

# Culturable endophytic bacteria from the salt marsh plant *Halimione portulacoides*: phylogenetic diversity, functional characterization, and influence of metal(loid) contamination

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**Abstract** *Halimione portulacoides* is abundant in salt marshes, accumulates mercury (Hg), and was proposed as useful for phytoremediation and pollution biomonitoring. Endophytic bacteria promote plant growth and provide compounds with industrial applications. Nevertheless, information about endophytic bacteria from *H. portulacoides* is scarce. Endophytic isolates ( $n=665$ ) were obtained from aboveground and belowground plant tissues, from two Hg-contaminated sites (sites E and B) and a noncontaminated site (site C), in the estuary *Ria de Aveiro*. Representative isolates ( $n=467$ ) were identified by 16S rRNA gene sequencing and subjected to functional assays. Isolates affiliated with Proteobacteria (64 %), Actinobacteria (23 %), Firmicutes (10 %), and Bacteroidetes (3 %). *Altererythrobacter* (7.4 %), *Marinilactibacillus* (6.4 %), *Microbacterium* (10.2 %), *Salinicola* (8.8 %), and *Vibrio* (7.8 %) were the most abundant genera. Notably, *Salinicola* ( $n=58$ ) were only isolated from site C; *Hoeflea* (17), *Labrenzia* (22), and *Microbacterium* (67) only from belowground tissues. This is the first report of *Marinilactibacillus* in the endosphere. Principal coordinate analysis showed that community composition changes with

the contamination gradient and tissue. Our results suggest that the endosphere of *H. portulacoides* represents a diverse bacterial hotspot including putative novel species. Many isolates, particularly those affiliated to *Altererythrobacter*, *Marinilactibacillus*, *Microbacterium*, and *Vibrio*, tested positive for enzymatic activities and plant growth promoters, exposing *H. portulacoides* as a source of bacteria and compounds with biotechnological applications.

**Keywords** Endophyte · Bacteria · *Halimione portulacoides* · Salt marsh plants · Plant growth promotion · Extracellular enzymes

## Introduction

Salt marshes are among the most productive ecosystems in terms of primary production and are a habitat to several species with commercial value (Alongi 1998). The vegetation in this type of ecosystem is usually subjected to harsh environmental conditions including periodic tidal flooding, high salinity levels, and contaminants such as metals from urban and industrial effluents. As a consequence, the typical salt marsh vegetation presents low species diversity and high specialization (Woerner and Hackney 1997).

*Ria de Aveiro*, a coastal lagoon on the northwest of Portugal, is one of the most mercury-contaminated coastal systems in Portugal. For over four decades (1950–1994), industrial effluents containing mercury (Hg), arsenic (As), zinc (Zn), lead (Pb), and other contaminants were discharged into natural water streams of this lagoon which led to contamination of water and sediments of adjacent marshes (Costa and Jesus-Rydyń 2001). Although discharges have substantially decreased over the last two decades, sediments in the Laranjo Bay salt marshes are still considerably contaminated, namely

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with Hg. This highly toxic nonessential metal is a hazardous contaminant with potential to be released to the water column and assimilated by the biota (Pereira et al. 2009).

The perennial halophyte *Halimione portulacoides* (L.) Aellen is one of the most productive and abundant species in European salt marshes (Bouchard et al. 1998), including those along the *Ria de Aveiro* lagoon. This Hg-tolerant halophyte allocates this toxic metal preferably to belowground tissues and was proposed as a bioindicator and biomonitor for Hg pollution (Válega et al. 2008a). Additionally, this halophyte has been proposed for phytoremediation of Hg (Anjum et al. 2011), tributyltin (Carvalho et al. 2010), and petroleum hydrocarbons (Couto et al. 2011).

Endophytic bacteria colonize internal plant tissues without causing symptoms of disease (Lodewyckx et al. 2002). These bacteria are highly diverse taxonomically, ubiquitous from herbaceous plants to trees, and contribute to plant health and productivity (Berg et al. 2014). Endophytes present a great potential for applications in agricultural, medical, biotechnological, and industrial fields. Industrially important enzymes such as amylases, cellulases, lipases, pectinases, proteases, and xylanases, among others, have previously been found in endophytic bacteria (e.g., Lodewyckx et al. 2002).

Plant-associated bacteria, including endophytic bacteria, are known to be very relevant for plant growth promotion (PGP; Hardoim et al. 2008), including in cases of biotic or abiotic stresses, such as contaminated soils (reviewed in Rajkumar et al. 2012). This PGP action may be conducted through direct or indirect mechanisms. Direct PGP mechanisms include enhancement of plant nutrient acquisition, by aiding in nitrogen fixation and phosphate solubilization; plant growth by production of the auxin indol-3-acetic acid (IAA); and lowering of ethylene-related plant stress by producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Indirect PGP is achieved by preventing phytopathogen prejudicial effects on plant development by producing metabolites involved in antimicrobial activities and iron chelation (reviewed in Bulgarelli et al. 2013). It is also known that plant-associated bacteria are able to aid in phytoremediation processes by phytoextraction (reviewed in Sessitsch et al. 2013). In fact, Oliveira et al. (2014a) have shown that endophytic bacteria isolated from *H. portulacoides* have potential to be used in detoxification of petroleum hydrocarbons.

Notwithstanding the acknowledged importance of endophytic bacteria, comprehensive studies regarding the basal culturable communities of these bacteria in the specific environment of salt marshes are scarce, as studies are usually directed toward studying contaminant-degrading bacteria. *H. portulacoides* is a highly relevant and ecologically important halophyte in salt marshes; however, the structure of endophytic bacterial communities of this plant has not yet been fully explored, only its fraction that is able to degrade hydrocarbons (Oliveira et al. 2014a).

This study aimed to characterize the culturable endophytic bacterial communities of this halophyte from a highly contaminated site, an intermediately contaminated site, and a noncontaminated site in *Ria de Aveiro*, regarding their phylogenetic diversity and traits associated with direct and indirect PGP effects.

## Methods

### Study area and sampling

The study was conducted in the estuary *Ria de Aveiro*, in Aveiro, Portugal. A confined area in this lagoon was subjected to Hg-rich industrial discharges for four decades (1950s–1990s, Pereira et al. 1998). Three locations in the salt marsh of *Ria de Aveiro* were assessed: one highly contaminated by industrial activity (site B, 450 m downstream from the contamination source in the estuary, 40° 43' 47.46" N, 8° 36' 45.40" W), one with intermediate levels of contamination (site E, 2500 m downstream from the contamination source, 40° 43' 15.90" N, 8° 38' 15.76" W) (Válega et al. 2008b), and a noncontaminated location (site C, 40° 38' 05.36" N, 8° 39' 38.87" W; 11.3 km from the contamination source).

Healthy specimens of the halophyte *H. portulacoides* were sampled in monospecific stands, at least in triplicate in each site. Adjacent sediment was sampled in triplicate with a 3 cm in diameter and 13-cm depth corer. Sampling was performed during low tide in November 2012 (average temperature 16 °C). Samples were transported to the laboratory under refrigerated conditions and promptly processed.

### Metal(loid) quantification

Composite samples of aboveground (AG) tissues (leaves, stems, and flowers; 1 g fresh weight), belowground (BG) tissues (roots; 1 g FW), and sediment (2 g FW) from each site were used for metal(loid) quantification, in triplicate. Chromium (Cr), nickel (Ni), copper (Cu), zinc (Zn), As (as total arsenic), and mercury (Hg) were quantified. Samples were subjected to acid digestion with concentrated nitric acid overnight at 115 °C (Figueira and Freitas 2013) and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) according to the International Standard ISO 17294 by a certified laboratory at the University of Aveiro.

One-way ANOVA (function *aov*) combined with post hoc Tukey's honest significant differences method (function *TukeyHSD*) were used to assess differences in metal(loid) concentrations in sediment and plant tissue samples on all sites. Welch two-sample *t* test (function *t.test*) was used to estimate differences in metal(loid) concentrations (i) among plant tissues, for each isolation site and (ii) between sediment and plant samples, for each isolation site. These analyses were

performed using the *stats* package in the free software R (R Core Team 2014) with abovementioned functions *aov*, *TukeyHSD*, and *t.test*, considering a 95 % confidence interval.

### Isolation of endophytic bacteria, growth media, and conditions

Eleven plants were processed at the laboratory: five from site C, three from site B, and three from site E. First, all plants were thoroughly washed with tap water. For each plant, 5 g (FW) of AG tissues and 2 g (FW) of BG tissues were used for endophytic bacteria isolation. Surface sterilization was optimized and performed as follows: samples were sequentially immersed in 50 mL of phosphate-buffered saline (PBS; 10 min), 96 % ethanol (1 min), 5 % NaOCl (30 min), 96 % ethanol (1 min), and rinsing in distilled sterile water three times. Surface sterilization was confirmed by inoculating water from the last wash in Tryptic Soy Agar (TSA, Merck, Germany), Marine Agar (MA, Difco Laboratories, France), and R2A (Merck, Germany) culture media. The plates were examined for bacterial growth after incubation at 28 °C for 72 h.

Tissues were then ground with a pestle in a mortar containing 10 mL of PBS. Serial dilutions were prepared in PBS and spread, in duplicate, on TSA, MA, and R2A media. The plates were incubated up to 72 h at 28 °C and were observed every 24 h for colony-forming unit (CFU) count. Morphologically distinct endophytic bacterial colonies were selected and purified by streaking, resulting in a collection of 665 endophytic bacterial isolates, and stored at −80 °C in 20 % glycerol. Function *aov* combined with *TukeyHSD* was used to assess differences in average CFU per gram of tissue (fresh weight) in different isolation sites and culture media, and *t.test* was used to assess tissue-based differences.

