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Bisphenol-A removal by the halophyte *Juncus acutus* in a phytoremediation pilot: Characterization and potential role of the endophytic community

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Abstract

A phytoremediation pilot emulating a shallow aquifer planted with *Juncus acutus* showed to be effective for remediating Bisphenol-A (BPA) contaminated groundwater. Biostimulation with root exudates, low molecular weight organic acids, of *J. acutus* did not improve BPA-degradation rates. Furthermore, the endophytic bacterial community of *J. acutus* was isolated and characterized. Many strains were found to possess increased tolerance to metals such as Zn, Ni, Pb and Cd. Moreover, several endophytic bacterial strains tolerated and even used BPA and/or two antibiotics (ciprofloxacin and sulfamethoxazole) as a sole carbon source. Our results demonstrate that the cultivable bacterial endophytic community of *J. acutus* is able to use organic contaminants as carbon sources, tolerates metals and is equipped with plant-growth promoting traits. Therefore, *J. acutus* has potential to be exploited in constructed wetlands when co-contamination is one of the restricting factors.

Keywords

Phytoremediation, halophyte, *Juncus acutus*, endophytic bacteria, Bisphenol-A, antibiotics.

1. Introduction

The high degree of industrialization led to the production of huge amounts of toxic wastes and contaminated soils and waters that need to be treated. Phytoremediation is a promising alternative in comparison to the conventional technologies since it is a low cost, solar powered technology with wide public acceptance [1]. However, the sometimes higher demand of time, the mixed nature of most pollutions and the potential accumulation of contaminants in the plant tissues which in turn may decrease plant fitness are some drawbacks of this technology. Constructed wetlands (CWs) are artificial wetlands that combine plants and their associated microbes for pollutant removal and they are characterized as a state of the art technology for water (including wastewater or groundwater) remediation [2]. Verlicchi & Zambello [3] suggested that this low cost technology represents a promising alternative for treating wastewater from small communities or as a final treatment step in tailored effluents such as from hospitals. Moreover, special attention was paid to this sustainable remediation option after that urban wastewater treatment plants were found to be the main point sources for the release of several emerging contaminants to the environment [4].

Bisphenol A [BPA, 2,2-bis(4-hydroxyphenyl)-propane] is a synthetic compound commonly used in the production of polymers, vinyl chloride, thermal paper, polyacrylates and lacquer coatings for tin cans [5]. It is produced worldwide in high volumes (three million tons each year) and wastewater treatment plants treating industrial effluents, sewage sludge and waste landfill leachates are considered the main sources of BPA release into the environment. Epidemiological studies revealed causal relationships between BPA exposure and chronic human diseases such as obesity, cardiovascular diseases, reproductive disorders, chronic kidney diseases, birth defects and development disorders, respiratory diseases, cancers and autoimmune diseases [6]. Several chemical methods have been established for treating BPA contaminated waters, along with the biological treatment which encompasses microbial and/or plant mediated degradation. Many bacterial strains have been employed for *in vitro* degradation experiments with promising results [7] next to the herbaceous plant species that showed an ability to decrease BPA concentrations from aqueous media [8].

The exploitation of plant-associated microorganisms may support a strategy towards enhanced degradation rates and improved performance of plants in CWs [9]. The

selected bacteria or fungi can colonize the rhizosphere or the endosphere and may play a major role in removal of organic and inorganic contaminants and in improving plant growth.

The water solubility, lipophilicity and $\log K_{ow}$ between 0.5 and 3.5 that characterize the majority of organic contaminants enables their translocation to the plant tissues [10]. As a result, endophytic bacteria may play an important role in plant detoxification. The term endophytic bacteria refers to bacteria that reside in the internal plant parts without negatively affecting the host [11]. Actually, many of them carry plant growth promoting characteristics such as nitrogen fixation, utilization of 1-aminocyclopropane-1-carboxylic acid as a sole nitrogen source and production of phytohormones along with their ability to tolerate high concentrations of metals [12,13]. In the case of organic contaminants, endophytic bacteria equipped with appropriate catabolic genes enhance the *in planta* degradation of xenobiotics [14]. Moreover, the roots can also take up the polar and highly water-soluble compounds. In this context, there exists a growing interest in isolating and characterizing the endophytic communities of wetland plants in terms of exploring strains capable of degrading/tolerating contaminants together with promoting plant growth [15–20].

