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RESEARCH LETTER

Rhizosphere effect and salinity competing to shape microbial communities in *Phragmites australis* (Cav.) Trin. ex-Steud

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Abstract

Rhizobacterial communities associated with *Phragmites australis* (Cav.) Trin. ex Steud. in a hypersaline pond close to Wuliangsuhai Lake (Inner Mongolia – China) were investigated and compared with the microbial communities in bulk sediments of the same pond. Microbiological analyses have been done by automated ribosomal intergenic spacer analysis (ARISA) and partial 16S rRNA gene 454 pyrosequencing. Although community richness was higher in the rhizosphere samples than in bulk sediments, the salinity seemed to be the major factor shaping the structure of the microbial communities. *Halanaerobiales* was the most abundant taxon found in all the different samples and *Desulfosalsimonas* was observed to be present more in the rhizosphere rather than in bulk sediment.

Introduction

Hypersaline systems are extreme environments with salt concentrations that approach or exceed saturation, globally distributed in marine and inland waters, springs and soils. These ecosystems are characterized by a low level of oxygen and a pH that can range from basic to acid (Paerl & Yannarell, 2010). The effect of salinity in sediments makes these environments suitable for the development of peculiar microbial communities adapted to survive in these extreme ecosystems. Microorganisms are indeed selected to thrive at different salinity levels and halophilic bacteria are the most common group found in such environments. Halophilic bacteria are present in different lineages of the phylogenetic branches, reflecting a high metabolic diversity (Das Sarma & Arora, 2002) ranging from aerobic to anaerobic respiration and phototrophic to heterotrophic nutrition (Ventosa *et al.*, 2012). They may use strategies to balance the osmotic pressure accumulating organic solutes into cytoplasm and to form biofilm with extracellular compounds containing water (Decho *et al.*, 2005; Roberts, 2005). Hypersaline environments can be also inhabited by plants that have evolved the capability to thrive on saline soils. For example, haplotype of *Phragmites australis*, an invasive species that increases its spatial distribution rapidly forming dense colonies along lake shores, channels, rivers and alkaline wetlands, have been found in salt environments (Marks *et al.*, 1994; Güsewell & Klötzli, 2000; Vasquez *et al.*, 2005). *Phragmites australis* is adapted to survive in salty ecosystems through a downward transportation mechanism that consists in limiting the entry of Na^+ into the shoots and the use of K^+ to balance the osmotic pressures in the leaves (Vasquez *et al.*, 2005). Therefore, the exudates released by roots also may modify the osmolarity increasing the salt stress tolerance conferred to the plant. These exudates, consisting in aminoacids, organic acids, proteins and others compounds, also play an important role in the organic input promoting the microbial activity (Bais *et al.*, 2003; Mayak *et al.*, 2004). The ecological niche intimately influenced by roots exudates is known as the rhizosphere and the above-mentioned physicochemical alterations occurring within the root–sphere are defined as the 'rhizosphere effect' (Antoun & Prevost,

2005). Moreover, it can be demonstrated that the rhizosphere effect is often species-specific. As a result, the same plant species have the ability to shape a microbial community structure in a variety of differing soil types (Smalla *et al.*, 2001; Mengoni *et al.*, 2004; Berg & Smalla, 2009). Although the effect of plant species and individuals on their rhizobacteria has been investigated extensively, especially as regards agricultural crops, very few studies have as yet investigated the microbial communities associated to roots of wild plants in hypersaline environments (e.g. Mapelli *et al.*, 2012). To the best of our knowledge there are no comparative studies focusing on the rhizobacterial communities associated with submerged plants in inland water sediments. Hence, the goals of our work were: (1) to compare the microbiota associated to *P. australis* in a hypersaline pond with the microbiota inhabiting bulk sediments; (2) to assess which is the main factor, the rhizosphere effect or saline stress, determining the overall genetic structure and taxonomic diversity of dwelling bacterial community.