### DNA extraction, PCR-based fingerprinting, and selection of representative isolates

DNA from all 665 isolates was extracted using the Genomic DNA Purification kit #0513 (Thermo Scientific, USA) according to the manufacturer's instructions. BOX-PCR with BOXA1 primer (5'-CTACGGCAAGGCGACGCTGAC-3'; Versalovic et al. 1994) was used for molecular typing of all isolates. PCR reactions were performed in a volume of 25 µL, using 5× Green GoTaq buffer, GoTaq DNA polymerase (5U µL<sup>-1</sup>), MgCl<sub>2</sub> (25 mM), and a dNTP mixture (2 mM) (Promega, USA). Conditions for amplification were as follows: one cycle at 95 °C (7 min), 30 cycles at 94 °C (1 min), 53 °C (1 min), 65 °C (8 min), and a final cycle at 65 °C (16 min). One hundred thirty-three isolates did not yield a clear profile with this methodology and were typed using ERIC-PCR with ERIC1R (5'-ATGTAAGCTCCTGGGGATTAC-3'; Versalovic et al. 1991) and ERIC2 (5'-AAGTAAGTACTGGGGTGAGCG-

3'; Versalovic et al. 1991) primers. Conditions for PCR amplification were the same as for BOX-PCR, except the annealing temperature, which was 52 °C.

Analysis of the genetic fingerprinting patterns was performed with GelCompar II software (Applied Maths, Belgium). The Pearson correlation coefficient was applied, and cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The resulting dendrograms were analyzed in order to obtain groups of isolates with at least 85 % similarity. This cutoff was determined so that patterns that were known to be equal (molecular-weight size markers) would be considered to be in the same group. Representative isolates of each group were randomly selected. A total of 467 representative isolates were obtained: 419 from molecular typing and 48 isolates that did not yield clear profiles by either BOX- or ERIC-PCR.

### 16S rRNA gene amplification, phylogenetic affiliation, and multivariate analyses

Four hundred sixty-seven representative isolates were subjected to PCR amplification of 16S rRNA gene using the universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3'; Lane 1991) and 1492r (5'-GGTTACCTTGTTACGACTT-3'; Lane 1991) and the NZYTaq 2× Green Master Mix (NZYTech, Portugal) in 25-µL reactions: one cycle at 94 °C (5 min), 30 cycles at 94 °C (1 min), 55 °C (1 min), 72 °C (1.5 min), and a final cycle at 72 °C (10 min). Five isolates did not yield quality sequences after several attempts and were excluded from the analysis. The remaining 462 amplification products were purified with the DNA Clean & Concentrator™-5 (Zymoresearch, USA) according to manufacturer's instructions. Amplicons were partially sequenced with primer 27f using GATC Biotech services (Germany) according to company specifications. The partial 16S rRNA gene sequences obtained in this study were submitted to GenBank and are available under the accession numbers KT324749 to KT325209.

Quality sequences with an overall average of 893 bp were examined and edited with FinchTV V1.4.0 software (Geospiza, USA). The Basic Local Alignment Search Tool (BLAST; Altschul et al. 1997) and the EzTaxon server (Kim et al. 2012) were used to obtain taxonomic classification up to the genus level. Information from the representative isolates was used to associate taxonomic affiliation to the respective 658 isolates from the collection. Sequences were then subjected to the Ribosomal Database Project (RDP) pyrosequencing pipeline (Cole et al. 2014), where they were aligned and clustered into operational taxonomic units (OTUs) considering a distance limit of 0.03 (97 % similarity). Consequently, an OTU table was built with abundances for 21 samples: five for CAG—community from AG tissues from site C; five for CBG—BG tissues from site C; two for EAG—AG tissues

from site E; three for EBG—BG tissues from site E; three for BAG—AG tissues from site B; and three for BBG—BG tissues from site B. Three AG samples were collected from site E; however, only two yielded culturable bacteria. As such, only those were considered for subsequent analyses.

Using R version 3.1.1 (R Core Team 2014), several analyses were performed to characterize the bacterial communities regarding their OTU abundance. Diversity indices Shannon's  $H'$  diversity and Pielou's evenness were assessed with functions *diversity* and with *diversity/log(specnumber)*, respectively, from the *vegan* package (Oksanen et al. 2015). The *multipatt* function from package *indicspecies* (De Caceres and Legendre 2009) was used to find indicator species (here, OTUs) in samples from different sites, tissues and communities, using a 10 % significance level for selecting indicators. The species-site group association parameter "IndVal.g" was used, since it corrects for unequal group sizes.

The variation in OTU composition among the samples was analyzed by assessing differences in a dissimilarity matrix, built with Bray-Curtis dissimilarity measure (function *vegdist* from package *vegan* in R) after transforming the OTU abundance table with square root (function *sqrt* in R). PERMANOVA was used to analyze variances in the Bray-Curtis dissimilarity matrix using the *adonis* function (from package *vegan* in R) on the interaction between isolation site and tissue, and to both variables independently. For this analysis, the number of permutations was 999 in all tests.

Information in the Bray-Curtis dissimilarity matrix was then graphically assessed with a principal coordinate analysis (PCoA) using *cmdscale* function (from package *vegan* in R) to create the PCoA ordination. The significance of the observed patterns in the PCoA was analyzed by assessing differences in sample scores across PCoA axis 1 (PCoA1) and PCoA axis 2 (PCoA2). The normality test *shapiro.test* (*stats* package in R) revealed nonnormality in PCoA1 (Shapiro-Wilk:  $W=0.8848$ ,  $p$  value  $<0.05$ ) and normality in PCoA2 (Shapiro-Wilk:  $W=9352$ ,  $p$  value  $>0.1$ ). As a consequence, patterns in PCoA1 were assessed with Kruskal-Wallis rank sum test (*kruskal.test* from *stats* package in R) combined with *posthoc.kruskal.nemenyi.test* (from *PMCMR* package in R; Pohlert 2005) for samples from different sites and communities, and with Wilcoxon rank sum test (*wilcox.test* from *stats* package in R) for samples from different tissues. PCoA2 patterns were analyzed with *aov* for samples from different sites and communities, and with *t.test* for samples from different tissues.

### Screening of endophytic bacteria for enzymatic activity

The 467 representative isolates were individually screened, in triplicate, for different enzymatic activities: amylolytic, cellulolytic, lipolytic, pectinolytic (at pH 5.0 and 7.0), proteolytic, and xylanolytic. The ability of the isolates to degrade a

substrate was evaluated using 0.2 % starch, 0.5 % carboxymethyl cellulose, 1 % Tween 20, 0.5 % pectin, 1 % skim milk, or 0.5 % xylan, respectively, on TSA or MA culture medium. The basal culture medium (TSA or MA) was selected for each isolate, according to where best bacterial growth was obtained. Tween 20 and skim milk solutions were sterilized separately and only added to the culture media after cooling down. The agar medium plates were inoculated with 5  $\mu$ L of a bacterial suspension with optical density (OD) at 600 nm = 0.8 U and incubated at 30 °C until sufficient growth was obtained, or up to 120 h.

The enzymatic activities were assessed according to the presence (positive result) or absence (negative result) of a halo surrounding the bacterial growth. Amylolytic activity was detected when a clear halo was observed upon addition of Lugol solution to the agar plate. Cellulolytic and xylanolytic activities were detected when a clear halo was observed upon incubation with a 1 % Congo red solution (with 10 % ethanol) and subsequent wash with 1 M NaCl. Lipolytic activity was observed by the formation of a white precipitate around the bacterial growth. Pectinolytic activity was detected by formation of a clear halo after addition of 1 % hexadecyltrimethylammonium bromide (CTAB) solution. Proteolytic activity was revealed by visualization of a clear halo against an opaque background. The results were interpreted according to the visibility of the halo obtained: negative, no halo observed; positive, faint to intense halo observed.

### Screening of endophytic bacteria for plant growth promotion traits

Representative isolates were individually screened for different PGP traits: ACC deaminase activity, IAA production, phosphate solubilization, and siderophore production. Isolates were incubated in adequate growth media at 30 °C until sufficient growth was obtained. Unless otherwise stated, strains for negative and positive controls were studied in Pereira et al. (2013).

To determine ACC deaminase activity, isolates were grown in DF salt minimal medium (Dworkin and Foster 1958) or variations of this medium. Basal DF was prepared as follows (per liter): 4 g  $\text{KH}_2\text{PO}_4$ , 6 g  $\text{Na}_2\text{HPO}_4$ , 0.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g glucose, 1 mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 10  $\mu\text{g}$   $\text{H}_3\text{BO}_3$ , 10  $\mu\text{g}$   $\text{MnSO}_4$ , 70  $\mu\text{g}$   $\text{ZnSO}_4$ , 50  $\mu\text{g}$   $\text{CuSO}_4$ , 10  $\mu\text{g}$   $\text{MoO}_3$ , 12 g agar, pH 7.2. This medium was either supplemented with 3 mM ACC (DF + ACC; ACC was sterilized by filtration and added to cooled down medium) or 2 g  $\text{L}^{-1}$   $(\text{NH}_4)_2\text{SO}_4$  (DF + ammonium sulfate) or no nitrogen source (DF). The ability to use ACC as a sole nitrogen source is a consequence of the activity of the enzyme ACC deaminase (Penrose and Glick 2003). *Pseudomonas putida* EAPC8 and *Arthrobacter rombi* EC32A were used as positive controls for ACC deaminase activity.

To test for IAA production, the method of Gordon and Weber (1951) was used: for each isolate, 500  $\mu\text{L}$  of supernatant obtained from cultures grown in MB or TSB + L-tryptophan (1 %) was mixed with 1 mL of Salper solution. After incubating in the dark for 30 min, OD was read at 535 nm. Using a calibration curve of pure IAA as a standard, IAA concentrations ( $\mu\text{g mL}^{-1}$ ) were obtained for each isolate. *Pseudomonas putida* EAPC8 and *Pseudomonas fluorescens* S3X were used as positive controls for IAA production.

The ability of isolates to solubilize phosphate was screened on National Botanical Research Institute's Phosphate (NBRIP) solid growth medium (Nautiyal 1999). After inoculation and appropriate incubation, phosphate solubilization was observed as a clear halo surrounding the bacterial growth. *Pseudomonas reactans* EDP28, *P. putida* EAPC8, *A. rombi* EC32A, and *P. fluorescens* S3X were used as negative controls for phosphate solubilization, and *Arthrobacter nicotinovorans* EAPAA and *Rhodococcus* sp. EC35 were used as positive controls.