Recently, the contribution of the endophytic community of *Phragmites australis* to carbamazepine degradation was evaluated; some strains could remove the psychotropic drug and at the same time demonstrated beneficial plant growth promoting traits [18]. Moreover, Dimitroula et al. [17] showed that some *Juncus acutus* endophytic strains can reduce Cr(VI) to Cr(III) assisting the detoxification of the halophyte exposed to Cr(VI). Along with the endophytic community, the plant can affect positively or negatively the degradation capabilities of the rhizospheric microorganisms through the excretion of various compounds [21,22].

In this study, a phytoremediation pilot (emulating a shallow aquifer) was constructed in order to investigate the capacity of the wetland plant *J. acutus* to remove BPA from contaminated groundwater. In addition, the potential impact of the presence of BPA on the pattern of organic acids exuded by the halophyte was also determined. Further, the cultivable endophytic community of *J. acutus* was assessed in terms of plant growth promoting traits, metal tolerance and resistance against emerging contaminants. In addition, the degradation potential was assessed leading to the selection of highly

promising candidates for bioaugmentation strategies. These are expected to enhance the performance of this halophyte in CWs.

2. Materials and Methods

2.1 Shallow Aquifer Phytoremediation Pilot Unit

The pilot unit emulated a shallow aquifer, treating contaminated groundwater. The total volume of the container was 1 m^3 and was filled with 20% gravel (110 L small-sized gravel at the bottom and 55 L of medium-sized gravel on top of it) and 80% soil (upper layer) (Fig.1). The pilot contained two *J. acutus* plants, collected and transplanted from the natural wetland of Morony at Souda bay (Chania, Greece). The soil mass in the system was 1040 kg and the total estimated water volume 315 L, at 100% saturation. An external reservoir of 75 L (working volume 60 L) was used for collection of the partially treated effluent water and with the help of a feed pump the water from this external reservoir was pumped back into the pilot. The external reservoir was also used for spiking the system with contaminant(s) at time zero. A peristaltic pump controlled the hydraulic retention time at 0.59 days corresponding to a flow rate of 17.9 L h^{-1}. Initial BPA concentration at 2667 μg L^{-1} was attained by diluting 160 mg of the compound in the external tank at day zero. BPA was separated and quantified using HPLC according to the protocol described elsewhere [23].

2.2 Collection of *J. acutus* root exudates

Ceramic pots were filled with 400 g gravel and 1200 g dry soil and planted with one *J. acutus* plant. In the bottom of each pot a sampling port with a valve was introduced. In order to increase the hydraulic conductivity and obtain a rapid infiltration of the water, the soil collected from the field in Akrotiri (Chania, GR) was mixed with beach sand prewashed with tap water. On the first day, pots with and without plants were spiked with 150 and 300 μg BPA diluted in 250 mL tap water (n=3). Pots with plants but without BPA addition were used as controls. The drainage was collected 2 h after spiking with BPA. The next two days, 150 mL tap water were added in each pot and the leachate from each pot was collected again 2 h after spiking the water in the reservoir. Subsequently, the leachates were evaporated until dryness under reduced pressure at 50°C, dissolved in 5 mL distilled water and then stored in a freezer at −20°C.

2.3 Identification of low molecular organic acids in root exudates

A solid-phase extraction procedure was applied to isolate low molecular organic acids from root exudates. A cartridge (SEP-PAK VAC, Accell Plus QMA cartridge, Waters) was activated with 10 mL 0.1 M sodium hydroxide solution (percolation rate 3 mL min^{-1}) and 40 mL of root exudates solution was passed at a flow-rate of 0.5 mL min^{-1}. Subsequently, the cartridge was rinsed with 10 mL water (3 mL min^{-1}) and organic acids were eluted with 4 mL 0.1 M sulfuric acid (0.5 mL min^{-1}). This solution was injected directly into the HPLC (Alliance 2690 series HPLC equipped with a UV-Vis detector) and the improved method of Cawthray [24] was used for the identification and quantification of the organic acids. Separation was achieved on a Nucleosil C18, reverse-phase column (250 mm x 4.6 mm, 5 μm) employing an isocratic elution program with one solvent that was ultrapure water adjusted to pH 2.5 with o-phosphoric acid. The flow rate was 0.5 mL min^{-1} for a total running time of 30 min. The detector was set at 210 nm. Standard solutions were used for the identification of all organic acids separately and as a mixture. Positive identification of them was accomplished by comparing standard retention times. All the reagents used for this study were purchased from Sigma–Aldrich (Germany).