Materials and methods

Study area

This study was conducted near Wuliangsuhai Lake in the western part of Inner Mongolia Autonomous Region (China) in 2011 June. Samples were collected in a hypersaline pond (40°47′005″N, 108°42′597″E, elevation 1019 m) with a surface area of about 70 m^2, part of which was covered with *P. australis* with a height of about 1 m. At the time of our sampling, water flux was completely motionless. Samples were taken in replicates from five different zones of the pond: B74a, B74b and B74c were collected from bulk sediments and R70a, R70b, R71a R71b, R72a, R72b, R73a and R73b from the area of the pond covered by *P. australis* (Table 1, Fig. 1). We defined rhizosphere samples as the tightly adhering particles within 1–3 mm of the roots. Bulk soil replicates were collected 2.5 m from *P. australis*. For all samples, 10 g was collected and transferred into sterile tubes at 4 °C for molecular analyses. Samples were immediately transported to nearby laboratories to allow a fast DNA extraction. The electrical conductivity (EC) of the sediments and pH were measured using an Accumet AP85 pH (Fisher Scientific Ltd., Pittsburgh, PA). EC is a parameter commonly used to measure the salinity because of the positive correlation with the salt concentration (Wollenhaupt *et al.*, 1986).

Table 1. Physical properties of the sediments

Parameter	R70	R71	R72	R73	B74
EC (mS cm^{-1})	56.3	30.1	50.2	32.5	80.0
pH_{H_2O}	8.3	8.2	8.3	8.1	8.4

EC, electrical conductivity.

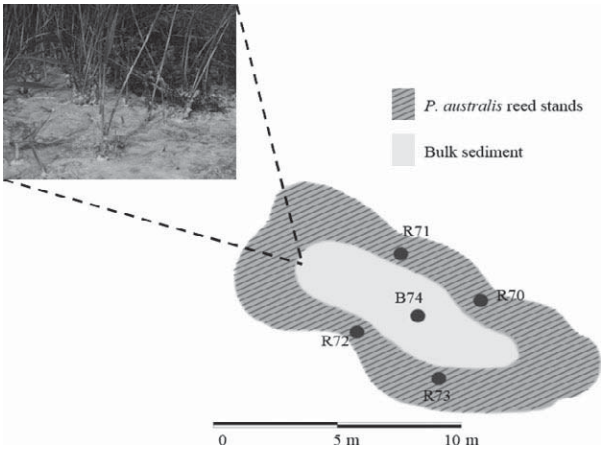

Fig. 1. Hypersaline pond and sampling scheme with the five sampling points. Bulk sediment (B) and rhizosphere sediment (R).

DNA extraction

The total DNA from 1 g (wet weight) of sediment was extracted through PowerSoil® DNA Isolation Kit (MoBio, Arcore, Italy) accordingly to the user manual. Quantification of DNA was performed using the NanoVue™ Plus spectrophotometer (GE Healthcare, NJ). All the reaction templates were normalized to the same DNA concentration of 30 ng per reaction.

Automated ribosomal intergenic spacer analysis

PCR reactions were carried out using primers ITSF and 6-FAM ITSReub, according to the chemical and thermal amplification protocol of Cardinale *et al.* (2004). PCR products were sent to STAB Vida Lda. (Caparica, Portugal), which performed the capillary electrophoresis using an ABI 310 genetic analyzer (Perkin-Elmer) with LIZ2500 as an internal size standard.

Fragment data were analyzed through PEAK SCANNER Software v1.0 (Applied Biosystems) setting a threshold at 40 fluorescent units, i.e. three times more than the highest peak detected by a blank DNA-free control. Output matrix was obtained as in Rees *et al.* (2004). The matrix was normalized and angular-transformed for statistical analysis.

454 pyrotag sequencing

Genomic DNA was pooled at equal molar ratio according to three groups identified through the ARISA-based

NMDS analysis: R7072 (R70a, R70b, R72a and R72b), R7173 (R71a, R71b, R72a and R72b) and B7474 (B74a, B74b and B74c) (Fig. 2). Samples were sent to Molecular Research LP (MR DNA™). PCR amplification of environmental 16S rRNA genes was performed using the extracted DNA with a primer set amplifying the V4–V6 variable regions (primers 518F 5′-CCAGCAGCYGCGGT AAN-3′ and 1046R 5′-CGACRRCCATGCANCACCT-3′). Samples were sequenced using the Roche 454 GS-FLX system, titanium chemistry, according to the protocols of that company.

