunity in its rhizosphere has been reported in several species (Houlden et al., 2008; Chaparro et al., 2014).

4.3. Differential abundances

A statistical analysis on the differential abundances of the bacterial diversity across all samples detected that 147 out of 1944 variants were significantly associated with seasonality. However, only 1 out of the 1944 variants was significantly associated with bulk soil and none with rhizosphere. These results point to salinity rather than the rhizosphere as the dominant factor determining the bacterial community in this system.

Across different plant species, the effect of rhizosphere on its surrounding bacterial community is variable. For some species, the composition of the bacterial community in their rhizosphere is consistently found to be different from adjacent bulk soil (Uroz et al., 2010), including some halophytes (Chaudhary et al., 2015; Fahmy and Al-Thani, 2008; Oliveira et al., 2014; Szymańska et al., 2016; Tian and Zhang, 2017; Rathore et al., 2017). Contrarily, other halophytes seem not to have any effect on the composition of the bacterial community in their rhizosphere (Chaudhary et al., 2015). However, special attention must be paid to the term "bulk soil". Sometimes, the different results could be related to differences in this term; while some authors define bulk soil as 'soil not tightly adhere to roots' or 'adjacent to plants', other authors define it as 'soil from an open space without plants'. We found no overall difference between rhizosphere and bacteria community using bulk soils 'adjacent to plants'. However, due to the specific conditions of the channel in July, the rhizosphere bacterial community in *A. macrostachyum* was very different to that found in the surrounding bulk soil (Fig. 5). These samples (jul1 vs jul2) had the highest salinity (22.0 mS/cm and 44.0 mS/cm, in rhizosphere and bulk soil, respectively; Table 1). This decrease in salinity associated with the effect of *A. macrostachyum* has already been reported in previous studies (Rubio-Casal et al., 2001), and could be related to the bioaccumulation of salts in high salinity soils in this species (Navarro-Torre et al., 2017). Such high salt concentrations have been shown to boost growth in *A. macrostachyum* (Redondo-Gómez et al., 2010).

5. Conclusions

We described large differences in diversity and abundance of bacteria according to the changing spatio-temporal saline conditions in the soil with season and site. However, the rhizosphere of the dominant halophyte in this community, *A. macrostachyum*, seemed to play little, or no influence in structuring the bacterial communities. Part of the alpha diversity was represented by bacterial groups from saline environments, but we did not detect an increase in alpha diversity with any of the factors studied. Beta diversity was low during the wet season, but high in the dry season, when soil salinity varied the most between sampling sites. Altogether, these results suggest salinity is the main factor structuring the bacterial community in these marshes, and that *A. macrostachyum* has no effect in most situations, although there could be an interaction with the phenology which deserves to be explored.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2020.103532.

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