In order to test for siderophore production, all glass materials used were deferrated by treating with 6 % nitric acid and extensively washing with sterile double-distilled water. Culture media deferration was conducted on TSA and MA medium as described by Cox (1994). After inoculation and incubation on either medium, the plates with bacterial growth were overlaid with CAS (O-CAS; Pérez-Miranda et al. 2007) and incubated with this overlay for 2 h. Siderophore production was detected as a change of color of the overlay from blue to orange or purple. This assay was only performed with TSA medium, since the O-CAS reacted with MA medium preventing a clear reading of the result. *Pseudomonas reactans* EDP28, *P. putida* EAPC8, *A. rombi* EC32A, *P. fluorescens* S3X, *A. nicotinovorans* EAPAA, and *Rhodococcus* sp. EC35 were used as positive controls for siderophore production.

The representative isolates were also assessed for their nitrogen fixing abilities by PCR-screening for the presence of *nifH* gene: the primers IGK3 and DVV (Ando et al. 2005), described as the best pair of primers to use in *nifH* amplification (Gaby and Buckley 2012) were used with the NZYTaq 2 $\times$  Green Master Mix (NZYTech, Portugal) in 25  $\mu\text{L}$  reactions. Conditions for PCR amplification were as follows: initial denaturing at 95  $^{\circ}\text{C}$  for 10 min, followed by 35 cycles of denaturing at 95  $^{\circ}\text{C}$  for 45 s, annealing at 56  $^{\circ}\text{C}$  for 30 s, and extending at 72  $^{\circ}\text{C}$  for 1 min. A final extension was performed at 72  $^{\circ}\text{C}$  for 10 min. *Rahnella aquatilis* M72troncoD (HQ538817; Proença et al. 2010) was used as a positive control, and *Escherichia coli* ATCC25922 was used as a negative control (Gaby and Buckley 2012). The resulting PCR products were observed in a 2 % agarose gel electrophoresis.

## Results

### Metal(loid) quantification of sediments and plant tissue

Five metals (Cr, Ni, Cu, Zn, and Hg) and the metalloid As were quantified from samples collected at sites C, E, and B, in sediment samples from monospecific stands of *H. portulacoides*, in AG and BG tissues. The results are presented in Table 1. For some replicates of Hg concentration determination in sites C and E, the obtained values were below detection limits ( $<2 \mu\text{g L}^{-1}$ ). In these cases, half of the lowest detectable concentration was used in statistical analyses.

Sediment samples collected from sites E and B presented concentrations at least 3-fold higher than samples from site C for all metal(loid)s tested ( $p$  value  $<0.05$ ). For As and Hg, a contamination gradient could be observed, where site B (closest to the effluent discharge) had the most contaminated sediment samples and site E presented intermediate contamination ( $p$  value  $<0.05$ ). Remarkably, sediment Hg concentration was, on average, 31 (site E) to 135 (site B) times higher than in samples from site C. All sediment samples were more contaminated than plant tissue samples, in all sites, for all tested metal(loid)s ( $p$  value  $<0.05$ ), except Hg in site C, where the differences in the metal concentration did not present statistical significance.

Considering metal(loid) contents in each site separately, we observed that in site B, metal(loid)s concentration in AG tissues were significantly higher than that in BG tissues. The same was observed for Ni in site E ( $p$  value  $<0.05$ ). Remarkably, Hg contamination of BG tissues in site B was 33-fold higher than in site C.

Considering metal(loid) contents in each type of tissue separately, we saw that BG tissues were significantly more contaminated with Cr, Ni, and Hg in site B than in site C. In the specific cases of As in AG tissues and Hg in both tissues, a significant gradient was observed:  $C < E < B$  ( $p$  value  $<0.05$ ).

### Phylogenetic diversity of endophytic bacteria, community structure, and multivariate analyses

Endophytic bacteria from sites C, E, and B were isolated from surface sterilized AG and BG tissues from eleven plants, in TSA, MA, and R2A culture media. Extensive results for CFU  $\text{g}^{-1}$  (FW) for all sampled plants are stated in Supplementary Table S1. AG tissues from site C presented significantly more CFU  $\text{g}^{-1}$  than BG tissues ( $p$  value  $<0.05$ ). The opposite was observed in sites E ( $p$  value  $<0.05$ ) and B (only for TSA culture medium;  $p$  value  $<0.05$ ).

The selection of morphologically distinct colonies from all culture media, plant tissues, and sampling sites resulted in a collection of 665 endophytic isolates. Molecular typing of the collection yielded 467 representative isolates (150 from CAG community, 74 from CBG, 18 from EAG, 94 from EBG, 33

**Table 1** Metal(loid) concentrations in sediment and plant tissue samples

Sample	Site	Cr	Ni	Cu	Zn	As	Hg
Sediment							
	C	4.97±0.67	3.01±0.39	3.44±0.30	12.35±1.51	3.05±0.37	0.02±0.01
	E	14.16±2.05	8.96±1.24	11.47±1.60	53.64±8.78	13.72±2.07	0.57±0.01
	B	13.74±1.90	9.13±1.32	15.43±2.10	67.82±10.75	20.93±3.34	2.48±0.17
BG tissues							
	C	0.76±0.31	0.51±0.22	1.82±0.65	8.69±3.82	0.86±0.55	0.01±0.00
	E	1.19±0.32	0.96±0.15	2.54±0.89	16.08±7.66	8.45±7.94	0.04±0.01
	B	1.89±0.20	1.60±0.28	3.24±0.38	22.47±3.92	4.20±0.80	0.25±0.03
AG tissues							
	C	0.69±0.21	0.47±0.23	1.77±1.57	5.31±1.59	0.40±0.13	0.01±0.00
	E	0.53±0.24	0.21±0.06	0.33±0.10	5.52±1.72	0.35±0.02	0.01±0.00
	B	0.82±0.15	0.52±0.15	0.53±0.12	7.26±2.19	1.03±0.25	0.07±0.02

Concentrations are given as mg kg<sup>-1</sup> (fresh weight) and presented as mean of three replicates ± standard deviation  
 AG aboveground, BG belowground

from BAG, and 97 from BBG), for which partial sequences of 16S rRNA gene were obtained. Five isolates did not yield quality sequences after several attempts and were excluded from the analysis, resulting in 462 amplification products. Taxonomic classification yielded matches with similarity ranging from 92.79 to 100 % similarity. Twenty-nine of 462 isolates (6.3 %) exhibited an identity percentage lower than 97 with the closest match, suggesting the potential for novel species in the collection. Taxonomic information was then affiliated to the respective 658 isolates.

The 658 isolates were distributed amongst class-level identification and grouped according to isolation site and tissue in Fig. 1. The phylum Proteobacteria (mainly classes alpha- and gamma-Proteobacteria) clearly dominated the collection of endophytic bacteria (64.29 % of 658 isolates; Fig. 1 and Supplementary Table S2), followed by Actinobacteria (22.8%) and Firmicutes (10.33%). The phylum Bacteroidetes was also present, but less represented (2.58 %). Figure 1 also shows that alpha-Proteobacteria are highly represented in all six communities, and that gamma-Proteobacteria, although present in all communities, are most abundant in site C. Isolates from classes Bacilli and Actinobacteria are present in all communities, except Actinobacteria in the community with the lower number of isolates (EAG, 22 isolates).

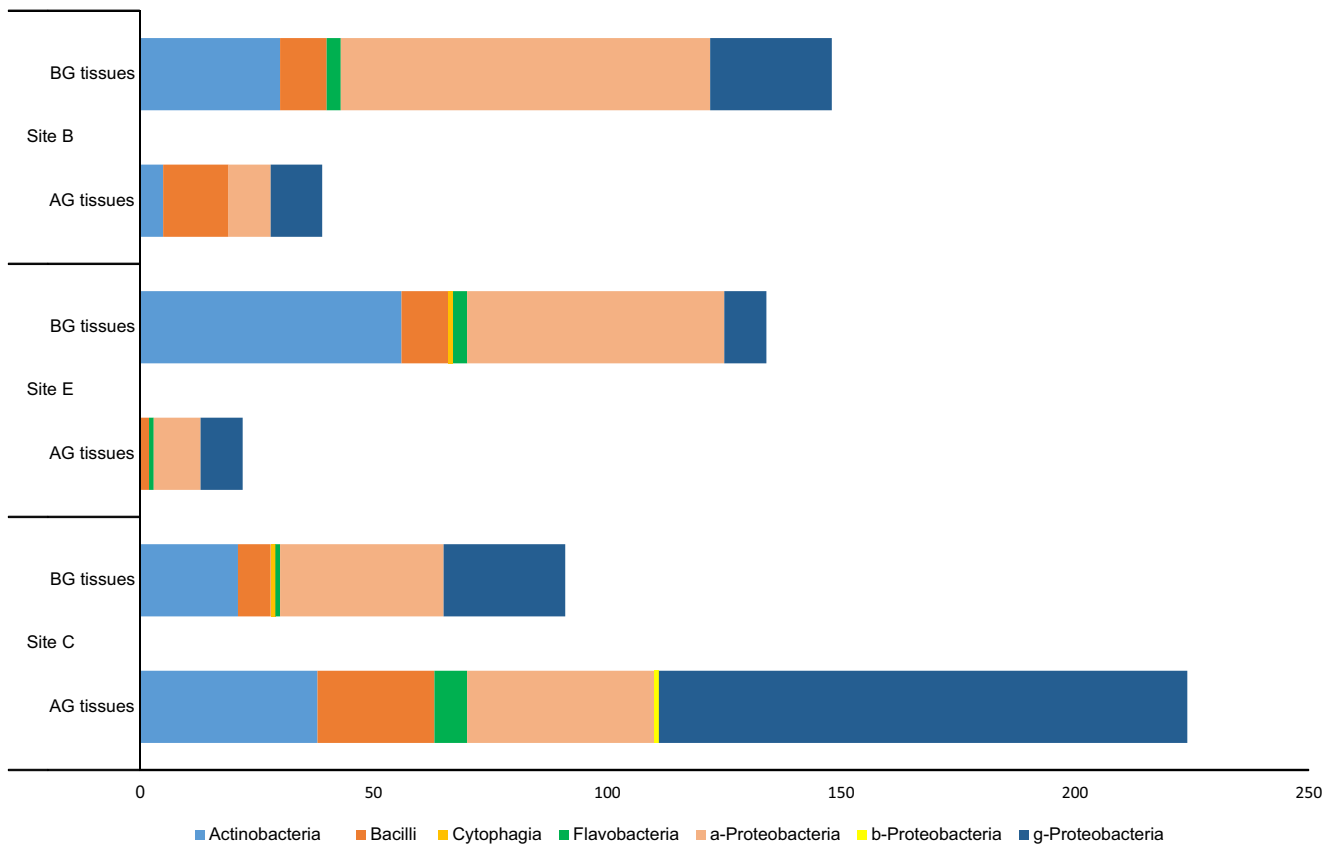
The most common families of bacteria in the collection were Vibrionaceae (51 of 658 isolates, 7.75 %) Microbacteriaceae (85/658 isolates, 12.92 %), Erythrobacteraceae (87/658 isolates, 13.22 %), and Halomonadaceae (98/658 isolates, 14.89 %). The complete distribution of the 658 isolates across 79 genera in all six communities is presented in Supplementary Table S2. Overall, the most abundant genera were *Altererythrobacter*, *Marinilactibacillus*, *Microbacterium*, *Salinicola*, and *Vibrio*, with 49, 42, 67, 58, and 51 isolates, respectively. *Altererythrobacter* was the only genus present in all six