Sequences with length < 200 bp or with ambiguous bases, and homopolymer runs exceeding 6 bp were removed before chimera checking. A redundancy control was performed, using a self-developed java script (https://github.com/combogenomics/DeUniFier), to obtain a single file containing only unique sequences. The sequences were then clustered using USEARCH (Edgar, 2010) with an identity cutoff value of 90%. After this step, all the centroid sequences were collected from the USEARCH output and classified using the RDP CLASSIFIER (Wang *et al.*, 2007). A confidence threshold of 80% was used in order to obtain only classification hits with high confidence.

FASTQ file sequences have been submitted to the EMBL/NCBI/DDBJ Short Read Archive under accession nos. ERS407985 (R7072), ERS407986 (R7173) and ERS407987 (B7474).

Statistical analysis

PAST and R software were used to perform the statistical analysis respectively on ARISA and 454 pyrosequencing data (Hammer *et al.*, 2001; R Core Team, 2012). The Chao1 index was calculated on metagenomic data assignments considering only reads assigned to genus level. Richness was calculated on the normalized non-transformed ARISA matrix. For the beta-diversity analysis, the transformed ARISA matrix was used to perform a non-metric multidimensional scaling (NMDS) using the Bray–Curtis measure.

Results

Physical and chemical characterization of the site

Sediment texture was a clayey soil with alkaline pH showing similar values across the five sampling points (Table 1). In contrast, we observed a variation in sediment electrical conductivity (EC), with a higher value in the bulk sediments (B74). Among the rhizosphere samples we noticed that the electrical conductivity of R70 and R72 was higher than R71 and R73 (Table 1). Direct observation indicated anaerobic conditions of the sediments, as inferred from their intense odor of hydrogen sulfide and dark color (Reiffenstein *et al.*, 1992).

Bacterial community structure and diversity

An average of 170.7 ± 34.4 of peaks per sample was found in ITS amplicons. The peak sizes ranged from 160 to 1200 bp. The lowest number of peaks was found in B74c and B74a, with values of 98 and 115, respectively. The highest values were found in R72b and in R70b, with values of 207 and 198, respectively. The similarity was found to be higher within the replicates of each sample than between samples (ANOSIM, $R = 0.87$, $P < 0.001$). The NMDS plot well separated microbial communities patterns as indicated by the goodness of fit (0.09) of the stress value for the ordination with two dimensions (Clarke, 1993). Three clusters were found: the first (B7474) contained samples collected in bulk sediments B74a, B74b and B74c; the second (R7072) contained samples collected in rhizospheres R70a, R70b R72a and R72b, and the third group (R7173) samples collected in R71a, R71b, R73a, R73b (Fig. 2). Taxa richness values were

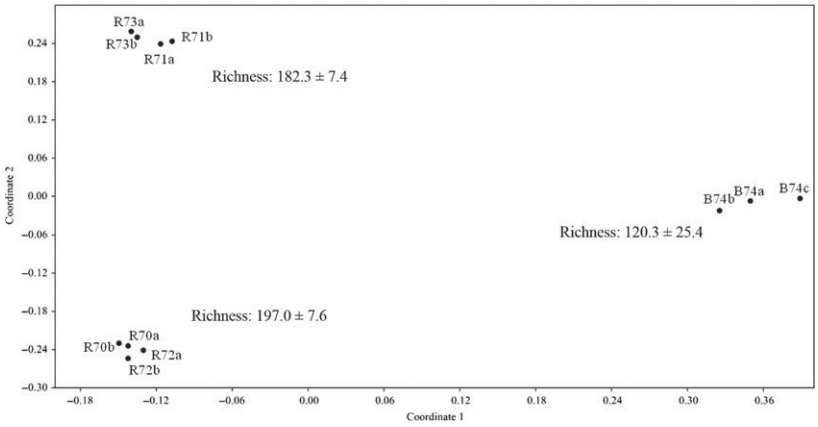

Fig. 2. NMDS ordination plot based on ARISA matrix for bacteria community rhizosphere sediments (R) and bulk sediments (B). Numbers indicate the sampling points and lowercase letters the replicates. Richness values and standard deviations are given for ITS regions of the three clusters. The values represent the cumulative averages for each cluster with the standard deviation.