2.4 Isolation of cultivable endophytic bacteria from *J. acutus*

Tissue samples (1 g from root and leaves respectively) were collected from *J. acutus* plants growing on the BPA-contaminated pilot. The samples were surface sterilized for 30 s in 70% ethanol followed by immersion in 2% NaClO solution supplemented with one droplet Tween 80 per 100 mL solution for 10 min. Finally, the samples were rinsed three times in sterile distilled water for 1 min; aliquots of the third rinsing solution were plated on 869 medium [25] and incubated for 7 days at 30^{o}C, in order to confirm surface sterility. Subsequently, the surface-sterile samples were macerated for 60 s in 10 mL of 10 mM $MgSO_4$ using a sterile mortar and pestle. Serial dilutions were plated on 1/10 strength 869 agar medium supplemented with 100 μg mL^{-1} of the fungicide cycloheximide to inhibit fungal growth. The plates were incubated at 30^{o}C for 7 days and the colony forming units (CFUs) were determined and calculated per gram fresh weight. All colonies with different morphology were picked off and spread onto new plates until pure colonies were formed. The selected isolates were preserved at −80°C in 15% (v/v) glycerol.

2.5 Genotypic characterization (DNA extraction, BOX-PCR genomic DNA profile, 16S rDNA amplification)

Total genomic DNA was extracted from purified strains using the DNeasy Blood and Tissue kit (Qiagen, Venlo, Netherlands) and was amplified using the BOX A1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′). PCR reactions, cycling conditions and separation of the PCR products were performed as described earlier [26]. The BOX profiles were analyzed and the isolates were grouped together according to their band patterns.

The 16S rDNA was amplified using the universal 1392R primer (5′-ACGGGCGGTGTGTRC-3′) and the bacteria-specific 26F primer (5′-AGAGTTTGATCCTGGCTCAG-3′) on one representative strain from each group and was further sequenced as previously described [27]. The sequences were compared with the nucleotide sequences deposited in GenBank using BLAST in the NCBI website. They were further aligned with Clustal X and the phylogenetic trees were constructed with the program MEGA 5.0 software [28]. The sequences of the strains are now available in the GenBank database under accession numbers KU598698- KU598764.

2.6 Screening for plant growth promoting characteristics

The isolated strains were tested for their ability to solubilize inorganic phosphate ($Ca_3(PO_4)_2$) in agar plates [29]. Indole acetic acid (IAA) production was assessed with the development of pink color after the addition of Salkowski reagent in 869 medium supplemented with 0.5 mg mL^{-1} tryptophan [30]. The 1-aminocyclopropane-1-carboxylate (ACC) deaminase capacity of the strains was determined as previously described by Belimov et al. [31]. Siderophore production was qualitatively estimated by the Chrome Azurol S assay [32]; when CAS binds to siderophores the color changes from blue to orange. The pH-sensitive color indicator Alizarine Red S was used in order to evaluate the organic acids production by bacteria [33].

2.7 Metal resistance

The tolerance of the isolated strains to metals was investigated using the selective 284 medium supplemented with 4 mM zinc, 1 mM nickel, 1 mM cadmium or 1 mM lead. The 284 medium contained per liter distilled water 6.06 g Tris–HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g $NaSO_4$, 0.20 g $MgCl_2$. $6H_2O$, 0.03 g $CaCl_2$. $2H_2O$, 40 mg

Na_2HPO_4. $2H_2O$, 0.48 mg Fe(III)NH_4 citrate and 1 mL microelements solution (1.3 mL 25% HCl, 144 mg $ZnSO_4$. $7H_2O$, 100 mg $MnCl_2$. $4H_2O$, 62 mg H_3BO_3, 190 mg $CoCl_2$. $6H_2O$, 17 mg $CuCl_2$. $2H_2O$, 24 mg $NiCl_2$. $6H_2O$ and 36 mg $NaMoO_4$. $2H_2O$) of final pH 7. Carbon sources (0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose, and 0.81 g succinate per liter medium) were added to the medium and the plates were incubated at 30^{o}C for 7 days.