communities. Isolates belonging to the genera *Altererythrobacter*, *Bacillus*, *Citromicrobium*, *Demequina*, *Labrenzia*, *Marinilactibacillus*, *Martellella*, *Microbacterium*, *Micrococcus*, *Pseudomonas*, *Sphingorabdus*, *Stakelama*, *Vibrio*, and *Zunongwangia* were present in all sites. Isolates belonging to 29 of the 79 genera were present in both BG and AG tissues.

Notably, all 58 isolates (8.8 % of the collection) associated with the genus *Salinicola* were exclusively isolated from site C. *Hoeflea*, *Labrenzia*, and *Microbacterium* were exclusive and considerably dominant in BG tissues, with 17 (2.6 % of the collection), 22 (3.3 %), and 67 (10.2 %) isolates, respectively.

An OTU table was built with abundances across all samples, using the RDP pyrosequencing pipeline. The OTUs were defined considering a distance limit of 0.03 (97 % similarity) and their distribution across communities is stated in Table 2. Analysis of indicator OTUs showed that the OTUs, here annotated with the corresponding genera, that contributed most for the characterization of each sampling site were as follows: OTU\_005 (*Salinicola* spp., 58 isolates, *p* value < 0.05) for site C, and OTU\_077 (*Bacillus* spp., 11 isolates, *p* value < 0.05) and OTU\_028 (*Rhizobium* spp., 6 isolates, *p* value < 0.05) for site B. This analysis also showed that indicator OTUs for the isolation tissue were as follows: OTU\_104 (*Zunongwangia* spp., 5 isolates, *p* value < 0.05) for AG tissues, and OTU\_033 (*Labrenzia* spp., 22 isolates, *p* value < 0.05) and OTU\_219 (*Microbacterium* spp., 44 isolates, *p* value < 0.05) for BG tissues.

A Bray-Curtis dissimilarity matrix was computed on a square root-transformed OTU table which allowed comparison of bacterial communities of all samples. The resulting matrix was tested for significant differences with PERMANOVA (*adonis* function in R). The interaction between isolation site and tissue was significant (*adonis*:



**Fig. 1** Distribution of 658 endophytic isolates by class amongst isolation sites (B, E and C) and plant tissues (aboveground and belowground). a-, b- and g-Proteobacteria refers to alpha-, beta- and gamma- Proteobacteria, respectively

$F_{2,15} = 1.38$ ,  $R^2 = 0.12$ ,  $p$  value  $< 0.05$ ), as was the test for each independent variable: isolation site (adonis:  $F_{2,18} = 1.65$ ,  $R^2 = 0.14$ ,  $p$  value  $< 0.01$ ) and tissue (adonis:  $F_{1,19} = 2.08$ ,  $R^2 = 0.09$ ,  $p$  value  $< 0.01$ ). However, only 23 % of the variation in the bacterial composition was explained by both variables and 12 % by their interaction.

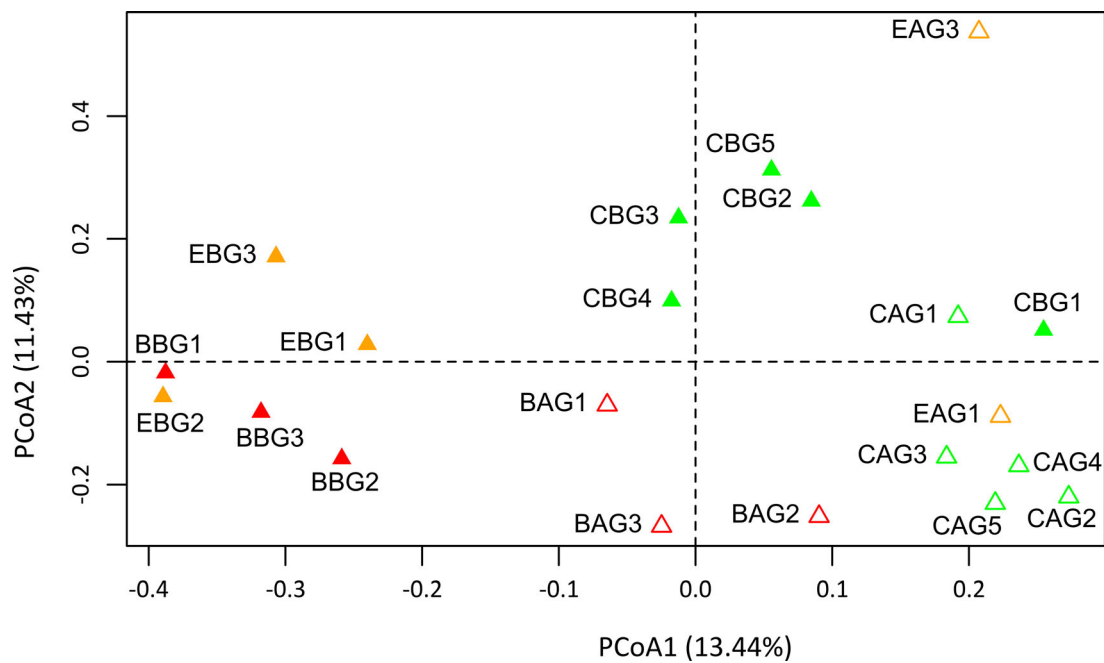
A PCoA plot was generated based on the Bray-Curtis dissimilarity matrix to compare all bacterial communities

(Fig. 2). The PCoA plot shows a clear gradient across axis PCoA1 which coincides with the presence of contamination, as samples with most contamination (BG tissues from sites B and E, see Table 1) are grouped in the left side of the plot. Analysis of sample scores across PCoA1 revealed that there are significant differences in OTU composition in isolation sites (Kruskal-Wallis chi-squared = 7.66,  $df = 2$ ,  $p$  value  $< 0.05$ ) and in isolation tissues (Wilcoxon rank sum test:

**Table 2** Analysis of 16S rRNA gene partial sequences with RDP pipeline

	CAG	CBG	EAG	EBG	BAG	BBG
Sequence information						
Number of isolates	224	91	22	134	39	148
Average 16S rRNA gene sequence length (bp)	851	876	961	837	937	896
Number of OTUs	52	31	10	41	18	38
Indices						
Average Shannon's $H'$	2.08	2.18	1.20	2.66	1.64	2.57
Average Pielou's evenness	0.84	0.90	0.76	0.89	0.92	0.88

Sequence and operational taxonomical unit (OTU) information for all isolation sites (C, E, and B) and tissues (aboveground (AG), belowground (BG)) are listed. Clustering was based on a 3 % divergence cutoff. Average values for Shannon's  $H'$  diversity index and Pielou's evenness, computed with R, are also presented for each community



**Fig. 2** Principal coordinates analysis (PCoA) plot based on 16S rRNA gene partial sequences from twenty one endophytic bacterial samples from all isolation sites and tissues. Distances between samples were calculated with Bray–Curtis dissimilarity measure on a square root-transformed OTU table. PCoA axes PCoA1 and PCoA2 are plotted

with the corresponding percentages of explained variation. *CAG* aboveground (AG) tissues from site C, *CBG* belowground (BG) tissues from site C, *EAG* AG tissues from site E, *EBG* BG tissues from site E, *BAG* AG tissues from site B, *BBG* BG tissues from site B

$W=93$ ,  $p$  value  $<0.01$ ). This was not observed in PCoA2 for either isolation sites (ANOVA:  $F_{2,18}=2.53$ ,  $p$  value  $>0.1$ ) nor tissues ( $t$  test:  $t=-1.8$ ,  $df=14.97$ ,  $p$  value  $>0.05$ ).

**Enzymatic activity and plant growth promotion traits in endophytic bacteria**

Assays for enzymatic activities and PGP traits were successful for 89–100 % of the representative isolates. Test success for siderophore production was 85.2 %; the remaining isolates ( $n=69$ ) were not able to grow in TSA medium, only MA, where a reaction occurred with the CAS-overlay and interfered with the interpretation of the result. Screening results are stated in Table 3 and the extensive list of results for all isolates is in Supplementary Table S3.

Only 5.6 to 22.1 % of isolates tested positive for pectinolytic activity. A great percentage of isolates tested positive for all other assays (up to 73.2 %), especially IAA production and ACC deaminase activity (98.9 and 51.2 %, respectively). IAA production was detected in 464 isolates, with concentrations ranging from 0.48 to 206  $\mu\text{g mL}^{-1}$ . Seventeen IAA producers presented high levels of IAA concentration, over 100  $\mu\text{g mL}^{-1}$ , 15 of which belonged to Proteobacteria phylum (5 *Pseudomonas* sp.).