higher in R7072 and R7173 (ANOVA, $P < 0.01$) than in B7474 (Fig. 2).

The yield of the pyrosequencing run, after quality checks, was of 7954, 4253 and 3437 pyrotags respectively from R7072, R7173 and B7474. The number of unique sequences obtained after the redundancy control step was 15 047 and the number of clusters acquired with the USEARCH algorithm was 1169, with a mean of 13 sequences per OTU. The Chao1 estimation indexes were 828.9 (R7072), 768.4 (R7173) and 717.1 (B7474). Rarefaction curves showed that the three samples have a different bacterial richness. In particular, the curve related to R7072 tends to be flatter, indicating a lower richness level than found for the other two samples (R7173 and B7474) (Fig. 3).

The composition at phylum level was dominated, in all the three clusters, by *Proteobacteria* (43% in R7173, 39% in R7072 and 36% in B7474), *Firmicutes* (26% in R7173, 44% in R7072 and 30% in B7474), *Bacteriodetes* (24% in R7173, 13% in R7072 and 20% in B7474), with lower abundances of several other phyla (Fig. 4). Analyses at a finer level revealed some differences among the samples. In particular, *Halanaerobiales* were present in high abundance in all samples (23% in R7072, 14% in R7173 and 12% in B7474; Fig. 5). *Desulfosalsimonas* was the second most abundant group detected in the different clusters, 19% in R7173 and 11% in R7072, but it was rarer in B7474 (3%).

Discussion

The similarity of the replicates in the different sampling points confirmed the low variability of microbial community structures when exposed to the same environmental conditions. In contrast, bulk sediment samples grouped separately from rhizosphere samples, which clustered in distinctive couples (Fig. 2). We expected the bacterial communities associated to rhizosphere to be very similar

LOW RESOLUTION FIG

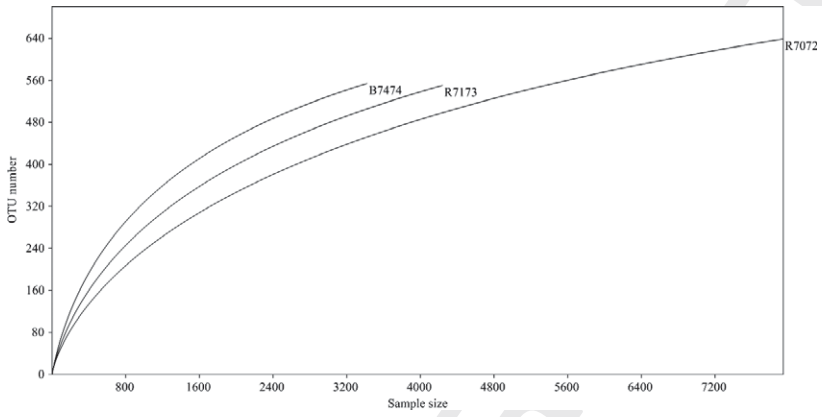

Fig. 3. Rarefaction curves describing the observed number 16S rRNA operational taxonomic units (OTUs). Samples were pooled according to the NMDS analysis on the ARISA matrix (see Fig. 2). 25