2.8 Antimicrobial susceptibility

The antibiotic disc assay was performed in order to investigate the tolerance of the isolated strains to selected antibiotics. Briefly, bacteria were streaked onto Mueller-Hinton Agar plates with sterile cotton swabs and four antibiotic discs (ciprofloxacin (1μg), erythromycin (15μg), sulfamethoxazole (25μg) and tetracycline (10μg)) were placed on the surface of the plate. Subsequently, the plates were incubated at 30^{o}C for 2 days. The zone of inhibition was calculated and the antibiotic resistance was evaluated according to the Kirby-Bauer chart.

2.9 Tolerance to Bisphenol-A by auxanography

Auxanography was used for the identification of the strains that could grow on 284 medium and utilize Bisphenol-A as a sole carbon source. Bacteria were cultivated in rich medium until they reached the late exponential phase, they were washed three times with 10 mM $MgSO_4$ and spread (concentration: 10^8 cfu mL^{-1}) on minimal medium agar plates without carbon source. The next day, one droplet of BPA was streaked out in one third of the plate and the plates were incubated at 30^{o}C for 7 days.

2.10 Degradation of organic contaminants

The ability of the isolates to use Bisphenol-A (BPA), ciprofloxacin (CIP), erythromycin (E), sulfamethoxazole (SMX) and tetracycline (TET) as a carbon source was investigated for the tolerant strains using Biolog MT2 plates (Biolog Inc., Hayward, CA, USA). The wells of the MT2MicroPlates contain only a buffered nutrient medium and tetrazolium dye as an indicator of carbon source utilization. Bacteria were grown until the late exponential phase and they were washed twice with PBS buffer. The cell suspension and each of the organic contaminants were inoculated in triplicates to the wells and the optical density was measured periodically with a microplate reader

(Biolog Inc.). A negative (PBS buffer) and a positive (bacterial suspension with glucose) control were used.

3. Results and Discussion

3. 1 Performance of the shallow aquifer phytoremediation pilot and quantification of organic acids excretion

Plant roots secrete organic compounds that interact with both biotic and abiotic factors in the rhizosphere and strongly influence them [21]. For example, root exudates can participate in mobilizing metal micronutrients or in competing for binding sites with anionic species; they can also serve as a substrate for co-metabolism or stimulation of the degradation of organic pollutants [34]. Among all the exudates, low molecular weight organic acids (LMWOAs) are the most active and abundant organic compounds.

In this context, the low molecular weight organic acids exuded by *J. acutus* roots were collected and identified in order to evaluate their potential contribution to BPA degradation. Monocarboxylic acids (formic and lactic acids), and di- and tricarboxylic acids (oxalic, malonic and tartaric acids) were found in the presence of BPA, while without BPA addition *J. acutus* rhizosphere did not contain any organic acid. Changes in the profile of exudates of rice roots have also been reported in response to Cr stress [35]. Similarly, the exudation pattern of two metallophytes was shown to be highly variable depending on the Cu concentration they were exposed to [36]; however, in the same study, the two agricultural plant species exuded similar quantities of citric acid at different Cu exposure levels.

Among the five organic acids detected, three of them (oxalic, formic and malonic acid) exhibited higher concentrations compared to tartaric acid while lactic acid was detected only at the first day of the experiment. The formic acid was the only acid that seemed affected by BPA exposure: it seems to be increased at lower BPA amounts in soil (from 105 μg to 22±6.5 μg and from 314±3.7 μg to 53±44 μg). Variability in exudates among plants of the same treatment was observed in this study. This intra-species variability in exudates was also mentioned in other studies [22,37]. Oxalate and malonate have also been identified in root exudates of *Juncus maritimus* growing on sandy and muddy

sites but their concentrations were significantly different between the two sites [37]. The authors suggested that the physico-chemical characteristics of the surrounding sediment might be responsible for these differences. In another study, no seasonal variations of the LMWOAs (oxalate, citrate, malate, malonate, succinate) exudation of *J. maritimus* were detected, but an increase of oxalic acid was demonstrated in presence of Cu [38]. It is well known that bacteria can also excrete organic acids in their environment; however, in this experiment no organic acids were measured in the leachates collected from the pots containing BPA-contaminated soil.