Isolates belonging to six genera (one *Chromohalobacter*, one *Halomonas*, one *Marinomonas*, one *Marinospirillum*, one *Micrococcus*, and seven *Vibrio*) tested positive for amylolytic, cellulolytic, lipolytic, pectinolytic (at pH 7), proteolytic, and

xylanolytic activities. From the 97 isolates that tested positive for *nifH* amplification, 74 belonged to the Proteobacteria phylum, including 19 *Vibrio* sp. isolates. The most noteworthy genera were *Altererythrobacter* (from the 31 isolates of this genus, 29 were lipolytic and 24 xylanolytic, 22 presented both of these activities), *Marinilactibacillus* (24 cellulolytic, 18 proteolytic and 39 xylanolytic isolates, out of 41 isolates from this genus), *Microbacterium* (32 amylolytic, 30 cellulolytic and 29 xylanolytic isolates, from 39 *Microbacterium* sp. isolates), and *Vibrio* (14 to 32 isolates producing every enzyme tested, from a total of 33 *Vibrio* sp. isolates). Isolates associated to five genera (one *Altererythrobacter*, one *Ensifer*, one *Oceanibulbus*, one *Stakelama*, and two *Vibrio*) displayed positive results for all tested PGP traits assays (Supplementary Table S3).

The community-based and class-level distribution of representative isolates that tested positive for the assayed enzymatic activities and PGP traits are represented in Fig. 3 and Supplementary Fig. S1, respectively. The class-level distribution of isolates that tested positive in all assayed enzymatic activities and PGPs was a reflection of the class-level and community-based distribution of all isolates of our collection (Fig. 1 and Table 2, respectively), i.e., the classes with higher representability in the endophytic collection were also those that presented more isolates that tested positive for the assayed enzymes and traits. This suggests that the probability of the presence of traits in a given class depends on the number of isolates in that class, instead of depending on the site or tissue of isolation.



**Table 3** Screening of bacterial endophytes for enzymatic activity and plant growth promotion traits

	Enzymatic activity						
	Amylolytic	Cellulolytic	Lipolytic	Pectinolytic (pH 5.0)	Pectinolytic (pH 7.0)	Proteolytic	Xylanolytic
Tested	450	442	416	72	230	446	450
Positive	186 (39.8 %)	281 (60.2 %)	215 (46.0 %)	26 (5.6 %)	103 (22.1 %)	161 (34.5 %)	342 (73.2 %)
Negative	264 (56.5 %)	161 (34.5 %)	201 (43.0 %)	46 (9.9 %)	127 (27.2 %)	285 (61.0 %)	108 (23.1 %)
	PGP trait						
	ACC deaminase activity	IAA production	Presence of <i>nifH</i> gene	Phosphate solubilization	Siderophore production		
Tested	465	465	467	465	398		
Positive	239 (51.2 %)	460 (98.5 %)	97 (20.8 %)	64 (13.7 %)	152 (32.5 %)		
Negative	226 (48.4 %)	5 (1.1 %)	370 (79.2 %)	401 (85.9 %)	246 (52.7 %)		

Number and percentage of isolates that were successfully tested out of 467 representative isolates (“tested”), isolates that tested positive (“positive”), and isolates that tested negative (“negative”) in trait assays

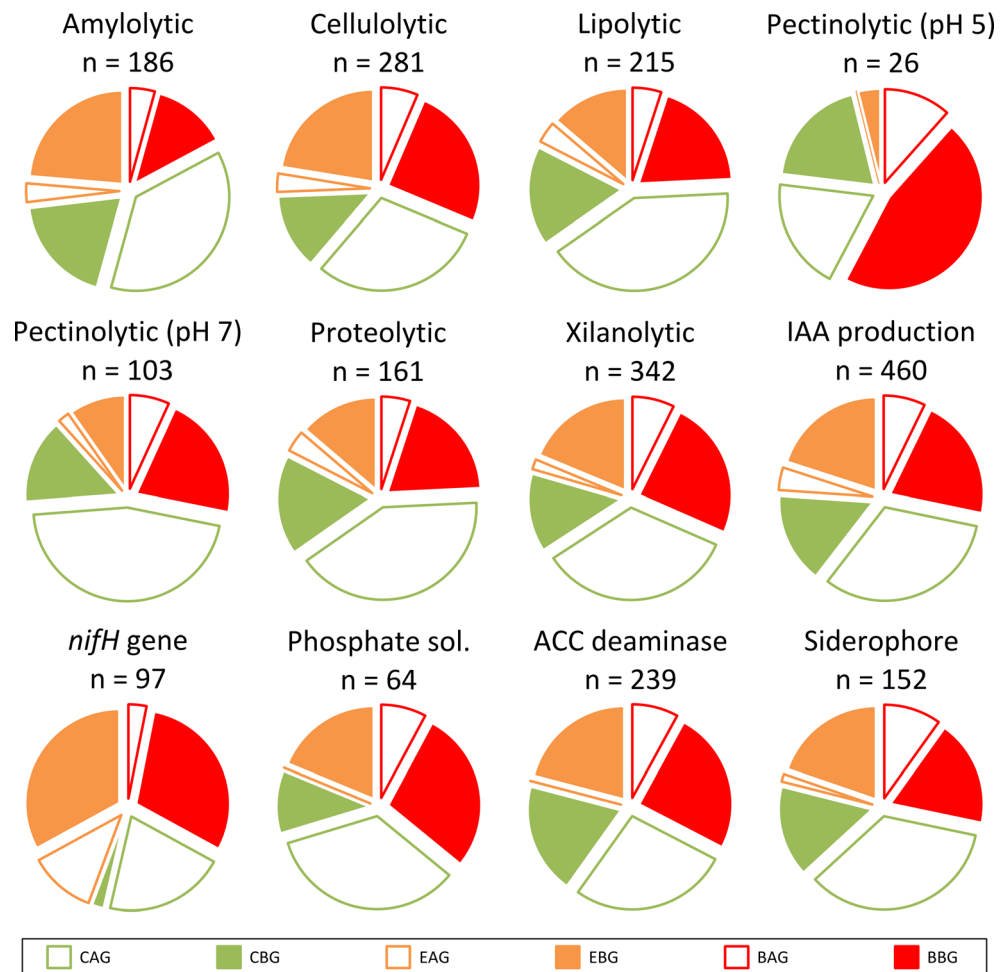
## Discussion

The main aim of this study was to elucidate the structure, diversity, and function of culturable endophytic bacterial communities from the salt marsh plant *H. portulacoides*. To our knowledge, this is the first study regarding the isolation, identification, and functional characterization of endophytic bacteria in

healthy specimens of the halophyte *H. portulacoides*. Bacteria were isolated from AG and BG tissues from the halophyte, collected from two sites contaminated with industrial effluents and one noncontaminated control site.

Several authors have verified that a gradient of contamination exists, from Laranjo Bay to nearshore, especially regarding sediment Hg contamination (reviewed in Pereira et al.

**Fig. 3** Distribution of isolates that tested positive for enzymatic activity assays and plant growth promotion traits from 467 representative isolates. Isolates are grouped according to isolation community (site and tissue). *n* represents the number of isolates that tested positive for each trait. Amylolytic, cellulolytic, lipolytic, pectinolytic (at pH 5.0 and 7.0), proteolytic, and xylanolytic activities are represented; ACC deaminase (as growth in DF + ACC medium), IAA production, presence of *nifH* gene, ability to solubilize phosphate, and siderophore production are also represented. *CAG* aboveground (AG) tissues from site C, *CBG* belowground (BG) tissues from site C, *EAG* AG tissues from site E, *EBG* BG tissues from site E, *BAG* AG tissues from site B, *BBG* BG tissues from site B



2009). In the present study, sediment samples presented higher metal(loid) contents in sites closest to the industrial effluent discharges (sites B and E). In fact, for As and Hg, a significant concentration gradient could be observed, where content in  $B > E > C$ . These metal(loid)s were among the known main contaminants in the industrial effluents in Laranjo Bay, as is stated in the report by Costa and Jesus-Rydyin (2001).

Several studies have found that *H. portulacoides* mainly accumulates metals in the BG tissues and that BG to AG translocation is very low (e.g., Válega et al. 2008a; Válega et al. 2008b; Anjum et al. 2011). The same pattern was observed in BG and AG tissues in this study, for all tested metals in site B (up to 6 times more metal content than in AG tissues) and for Ni in site E (average of 4.5 times more Ni in BG than in AG tissues). This allocation pattern was described by Lozano-Rodriguez et al. (1997) as a strategy for protecting the more sensitive AG tissues from the effects of metal-induced stress.

We analyzed a much higher number of isolates than what is usually observed in studies focusing endophytic bacteria. This could be attributed to the fact that we used three culture media with different nutritional compositions and that the plate incubation period for the isolation of the endophytic bacteria was long (72 h). Sample sizes were considerably smaller for EAG and BAG communities and, as a consequence of this unbalance dataset, analyzing indicator OTUs may raise concerns. However, when using the parameter “IndVal.g” in the *indicspecies* package in R, there is a correction for unequal group sizes in the calculations for indicator species (OTUs in our case). The quantity “A,” used in calculating the IndVal, is obtained by using the mean abundance of the species in the target site group divided by the sum of the mean abundance values over all groups. In using the sum of the mean abundance values over all groups, instead of using real abundances from all groups, the unequal sizes of the site groups are controlled for (De Caceres and Legendre 2009). Regarding the statistical analysis performed based on a dissimilarity measure (Bray-Curtis), we minimized the most relevant intrinsic bias of this measure (giving too much importance to dominant OTUs and overseeing rare ones) by applying a square root transformation to our OTU abundance table prior to obtaining the Bray-Curtis dissimilarity matrix. The selection of the index at hand was based on (i) its applicability to assess differences between communities using abundance data, (ii) its wide use in the field of ecology and microbiology for this purpose, and (iii) its suitability to answer our questions.

We found considerable culturable bacterial diversity in the internal tissues of *H. portulacoides* from all sampled sites. It is widely accepted that the culturable fraction of plant-associated microbes corresponds to a small fraction of the real diversity, as a consequence of growth medium inadequacy (Tanaka et al.

2014), and limitations to reproduce optimal growth conditions. Nevertheless, the culture-dependent approach allows for extensive functional characterization of the isolates in the collection, which is not possible with culture-independent approaches. Additionally, traditional methods of culture-dependent techniques are useful for the determination of the overall effect of metal contaminants in bacterial communities (Ellis et al. 2001).