LOW RESOLUTION FIG

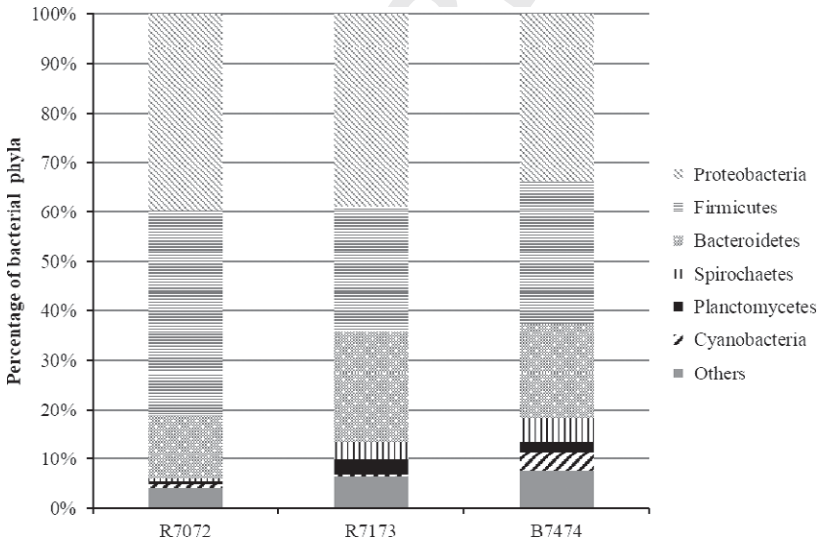

Fig. 4. Bacterial classification using the RDP classifier at phylum level. Phyla abundances lower than 5% were shown as 'others' (*Chloroflexi*, *Lentisphaerae*, *Actinobacteria*, *Acidobacteria* and *Verrucomicrobia*). 26

LOW RESOLUTION FIG

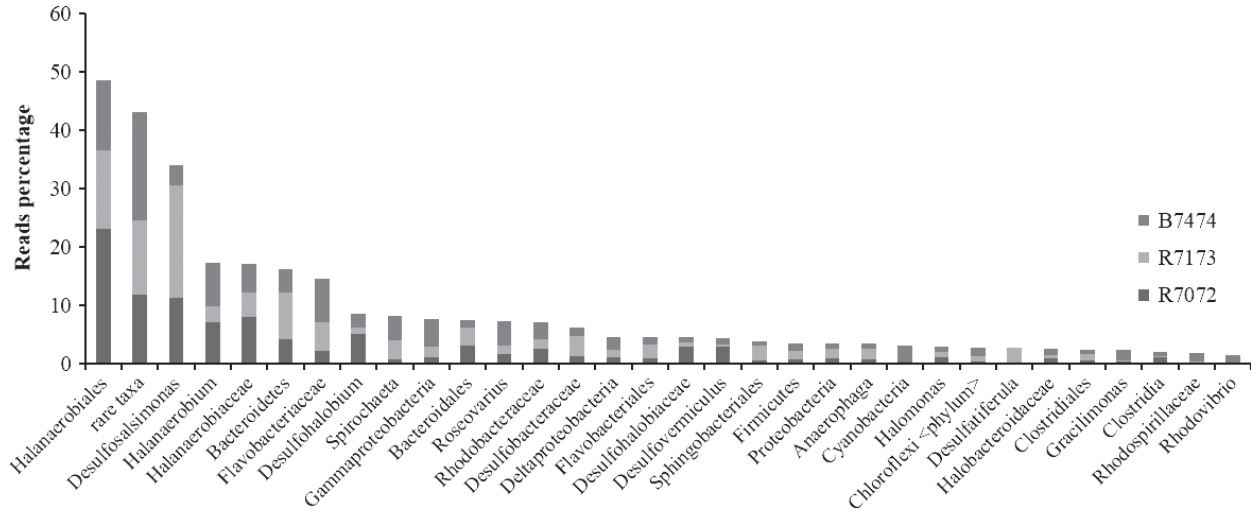

Fig. 5. RDP classification at a finer level: taxon name for each sample is shown on *x*-axis and the percentage of the reads on *y*-axis. Taxa abundances lower than 1% were pooled and shown as 'rare taxa'. 27