After identifying the exuded organic acids, a biostimulation run was conducted in order to quantify potential benefits of these organic acids on the removal of BPA. In particular, the following amounts were added: 4.99 g oxalic, 18.75 mg tartaric, 9.64 g formic, 6.47 g malonic and 6.48 g lactic acid which correspond to 5x the maximum concentration found in the pot experiments. However, as seen in Fig. 2, the amount of BPA in the soil decreased with similar rates in both, the tank with and the one without supplementation of organic acids. Likewise, the addition of a nutrient solution was not effective for improving the efficiency of *Juncus* to remediate a soil contaminated with petroleum hydrocarbons [39]. In particular, after adding nutrients, *J. maritimus* stems looked less healthy than without any additional nutrients. Moreover, root biomass and hydrocarbons degradation potential were negatively affected.

3. 2 Isolation and identification of the cultivable endophytic bacteria associated with *J. acutus*

Since biostimulation with low molecular organic acids did not appear to be effective and also the fact that Bisphenol A can be taken up by the plant, the endophytic bacteria of *J. acutus* were isolated and characterized. After assuring surface sterilization of the plant parts, colonies 110 morphologically distinct were obtained which formed 67 groups according to their variable BOX-PCR profiles. DNA extraction and sequencing of the 16s rRNA encoding genes were performed for one representative strain of each group.

Phylogenetic analysis showed the presence of the phyla Actinobacteria, Firmicutes and Proteobacteria in both plant organs, but the composition of the communities differed (Fig.3). In general, the phylum Proteobacteria dominated the cultivable endophytic

community of roots and leaves (75% and 46% respectively). In the leaves, the Alpha-proteobacteria were dominated by the genus *Sphingomonas* (15% of the total isolates) while the Beta-proteobacteria were mainly represented by the genus *Ralstonia* (14%) followed by the genus *Herbaspirillum* (7%). The Firmicutes were only represented by the genus *Bacillus* (39%) and the Actinobacteria were almost exclusively members of the genus *Nocardioides* (15% of isolates). With respect to the root community, the genus *Ralstonia* (49%) was the most abundant followed by *Ochrobactrum* (24%) and *Bacillus* (23%). The remaining isolates (*Aeromonas*, *Arthrobacter*, *Herbaspirillum*, *Hyphomicrobium*, *Yonghaparkia*, *Microbacterium*, *Pelomonas*, *Promicromonospora*, *Pseudomonas*, *Rhizobium*, *Sphingomonas*, *Virgibacillus*) represented only 4% of the total number of root isolates.

Endophytic bacteria enter the plant mainly via the roots and their establishment in the root interior depends on their ability to colonize the plant and establish a population. In polluted environments, the concentration of the contaminant inside is a factor that contributes to shape the community together with the plant genotype, the age and other environmental factors [14,40]. The *J. acutus* endophytic community contains many common genera that were also found in other plant species growing on contaminated sites [15,41–43].

The genus *Ralstonia*, dominating the cultivable *J. acutus* root endophytes consists of aerobic bacteria that were found in water, soil and inside plants. They have been shown able to degrade a wide range of xenobiotics such as benzene, phenol and TCE [44] and during the years this genus became a model genus for the study of metal tolerance mechanisms [45]. Moreover, bioaugmentation with *Ralstonia* strains enhanced the uptake of Cd and Zn by *Helianthus annuus* [46] and Cr and Pb translocation to maize shoots [47]. Many members of the genus *Bacillus* (the dominant genus among the leaf isolates) have been isolated from several plant species and carry appropriate characteristics for bioremediation. For example, the endophytic *Bacillus* sp. L14 isolated from the leaves of the Cd hyperaccumulator *Solanum nigrum* L. did not only tolerate high concentrations of Cu (II), Cd (II) and Cr (VI), but also removed metals, especially Cd (II) and Pb (II) [48]. Shin et al. [49] demonstrated the ability of another root endophytic strain identified as *Bacillus* sp. to promote plant growth in combination

with high Pb resistance. After inoculation of this strain in *B. juncea* seedlings, root elongation was significantly stimulated in the presence of lead compared to the control.