As stated above, in our study, the number of isolates obtained from EAG and BAG samples was considerably lower than for other samples. Since the method for obtaining endophytic bacteria includes a harsh surface sterilization process, the viability of endophytes in the samples could be compromised. However, we do not believe this to be the main reason for such a disparity in the number of isolates in the different samples, since the same process was applied to all samples. The isolation efforts were the same for all samples, and as such, the difference in numbers of obtained endophytic isolates may be a reflection of these AG tissues being less populated, since the highest bacterial densities are usually observed in BG tissues, and decrease toward AG tissues (Lodewyckx et al. 2002). In fact, we observed a significantly lower average number of CFU per gram (FW) obtained from EAG and BAG samples, when compared with the respective BG tissues (Supplementary Table S1). This pattern was not, however, observed for AG and BG tissues from site C, where the CAG community presented a significantly higher average number of CFU per gram (FW) than CBG. This suggests that the degree of soil and plant tissue contamination could also play a role in the average number of culturable bacteria. This latter effect has been previously reported for bacterial soil communities, where the presence of metal contaminants negatively influenced the ability of readily culturable bacteria to replicate in laboratory media (Ellis et al. 2003).

Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were the phyla recognized in the endophytic collection from this study. This result is in accordance with studies describing the phyllosphere and endosphere assemblages in other plant hosts (reviewed in Bulgarelli et al. 2013), including halophytes (Mora-Ruiz et al. 2015), and the rhizosphere of *H. portulacoides* (Oliveira et al. 2014b). The genera *Altererythrobacter*, *Marinilactibacillus*, *Microbacterium*, *Salinicola*, and *Vibrio* were the most abundant in our collection. To our knowledge, this is the first time an isolate belonging to the genus *Marinilactibacillus* was obtained from the endosphere.

Isolates affiliated to the genus *Altererythrobacter* have been previously identified in the endosphere (e.g., Liu et al. 2014). In our collection, 73.5 % of isolated *Altererythrobacter* spp. were collected from contaminated sites (E and B). Some species of *Altererythrobacter* are described as able to resist metals (Wu et al. 2014), a trait that could be useful in the endosphere of plants colonizing contaminated locations.

*Marinilactibacillus* spp. have been detected in marine environments (e.g., Toffin et al. 2005) and detected with culture-independent methods in olive fermentation brine (Lucena-Padrós et al. 2015). Isolates belonging to this genus have also been isolated from many types of cheese, including as part of consortia that were able to reduce the counts of the human pathogen *Listeria monocytogenes* (e.g., Roth et al. 2010), indicating antimicrobial activities which could be useful in the endosphere environment.

Isolates from the genus *Microbacterium*, exclusively isolated from BG tissues in this study, have been thoroughly characterized and detected in many environments, including the rhizosphere of ginseng (Kim et al. 2015) and the endosphere of crops and prairie plants (Zinniel et al. 2002). Recently, three novel species from this genus were described from the endosphere of *H. portulacoides* (Alves et al. 2014; Alves et al. 2015). PGP traits have been previously associated with this genus (see below), suggesting a high importance in the endosphere.

Isolates from the genus *Salinicola* have been previously obtained from diverse environments (e.g., deep sea surface sediments, Romanenko et al. 2013) including the endosphere of a halophyte (Mora-Ruiz et al. 2015). To the best of our knowledge, PGP traits have not been explored in this genus. The prosperity of *Salinicola* spp. in marine environments could be related to their presence in the endosphere of the halophyte at study.

*Vibrio* spp. have been abundantly detected in aquatic environments and organisms (Thompson et al. 2004) and in the endosphere (e.g., seagrass, Jose et al. 2014). PGP traits have been associated with this genus (see below), suggesting that this widely known pathogen in humans and other animals may have a positive role in the endosphere.

The genera *Pseudomonas* and *Micrococcus*, less represented in this study (15 and 9 isolates, respectively), were previously detected in the endosphere of *H. portulacoides* in a study regarding hydrocarbon-degrading bacteria (Oliveira et al. 2014a).

From the 462 isolates retrieved in this study, 29 isolates presented less than 97 % similarity with the closest 16S rRNA gene sequence in GenBank. These isolates may potentially belong to novel species, revealing the endosphere of *H. portulacoides* as a source for undescribed phylogenetic groups.

As reviewed in Bulgarelli et al. (2013) for many plant hosts and discussed in Oliveira et al. (2014b) for *H. portulacoides*, plant-related factors contribute to the composition of plant-associated bacterial communities. In this work, 14 genera were isolated from all sites, suggesting the presence of a common structure associated to the studied halophyte. On the other hand, differences were observed, where considerably abundant genera were exclusively isolated from one site (e.g., *Salinicola* spp. from site C).

In the present study, *Altererythrobacter* spp., *Marinilactibacillus* spp., *Microbacterium* spp., and *Vibrio* spp. isolates were abundantly detected in all sites, suggesting an ability to resist metals, which has been previously described in these genera (Wu et al. 2014; Sheng et al. 2008; Jafari et al. 2015) except, to the best of our knowledge, for the genus *Marinilactibacillus*. OTUs belonging to the genera *Bacillus* and *Rhizobium*, albeit less represented in the endophyte collection (11 and 6 isolates, respectively), were considered indicator OTUs for site B, and metal resistance has previously been associated with these genera (Ma et al. 2015; Aafi et al. 2015). Isolates belonging to *Salinicola* spp. ( $n=58$ ) were exclusively detected in site C, and the indicator OTU for site C was also attributed to this genus. Information on whether *Salinicola* spp. can tolerate metal contamination is lacking in the literature; however, considering the number of isolates obtained from this genus and the extension of our collection, we suggest that it should be further investigated as to its ability as a bioindicator.

Usually, bacteria access plant tissues through natural entry points in the root tips and lateral roots emergence points, as a consequence of root growth. Once inside the plant, endophytes may colonize the plant by migration from BG to AG tissues. Additional penetration may occur through natural openings on leaves such as stomata, or through leaf wounds (reviewed in Lodewyckx et al. 2002). Here, we found that BG and AG tissues shared 20 bacterial genera, suggesting that migration from BG to AG tissues may occur in *H. portulacoides*. On the other hand, isolates belonging to the genera *Hoeflea* (17), *Labrenzia* (22), and *Microbacterium* (67) were only isolated from BG tissues, and the two latter genera were identified as indicator OTUs for these tissues by our indicator species analysis. The genera *Hoeflea* and *Labrenzia* have been isolated from root endosphere of a salt marsh halophyte (Bibi et al. 2014) and, to the best of our knowledge, have not been detected in AG tissues of plants, as was the case in our study. On the other hand, *Microbacterium* spp. has been detected in AG tissues of *H. portulacoides* (e.g., Alves et al. 2014). An OTU attributed to the genus *Zunongwangia* was considered indicator OTU for AG tissues in our analysis. Although this genus has been isolated from the rhizosphere of mangrove trees (Rameshkumar et al. 2014) and detected by pyrosequencing in the endosphere (Mora-Ruiz et al. 2015), it had not been previously isolated in the endosphere.

We found that the bacterial composition across our collection is significantly affected by the isolation site and tissue, although these factors only account for 23 % of the total variation observed in our data. Nevertheless, a variation in composition could be observed across the gradient of contamination in our sampling sites, suggesting that the contamination levels do, in fact, affect the composition of the culturable endosphere of *H. portulacoides*. Other variation parameters

(e.g., soil water content, organic matter content, fine particle content, redox potential, pH, and conductivity; Válega et al. 2008b) could be explored in order to further understand the reasons for composition variation in the culturable endosphere of this halophyte.

Many isolates from the collection obtained in this study tested positive for several enzymatic activities and PGP traits, exposing the endosphere of the halophyte *H. portulacoides* as a novel source of bacteria and bacterial compounds with potential biotechnological applications. The ability to produce these extracellular enzymes and PGP traits is well distributed across phylogenetic groups, as the most represented classes (Fig. 1) amply exhibited all tested traits, except Bacilli for phosphate solubilization (Supplementary Fig. S1). This ability is also well represented, overall, in all isolation sites and tissues (Fig. 3). The ability to produce industrially important extracellular enzymes was abundantly detected in isolates associated to the genera *Altererythrobacter*, *Marinilactibacillus*, *Microbacterium*, and *Vibrio*. To our knowledge, the production of the tested enzymes had not been previously described in *Altererythrobacter* spp. and *Marinilactibacillus* spp.

To the best of our knowledge, the PGP traits tested in the presented study were not previously evaluated in *Altererythrobacter* spp. isolates. Here, we found that isolates from this genus tested positive for one to six extracellular enzymatic activities and presented one to five PGP traits. Overall, all tested enzymes and traits were produced by isolates belonging to his genus.

Traits for *Marinilactibacillus* spp. isolates from other studies have not been deeply characterized. Here, we found that *Marinilactibacillus* spp. isolates tested positive for one to five extracellular enzymatic activities, and presented one to four PGP traits. Considering all the isolates from this genus, all of the tested enzymes and traits were produced, except pectinase at pH 5.

The ability to promote plant growth has been previously detected in the genus *Microbacterium* with traits as ACC deaminase (Sheng et al. 2008), IAA production, phosphate solubilization (Kukla et al. 2014), and siderophore production (Pereira et al. 2013). The presence of the *nifH* gene was also detected (Zakhia et al. 2006). Production of protease and lipase (Pereira et al. 2013), xylanase, amylase (Park et al. 2006), cellulase (Kukla et al. 2014), and pectinase (Vinod et al. 2014) has been characterized in *Microbacterium* spp. Antimicrobial activity and resistance to metals have also been reported in this genus (Sheng et al. 2008; Passari et al. 2015). In the present study, one to five of the abovementioned extracellular enzymes were also detected in all *Microbacterium* spp. isolates, as were one to four PGP traits. Considering all the isolates from this genus, all of the tested enzymes and traits were present.

Previously studied *Vibrio* spp. exhibited enzymatic activities such as proteolytic, cellulolytic, amylolytic (Jose et al.