in terms of structure, compared with samples from bulk sediment. Indeed there are several reports of a plant species-specificity of rhizobacterial communities. Plant root exudates differ among species. As a consequence, microbial communities differ according to the plant species examined. Because of plant species-specificity, similar rhizobacterial communities can be found in different environments where the same plant is present (Smalla *et al.*, 2001; Berg & Smalla, 2009). A possible interpretation that could explain this outcome is that in specific environments such as a hypersaline pond, the rhizosphere effect has only a minor role in shaping its microbial community structure. In such extreme environments other variables could play a stronger role with respect to the biotic effect of rhizosphere in selecting the microbial communities. This hypothesis seems to be supported by similar findings of a smaller influence of biotic interactions than of abiotic stresses on ecosystem functions in biological soil crusts that have developed on desert soils (Li *et al.*, 2013). In our study we found that samples clustered according to the different values of salinity. Salinity is indeed one of the most important abiotic factors that affect the shaping of microbial community composition (Lozupone & Knight, 2007). To our knowledge the only report concerning the rhizosphere effect in a hypersaline environment was done by Mapelli *et al.* (2013). Those 14 authors found in rhizosphere of *Salicornia* sp. a higher similarity among the rhizobacterial communities collected in different hypersaline soils, showing that the composition of microbial communities was influenced more by root activity than by soil composition. The lack of agreement between such data on *Salicornia* sp. and our results on *P. australis* could be linked to differences in soil texture characteristics (sand vs. clay), or more directly to the different chemical exudate patterns of the two plant species (Garbeva *et al.*, ~~2004~~). ~~In the first case,~~ Marschner 15 *et al.* (2001) demonstrated that microbial communities in sandy soil and loam were affected more by root exudates than were communities inhabiting clay matrices. They hypothesized a dilution of the rhizosphere effect due to the greater amount of clay particles adhering to root surfaces compared with sand and loam particles. ~~In the second case,~~ ~~it~~ is well known how different plant species can 16 produce different exudation patterns, which have deep consequences for the selection of the surrounding microbial community composition and structure. Another hypothesis to explain such differences can be derived by the investigative techniques chosen: microbial communities of *Salicornia* sp. were analyzed through 16S rRNA gene PCR and denaturing gradient gel electrophoresis (DGGE), a technique with a lower resolution compared with 16S-23S rRNA gene PCR and ARISA (Fisher & Triplett, 1999; Cardinale *et al.*, 2004). DGGE investigates a microbial community at the genus/species level, depending on how much a taxonomic group has already been studied phylogenetically, whereas ARISA, based on the higher variable intergenic spacer region of ribosomal operon, can investigate at the subspecies level (Daffonchio *et al.*, 1998, 2000; Brusetti *et al.*, 2004), because even bacterial genomes usually harbor multiple ribosomal operons (Johansen *et al.*, 1996; Nubel *et al.*, 1996). Consequently, we found many more peaks than using a standard DGGE electrophoretic gel, obtaining more information at a finer resolution scale (Brusetti *et al.*, 2004). Basically, at this

scale, we can obtain semi-quantitative information on rare bacterial subspecies, which are affected mostly by even weak environmental changes.

Even though we observed that the rhizosphere played a minor role in shaping the microbial community structure in a similar way, we did found that it promoted richness diversity (Fig. 2). Since about 40% of photosynthates of plants are released into rhizosphere, it is not uncommon to find a higher microbial density in such an ecological niche (Egamberdieva *et al.*, 2008; Berendsen *et al.*, 2012). Moreover, root exudates are also responsible for microbiota chemotactical attraction from the surrounding root-free soil to the rhizosphere (Bais *et al.*, 2003).