3. 3 Plant growth promoting properties of the isolated endophytic strains

Plant growth promoting bacteria can improve plant growth via direct or indirect mechanisms, they can assist plants to cope with pollutants and enhance the remediation capabilities of plants [11,50,51]. The isolated endophytic strains were investigated *in vitro* for their potential to express plant growth promoting (PGP) traits. The majority of the strains (more than 88%) showed at least one PGP characteristic out of the five tested, but none of them exhibited all the characteristics. In general, the community was rich in isolates able to solubilize mineral phosphate (40% of the total isolates), produce indole-acetic acid (46%), produce organic acids (27%) and secrete siderophores into the medium (55%).

The distribution of the traits differed between leaf and root isolates (Fig.4). For example, the roots harbored many mineral phosphate solubilizers (49% of the root isolates) while the majority of the strains producing organic acids were residing in the leaves (61%). IAA-producers were similarly represented in the cultivable root and leaf endophyte populations. Only four leaf isolates could utilize ACC as a sole N source and were belonging to the genera *Bacillus, Microbacterium* and *Ralstonia* while seven root isolates exhibited this trait and belonged to *Bacillus*, *Yonghaparkia*, *Ochrobactrum*, *Rhizobium* and *Sphingomonas*.

Among all isolates, two leaf (belonging to *Bacillus* and *Ralstonia*) and four root strains (belonging to *Bacillus, Yonghaparkia, Microbacterium* and *Ralstonia*) showed positive for four out of the five tested PGP traits. IAA-production was the common PGP trait for the above-mentioned strains. The leaf isolates shared phosphate solubilization and siderophore secretion, while the root isolates shared organic acids production.

3. 4 Tolerance to metals

Since *J. acutus* is a commonly used macrophyte in CWs worldwide [52], the ability of its endophytic community to tolerate trace metals was investigated. In terms of prolonging the lifetime of the substrate and generally the CW retention, plant harvesting/cutting should be performed frequently especially in case of mixed contaminations. Therefore, it is of high importance to find strains able to enhance the

metal uptake by plants and/or, even better, the translocation of metals to the aboveground tissues. In this context, the ability of the endophytic isolates to grow in the presence of metals was investigated. A high number of isolates showed able to form colonies on minimal medium supplemented with 4 mM Zn or 1 mM Ni (78% and 81% respectively). The majority of the resistant strains originated from leaves and it is noteworthy that among all leaf isolates only one strain (identified as *Bacillus*) did not show increased resistance to zinc and nickel. 68% of the endophytic strains were characterized as Pb-tolerant and 46% as Cd-tolerant. These high percentages of tolerance to Pb and Cd were not observed for the root isolates. From the latter, respectively 56% and 36% tolerated exposure to increased concentrations of respectively Pb and Cd.

Remarkably, although, *J. acutus* is a salt marsh plant that shows a tendency to accumulate metals in the belowground tissues [53], it was not the root but the leaf community that was dominated by metal tolerant strains. In the leaves, 54% of the isolates exhibited resistance to all 4 metals tested and 35% exhibited resistance to 3 metals (Fig.5). In case of the root endophytic community, a large fraction (39%) of isolates tolerated 3 metals and only a smaller amount showed increased tolerance to 4 metals (18%).

3. 5 Tolerance to emerging organic pollutants and potential degradation capacity

Plants can selectively stimulate the growth of indigenous endophytic microbial strains equipped with specific catabolic genes in order to cope with pollutants [54]. In this context, many studies [27,40,55,56] have demonstrated that host plants growing on a contaminated soil harbor many tolerant endophytic strains. Besides the plant genotype, the concentration of the contaminant may influence the metabolic potential of the *in planta* community.

In this study, the ability of the cultivable endophytic community of *J. acutus* growing on a BPA-contaminated pilot to tolerate high BPA concentrations was assessed. The majority of the strains (75%) could grow on minimal medium supplemented with 100mg L^{-1} BPA. To our knowledge, this is the first report of BPA-tolerant endophytic bacteria; previous studies focused on rhizosphere populations [57,58]. For example, from the rhizosphere of the tropical plant *Dracaena sanderiana* growing hydroponically with various concentrations of BPA, six bacterial strains were isolated

that showed tolerant to 20μM BPA [58]. Furthermore, this study suggested that BPA could not only be taken up by the plant but that there was also a tendency to be accumulated in the stems after increasing the duration of exposure. In our study, higher numbers of leaf (86%) isolates exhibited BPA resistance compared to root (67%) isolates (Fig.6), indicating that *J. acutus* might employ a similar mechanism of BPA translocation to the aerial parts but further experiments should be performed in order to confirm this hypothesis.