2014), xylanolytic (Kiyohara et al. 2005), and lipolytic (Ray et al. 2012), showed potential for plant growth promotion by solubilizing phosphate, fixing atmospheric nitrogen, producing IAA (Jose et al. 2014), and siderophores (Thompson et al. 2004). *Vibrio* spp. isolates from our collection tested positive for these activities and traits and, in addition, pectinolytic activity (at both pH=5.0 and 7.0) and ACC deaminase activity. Other studies have also detected antimicrobial activity (Kumar and Nair 2007) and considerable Hg resistance and ability for Hg bioremediation in *Vibrio* spp. isolates (Jafari et al. 2015).

IAA production was detected in 460 isolates, 17 of which produced over 100  $\mu\text{g mL}^{-1}$ . The most represented IAA producing genus is *Pseudomonas* with five isolates, which has previously been associated with IAA producing potential in rice root exudates (Karnwal 2009). Phosphate solubilization was detected in 64 isolates, 10 of which belong to the genus *Microbacterium*, and 15 belong to the genus *Salinicola*. While *Microbacterium* spp. have been associated with phosphate solubilizing activity (Kukla et al. 2014), this is, to our knowledge, the first report of such activity in the *Salinicola* genus. Using PCR amplification, 20.8 % of representative isolates tested positive for the presence of the *nifH* gene. Of the 97 *nifH*-positive isolates, 63 originated from BG tissues and 17 belonged to the genus *Vibrio*, which has previously been associated with this trait (Jose et al. 2014). According to Penrose and Glick (2003), the ability to use ACC as a sole nitrogen source is a consequence of the activity of the enzyme ACC deaminase. From the 239 isolates that were able to grow in DF minimal medium supplemented with ACC, 155 (64.9 %) originated from BG tissues, and 60 % of isolates sampled in site B tested positive for this activity. Isolates associated with the genera *Microbacterium* (33) and *Altererythrobacter* (25) contributed the most for this PGP trait. Isolates from *Microbacterium* genus have previously been associated with ACC deaminase activity (Sheng et al. 2008); however, to our knowledge, this is the first description of such activity in the genus *Altererythrobacter*. Siderophore production was observed in 152 isolates (32.5 % of the collection), 19 of which belong to the genus *Vibrio*, which had been previously linked to this activity (Thompson et al. 2004). Nineteen *Marinilactibacillus* spp. isolates also tested positive, which is, to our knowledge, the first time such activity was described in this genus.

This work revealed that the endosphere of *H. portulacoides* represents a bacterial hot spot harboring a diverse microbiota, including putative novel species and compounds with biotechnological relevance. Endophytic bacteria were isolated from AG and BG tissues of the halophyte from contaminated and noncontaminated sites. Seventy-nine different genera were found using traditional cultivation methods. The phylogenetically diverse collection presented structural differences among sampling tissues and sites, and many of the isolates revealed their ability to produce extracellular enzymes and

PGP traits. The PGP potential of these endophytes should be further characterized, aiming toward enhancing phytoextraction of metal-contaminated soils by *H. portulacoides*. The possibility of monitoring *Salinicola* spp. as a bioindicator of lack of contamination should also be further studied. Metal resistance and antimicrobial properties of these isolates should also be assessed to further characterize functional capabilities of the collection.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

- Aafi NE, Saidi N, Maltouf AF, Perez-Palacios P, Dary M, Brhada F, Pajuelo E (2015) Prospecting metal-tolerant rhizobia for phytoremediation of mining soils from Morocco using *Anthyllis vulneraria* L. *Environ Sci Pollut Res* 22:4500–4512. doi:10.1007/s11356-014-3596-y
- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402. doi:10.1093/nar/25.17.3389
- Alongi DM (1998) Mangroves and salt marshes. In: Kennish MJ, Lutz PL (eds) Coastal ecosystem processes. CRC, Florida, 419 pp. ISBN 0-8493-8426-5
- Alves A, Correia A, Igual JM, Trujillo ME (2014) *Microbacterium endophyticum* sp. nov. and *Microbacterium halimionae* sp. nov., endophytes isolated from the salt-marsh plant *Halimione portulacoides* and emended description of the genus *Microbacterium*. *Syst Appl Microbiol* 37:474–479. doi:10.1016/j.syapm.2014.08.004
- Alves A, Riesco R, Correia A, Trujillo ME (2015) *Microbacterium proteolyticum* sp. nov. isolated from roots of *Halimione portulacoides*. *Int J Syst Evol Microbiol* 65:1794–1798. doi:10.1099/ijms.0.000177
- Ando S, Goto M, Meunchang S, Thongra-ar P, Fujiwara T, Hayashi H, Yoneyama T (2005) Detection of *nifH* sequences in sugarcane (*Saccharum officinarum* L.) and pineapple (*Ananas comosus* [L.] Merr.). *Soil Sci Plant Nutr* 51:303–308. doi:10.1111/j.1747-0765.2005.tb00034.x
- Anjum NA, Ahmad I, Válega M, Pacheco M, Figueira E, Duarte AC, Pereira E (2011) Impact of seasonal fluctuations on the sediment-mercury, its accumulation and partitioning in *Halimione portulacoides* and *Juncus maritimus* collected from Ria de Aveiro coastal lagoon (Portugal). *Water Air Soil Pollut* 222:1–15. doi:10.1007/s11270-011-0799-4
- Berg G, Grube M, Schloter M, Smalla K (2014) Unraveling the plant microbiome: looking back and future perspectives. *Front Microbiol* 5:148. doi:10.3389/fmicb.2014.00148
- Bibi F, Jeong JH, Chung EJ, Jeon CO, Chung YR (2014) *Labrenzia suaedae* sp. nov., a marine bacterium isolated from a halophyte, and emended description of the genus *Labrenzia*. *Int J Syst Evol Microbiol* 64:1116–1122. doi:10.1099/ijms.0.052860-0
- Bouchard V, Creach V, Lefeuvre JC, Bertru G, Mariotti A (1998) Fate of plant detritus in a European salt marsh dominated by *Atriplex portulacoides* (L.) Aellen. *Hydrobiologia* 373:75–87
- Bulgarelli D, Schlaeppi K, Spaepen S, van Themaat EVL, Schulze-Lefert P (2013) Structure and functions of the bacterial microbiota of plants. *Annu Rev Plant Biol* 64:807–838. doi:10.1146/annurev-arplant-050312-120106
- Carvalho PN, Basto MCP, Silva MFGM, Machado A, Bordalo AA, Vasconcelos TSD (2010) Ability of salt marsh plants for TBT remediation in sediments. *Environ Sci Pollut Res* 17:1279–1286. doi:10.1007/s11356-010-0307-1
- Costa C, Jesus-Rydyin C (2001) Site investigation on heavy metals contaminated ground in Estarreja – Portugal. *Eng Geol* 60:39–47. doi:10.1016/S0013-7952(00)00087-9
- Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM (2014) Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res* 41:D633–D642. doi:10.1093/nar/gkt1244
- Couto MNPFS, Basto MCP, Vasconcelos MTS (2011) Suitability of different salt marsh plants for petroleum hydrocarbons remediation. *Chemosphere* 84:1052–1057. doi:10.1016/j.chemosphere.2011.04.069
- Cox CD (1994) Deferration of laboratory media and assays for ferric and ferrous ions. *Method Enzymol* 235:315–329. doi:10.1016/0076-6879(94)35150-3
- De Caceres M, Legendre P (2009) Associations between species and groups of sites: indices and statistical inference. *Ecology* 90:3566–3574. doi:10.1890/08-1823.1
- Dworkin M, Foster JW (1958) Experiments with some microorganisms which utilize ethane and hydrogen. *J Bacteriol* 75:592–603
- Ellis RJ, Neish B, Trett MW, Best JG, Weightman AJ, Morgan P, Fry JC (2001) Comparison of microbial and meiofaunal community analyses for determining impact of heavy metal contamination. *J Microbiol Meth* 45:171–185. doi:10.1016/S0167-7012(01)00245-7
- Ellis RJ, Morgan P, Weightman AJ, Fry JC (2003) Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Appl Environ Microb* 69:3223–3230. doi:10.1128/AEM.69.6.3223-3230.2003
- Figueira E, Freitas R (2013) Consumption of *Ruditapes philippinarum* and *Ruditapes decussatus*: comparison of element accumulation and health risk. *Environ Sci Pollut Res* 20:5682–5691. doi:10.1007/s11356-013-1587-z
- Gaby JC, Buckley DH (2012) A comprehensive evaluation of PCR primers to amplify the *nifH* gene of nitrogenase. *PLoS ONE* 7:7. doi:10.1371/journal.pone.0042149
- Gordon SA, Weber RP (1951) Colorimetric estimation of indoleacetic acid. *Plant Physiol* 26:192–195
- Hardoim PR, van Overbeek LS, van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* 16:463–471. doi:10.1016/j.tim.2008.07.008
- Jafari SA, Cheraghi S, Mirbakhsh M, Mirza R, Maryamabadi A (2015) Employing response surface methodology for optimization of mercury bioremediation by *Vibrio parahaemolyticus* PG02 in Coastal Sediments of Bushehr, Iran. *CLEAN* 43:118–126. doi:10.1002/clean.201300616