We integrated the comparisons among the different microbial communities structures with pyrosequencing data to get a snapshot of the different taxon distribution in the samples, information that it is not possible to obtain through ARISA. *Firmicutes* and *Proteobacteria* belonging to the subphylum Gamma were found to be preponderant in the rhizosphere and bulk sediments, as has also commonly been found in similar hypersaline environments in China, such as in Sichuan province (Xiang *et al.*, 2008; Wen *et al.*, 2009; Tang *et al.*, 2011), confirming the importance of these two taxa in the overall diversity of Chinese hypersaline environments (Fig. 3). Due to the low level of oxygen in sediments it is not surprising to find a considerable number of anaerobic halophilic bacteria such as *Halanaerobiales*. This order is composed of microorganisms with an obligate anaerobic fermentative or homoacetogenic metabolism (Fig. 4) that is able to accumulate KCl in cytoplasm, instead of organic solutes, to balance the osmotic pressure (Oren, 2008). *Halanaerobiales* has been detected in several hypersaline environments such as the Dead Sea, hypersaline lakes in Tunisia, and salty ponds in France (Cayol *et al.*, 1994; Ollivier *et al.*, 1994; Oren *et al.*, 2005). Species of the *Desulfosalsimonas* genus are commonly found in black sulfide-containing hypersaline sediments and may grow with NaCl concentrations of up to 100 g NaCl L^{-1} using sulfate as terminal electron acceptor and producing hydrogen sulfide (Kjeldsen *et al.*, 2010). The major presence of sulfate-reducing bacteria in rhizosphere compared with bulk sediments could be due to the presence of root exudates and plant material (Fig. 4). Furthermore, the microenvironments could be richer in sulfate compared with the bulk sediment as there is evidence that the *P. australis* root system can increase oxygen content in the rhizosphere (Armstrong, 1992; Vladár *et al.*, 2008).

In conclusion, we observed a partially masked rhizosphere effect probably because of softening by the high salt concentrations of the hypersaline sediments. We can deduce that in extreme environmental conditions, where one or more ecological parameters reach the lower or the upper limit for cellular life, these parameters are bigger constraints in the shaping of bacterial communities.

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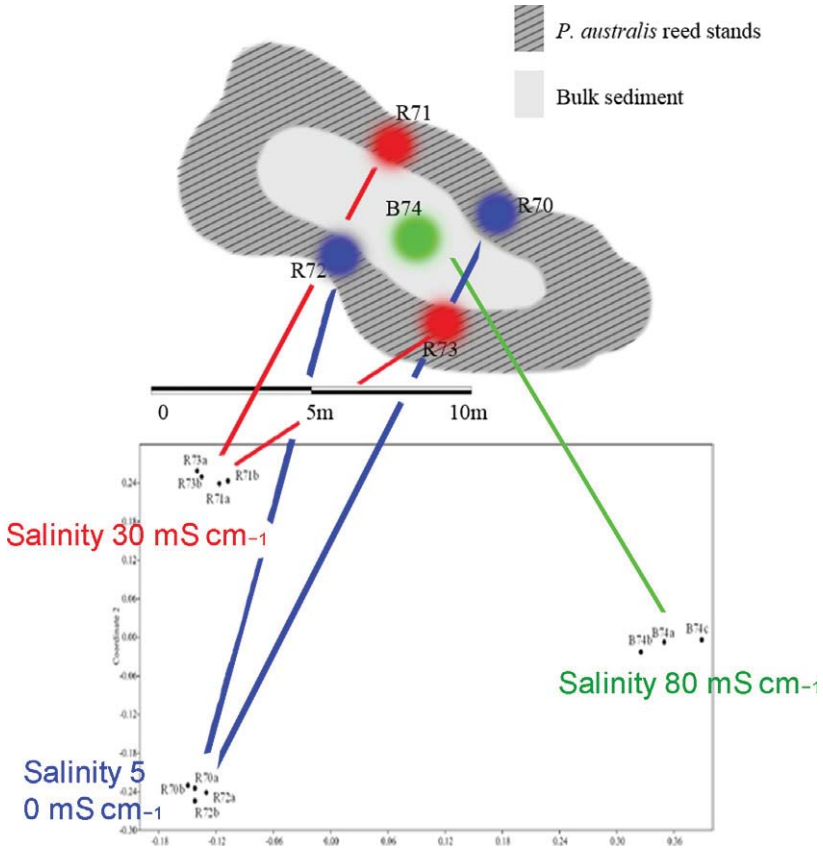

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