In order to assess their contribution to attenuate plant stress, the BPA-tolerant strains were further tested for their capacity to degrade BPA. Using Biolog MT2 plates, nine strains belonging to the genera *Ralstonia*, *Microbacterium* and *Nocardioides*) isolated from *J. acutus* leaves and 65% of the BPA- tolerant root isolates were characterized as potential BPA degraders. All these strains changed the color of the medium from white to purple after 7 days of cultivation. Many gram positive and gram negative bacteria that exhibit BPA degradation capacity with different metabolic pathways have been isolated from different environments [7]. A *Novosphingobium* sp. strain TYA-1 was isolated from the rhizosphere of *Phragmites australis* and could completely degrade 22.8 - 228.3 mg L^{-1} BPA in cultures with minimal medium and use BPA as the sole carbon source [57]. Moreover, two *Enterobacter* sp. strains and one *Bacillus* sp. strain associated with *Dracaena sanderiana*, enhanced BPA removal from hydroponic systems and mixed cultures [58].

In order to investigate the potential antibiotic resistance, four commonly used antibiotics were selected: ciprofloxacin (Quinolones), sulfamethoxazole (Sulfonamides), tetracycline (Tetracyclines) and erythromycin (Macrolides). For this purpose, a disc diffusion test was performed and the use of the antibiotics as a carbon source was examined. Antibiotics have been found inside plants but the concentrations in the different plant parts depend on several factors such as the plant species and the growth stage. For example, the concentration of antibiotics was higher in leaves and decreased from stems to roots in some vegetables [59], while in the wetland plant *Phragmites australis*, higher levels of antibiotics were found in roots and concentrations decreased from leaves to stems [60].

About 50% of the isolates from roots and leaves of *J. acutus* showed resistance to sulfamethoxazole (Fig.6). Among the resistant leaf isolates, five strains (identified as

Acidovorax, *Bacillus*, *Nocardioides*, *Ralstonia* and *Sphingomonas*) were characterized as potential sulfamethoxazole degraders. With respect to the root community, 11 strains belonging to the genera *Bacillus*, *Microbacterium*, *Ochrobactrum*, *Pseudomonas*, *Ralstonia* and *Virgibacillus* possessed the potential to degrade sulfamethoxazole; they could grow and change the color of the medium from white to purple when cultured in presence of 20 mg L^{-1} sulfamethoxazole. In most of the studies, consortia originating from activated sludge were used for aerobic SMX degradation tests but in recent years the exploitation of single microorganisms has been explored [61]. Nine bacterial strains isolated from activated sludge were tested individually for their ability to degrade SMX in cultures with an initial SMX concentration of 10 mg L^{-1} and a concentration range of carbon and nitrogen sources [62]. This study demonstrated that the biodegradation rates by the microorganisms were lower when sulfamethoxazole was the sole nutrient source in the medium; however, after 10 days of incubation the concentration was still below detection limit. In another experiment, the potential mineralization of SMX (initial concentration: 127mg L^{-1}) by enriched cultures originating from an acclimated lab scale MBR was investigated [63]. After 24 days of incubation, a 58% decreased SMX concentration was observed. However, when the five strains were separated, they could individually mineralize 24 - 44% of SMX in 16 days incubation.

Regarding ciprofloxacin (CIP), 16% of the isolates were considered as CIP-resistant since no halo zone was formed around the antibiotic disc. The six leaf and five root CIP-tolerant strains were further tested for their ability to degrade ciprofloxacin in Biolog plates. Based on the results, the *Nocardioides* sp. and *Sphingomonas* sp. leaf strains and the root-associated *Promicromonospora* sp. strain should be considered as potential ciprofloxacin degraders. Earlier reports concerning bacterial degradation of ciprofloxacin are scarce. Amorim et al. [64] investigated the capability of the soil bacterium *Labrys portucalensis* F11 to degrade a range of fluoroquinolones such as ciprofloxacin in minimal medium supplemented with acetate as an additional carbon source. They demonstrated that the concentration of the antibiotic in the medium decreased by 85% after 28 days due to the presence of the bacterium.

Only four root isolates (belonging to the genera *Aeromonas*, *Ochrobactrum*, *Pseudomonas* and *Ralstonia*) were tolerant to tetracycline while none of the isolates resisted erythromycin (Fig. 6).