- Jose PA, Sundari IS, Sivakala KK, Jebahumar SRD (2014) Molecular phylogeny and plant growth promoting traits of endophytic bacteria isolated from roots of seagrass *Cymodocea serrulata*. *Indian J Geomorphol Sci* 43:571–579
- Karnwal A (2009) Production of indole acetic acid by fluorescent *Pseudomonas* in the presence of L-tryptophan and rice root exudates. *J of Plant Pathol* 91:61–63
- Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Micr* 62:716–721. doi:10.1099/ijs.0.038075-0
- Kim Y-J, Nguyen N-L, Hoang V-A, Min J-W, Hwang K-H, Yang D-C (2015) *Microbacterium panaciterrae* sp. nov., isolated from the rhizosphere of ginseng. *Int J Syst Evol Micr* 65:927–933. doi:10.1099/ijs.0.000041
- Kiyohara M, Sakaguchi K, Yamaguchi K, Akari T, Nakamura T, Ito M (2005) Molecular cloning and characterization of a novel  $\beta$ -1,3-xylanase possessing two putative carbohydrate-binding modules from a marine bacterium *Vibrio* sp. strain AX-4. *Biochem J* 388:949–957. doi:10.1042/BJ20050190
- Kukla M, Płociniczak T, Piotrowska-Seget Z (2014) Diversity of endophytic bacteria in *Lolium perenne* and their potential to degrade petroleum hydrocarbons and promote plant growth. *Chemosphere* 117:40–46. doi:10.1016/j.chemosphere.2014.05.055
- Kumar NR, Nair S (2007) *Vibrio rhizosphaerae* sp. nov., a red-pigmented bacterium that antagonizes phytopathogenic bacteria. *Int J Syst Evol Micr* 57:2241–2246. doi:10.1099/ijs.0.65017-0
- Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. John Wiley and Sons, New York, pp 115–175
- Liu XL, Liu SL, Liu M, Kong BH, Liu L, Li YH (2014) A primary assessment of the endophytic bacterial community in a xerophilous moss (*Grimmia montana*) using molecular method and cultivated isolates. *Braz J Microbiol* 45:163–173
- Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgey M, van der Lelie D (2002) Endophytic bacteria and their potential applications. *Crit Rev Plant Sci* 21:583–606. doi:10.1080/0735-260291044377
- Lozano-Rodríguez E, Hernández LE, Bonay P, Carpena-Ruiz RO (1997) Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances. *J Exp Bot* 48:123–128
- Lucena-Padrós H, Jiménez E, Maldonado-Barragán A, Rodríguez JM, Ruiz-Barba JL (2015) PCR-DGGE assessment of the bacterial diversity in Spanish-style green table-olive fermentations. *Int J Food Microbiol* 205:47–53. doi:10.1016/j.ijfoodmicro.2015.03.033
- Ma Y, Oliveira RS, Nai F, Rajkumar M, Luo Y, Rocha I, Freitas H (2015) The hyperaccumulator *Sedum plumbizincicola* harbors metal-resistant endophytic bacteria that improve its phytoextraction capacity in multi-metal contaminated soil. *J Environ Manage* 156:62–69. doi:10.1016/j.jenvman.2015.03.024
- Mora-Ruiz MR, Font-Verdera F, Díaz-Gil C, Urdiain M, Rodríguez-Valdecantos G, González B, Orfila A, Rosselló-Móra R (2015) Moderate halophilic bacteria colonizing the phylloplane of halophytes of the subfamily *Salicornioideae* (*Amaranthaceae*). *Syst Appl Microbiol* 38:406–416. doi:10.1016/j.syapm.2015.05.004
- Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol Lett* 170:265–270. doi:10.1111/j.1574-6968.1999.tb13383.x
- Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H (2015) *vegan*: Community Ecology Package. R package version 2.2-1. <http://CRAN.R-project.org/package=vegan>
- Oliveira V, Gomes NCM, Almeida A, Silva AMS, Simões MQM, Smalla K, Cunha A (2014a) Hydrocarbon contamination and plant species determine the phylogenetic and functional diversity of endophytic degrading bacteria. *Mol Ecol* 23:1392–1404. doi:10.1111/mec.12559
- Oliveira V, Gomes NCM, Cleary DFR, Almeida A, Silva AMS, Simões MMQ, Silva H, Cunha A (2014b) Halophyte plant colonization as a driver of the composition of bacterial communities in salt marshes chronically exposed to oil hydrocarbons. *FEMS Microbiol Ecol* 90:647–662. doi:10.1111/1574-6941.12425
- Park HY, Kim KK, Jin L, Lee S-T (2006) *Microbacterium paludicola* sp. nov., a novel xylanolytic bacterium isolated from swamp forest. *Int J Syst Evol Micr* 56:535–539. doi:10.1099/ijs.0.63945-0
- Passari AK, Mishra VK, Saikia R, Gupta VK, Singh BP (2015) Isolation, abundance and phylogenetic affiliation of endophytic actinomycetes associated with medicinal plants and screening for their in vitro antimicrobial biosynthetic potential. *Front Microbiol* 6:273. doi:10.3389/fmicb.2015.00273
- Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol Plantarum* 118:10–15. doi:10.1034/j.1399-3054.2003.00086.x
- Pereira ME, Duarte AC, Millward GE, Vale C, Abreu SN (1998) Tidal export of particulate mercury from the most contaminated area of Aveiro's Lagoon, Portugal. *Sci Total Environ* 213:157–163. doi:10.1016/S0048-9697(98)00087-4
- Pereira ME, Lillebø AI, Pato P, Válega M, Coelho JP, Lopes CB, Rodrigues S, Chachada A, Otero M, Pardal MA, Duarte AC (2009) Mercury pollution in *Ria de Aveiro* (Portugal): a review of the system assessment. *Environ Monit Assess* 155:39–49. doi:10.1007/s10661-008-0416-1
- Pereira SIA, Barbosa L, Castro PML (2013) Rhizobacteria isolated from a metal-polluted area enhance plant growth in zinc and cadmium-contaminated soil. *Int J Environ Sci Technol* 12:2127–2142. doi:10.1007/s13762-014-0614-z
- Pérez-Miranda S, Cabirol N, George-Téllez R, Zamudio-Rivera LS, Fernández FJ (2007) O-CAS, a fast and universal method for siderophore detection. *J Microbiol Meth* 70:127–131. doi:10.1016/j.mimet.2007.03.023
- Pohlert T (2005) PNCMR: Calculate Pairwise Multiple Comparisons of Mean Rank Sums. R package version 1.1. <http://CRAN.R-project.org/package=PNCMR>
- Proença DN, Francisco R, Santos CV, Lopes A, Fonseca L, Abrantes IMO, Morais PV (2010) Diversity of bacteria associated with *Bursaphelenchus xylophilus* and other nematodes isolated from *Pinus pinaster* trees with pine wilt disease. *PLoS One* 5:12. doi:10.1371/journal.pone.0015191
- R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>
- Rajkumar M, Sandhya S, Prasad MNV, Freitas H (2012) Perspectives of plant-associated microbes in heavy metal phytoremediation. *Biotechnol Adv* 30:1562–1574. doi:10.1016/j.biotechadv.2012.04.011
- Rameshkumar N, Krishnan R, Lang E, Matsumura Y, Sawabe T, Sawabe T (2014) *Zunongwangia mangrovi* sp. nov., isolated from mangrove (*Avicennia marina*) rhizosphere, and emended description of the genus *Zunongwangia*. *Int J Syst Evol Microbiol* 64:545–550. doi:10.1099/ijs.0.053512-0
- Ray AK, Ghosh K, Ringø E (2012) Enzyme-producing bacteria isolated from fish gut: a review. *Aquacult Nutr* 18:465–492. doi:10.1111/j.1365-2095.2012.00943.x
- Romanenko LA, Tanaka N, Kalinovskaya NI, Mikhailov VV (2013) Antimicrobial potential of deep surface sediment associated bacteria from the Sea of Japan. *World J Microb Biot* 29:1169–1177. doi:10.1007/s11274-013-1276-6
- Roth E, Schwenninger SM, Hasler M, Eugster-Meier E, Lacroix C (2010) Population dynamics of two antilisterial cheese surface consortia

- revealed by temporal temperature gradient gel electrophoresis. *BMC Microbiol* 10:74. doi:10.1186/1471-2180-10-74
- Sessitsch A, Kuffner M, Kidd P, Vangronsveld J, Wenzel WW, Fallmann K, Puschenreiter M (2013) The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. *Soil Biol Biochem* 60:182–194. doi:10.1016/j.soilbio.2013.01.012
- Sheng XF, Xia JJ, Jiang CY, He LY, Qian M (2008) Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. *Environ Pollut* 156:1164–1170. doi:10.1016/j.envpol.2008.04.007
- Tanaka T, Kawasaki K, Daimon S, Kitagawa W, Yamamoto K, Tamaki H, Tanaka M, Nakatsu CH, Kamagata Y (2014) A hidden pitfall in agar media preparation undermines cultivability of microorganisms. *Appl Environ Microbiol* 80:7659–7666. doi:10.1128/AEM.02741-14
- Thompson FL, Iida T, Swings J (2004) Biodiversity of Vibrios. *Microbiol Mol Biol R* 68:403–431. doi:10.1128/MMBR.68.3.403-431.2004
- Toffin L, Zink K, Kato C, Pignet P, Bidault A, Bienvenu N, Birrien J-L, Prieur D (2005) *Marinilactibacillus piezotolerans* sp. nov., a novel marine lactic acid bacterium isolated from deep sub-seafloor sediment of the Nankai Trough. *Int J Syst Evol Microbiol* 55:345–351. doi:10.1099/ijs.0.63236-0
- Válega M, Lillebø AI, Pereira ME, Caçador I, Duarte AC, Pardal MA (2008a) Mercury in salt marshes ecosystems: *Halimione portulacoides* as biomonitor. *Chemosphere* 73:1224–1229. doi:10.1016/j.chemosphere.2008.07.053
- Válega M, Lillebø AI, Pereira ME, Duarte AC, Pardal MA (2008b) Long-term effects of Mercury in a salt marsh: hysteresis in the distribution of vegetation following recovery from contamination. *Chemosphere* 71:765–772. doi:10.1016/j.chemosphere.2007.10.013
- Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* 19:6823–6831
- Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Method Mol Cell Biol* 5:25–40
- Vinod V, Kumar A, Zachariah TJ (2014) Isolation, characterization and identification of pericarp-degrading bacteria for the production of off-odour-free white pepper from fresh berries of *Piper nigrum* L. *J Appl Microbiol* 116:890–902. doi:10.1111/jam.12431
- Woerner LS, Hackney CT (1997) Distribution of *Juncus roemerianus* in North Carolina tidal marshes: the importance of physical and biotic variables. *Wetlands* 17:284–291
- Wu YH, Xu L, Meng FX, Zhang DS, Wang CS, Oren A, Xu X-W (2014) *Altererythrobacter atlanticus* sp. nov., isolated from deep-sea sediment. *Int J Syst Evol Microbiol* 64:116–121. doi:10.1099/ijs.0.052951-0
- Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B, de Lajudie P (2006) Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for *nifH*-like gene within the genera *Microbacterium* and *Starkeya*. *Microbial Ecol* 51:375–393. doi:10.1007/s00248-006-9025-0
- Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG, Vidaver AK (2002) Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl Environ Microb* 68:2198–2208. doi:10.1128/AEM.68.5.2198-2208.2002