Some endophytic strains showed resistance to more than one of the tested organic contaminants, for example five leaf isolates tolerated BPA, CIP and SMX. With respect to root isolates, it was observed that all the tetracycline-resistant strains were also resistant to BPA and SMX. A few strains demonstrated a potential ability to degrade two pollutants (one leaf and four root isolates) and only one *Nocardioides* strain from the leaves could degrade BPA, CIP and SMX in 7 days.

A wide range of organic and inorganic pollutants should be treated in CWs but the co-contamination decreases the fitness of the plants and their associated microorganisms. As a result, it is of high importance to find microbial strains that combine many of the desired characteristics and that can subsequently be used in bioaugmentation strategies to enhance the efficiency of CWs. From the cultivable *J. acutus* endophytic community, few endophytic strains showed PGP characteristics together with tolerance to various metals, resistance to BPA and antibiotics and potential degradation (Fig.7). The above results suggest that these isolates could potentially enhance the capacity of wetland plants to take up metals and organic contaminants from wastewater, degrade the organic contaminants and at the same time increase the plant biomass. In this context, depending on the type of effluents (domestic or industrial) where many of these compounds usually co-exist [65,58], the appropriate endophytic strains equipped with the desired characteristics may be employed.

4. Conclusions

In a pilot CW study the successful treatment of BPA-contaminated groundwater was demonstrated using the salt-tolerant wetland plant *J. acutus*. Biostimulation with the excreted organic acids did not have a beneficial effect on the BPA degradation rate. However, it appears that the plant harbors a microbial community strongly enriched with strains able to degrade organic compounds (BPA, CIP, SMX) and tolerate high concentrations of metals (Zn, Ni, Pb, Cd), together with PGP properties. To our knowledge, this is the first study that demonstrates the potential of endophytic bacteria and found some strains that possess the ability to degrade BPA, CIP and SMX.

5. Acknowledgements

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List of Figures

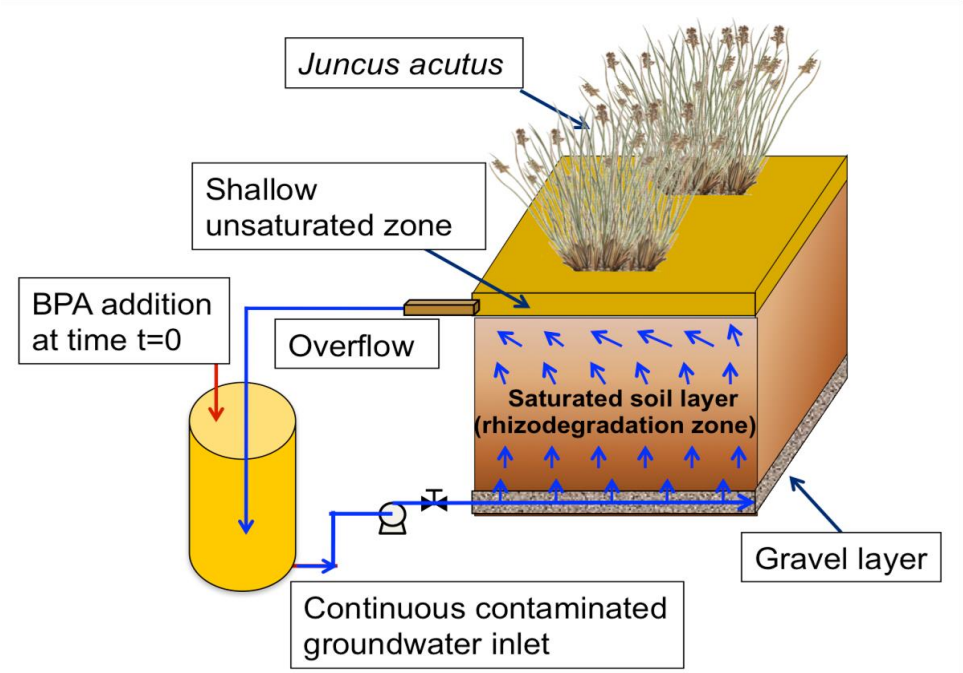

Figure 1. Schematic representation of the Shallow Aquifer Phytoremediation Pilot, planted with two *Juncus acutus* plants.

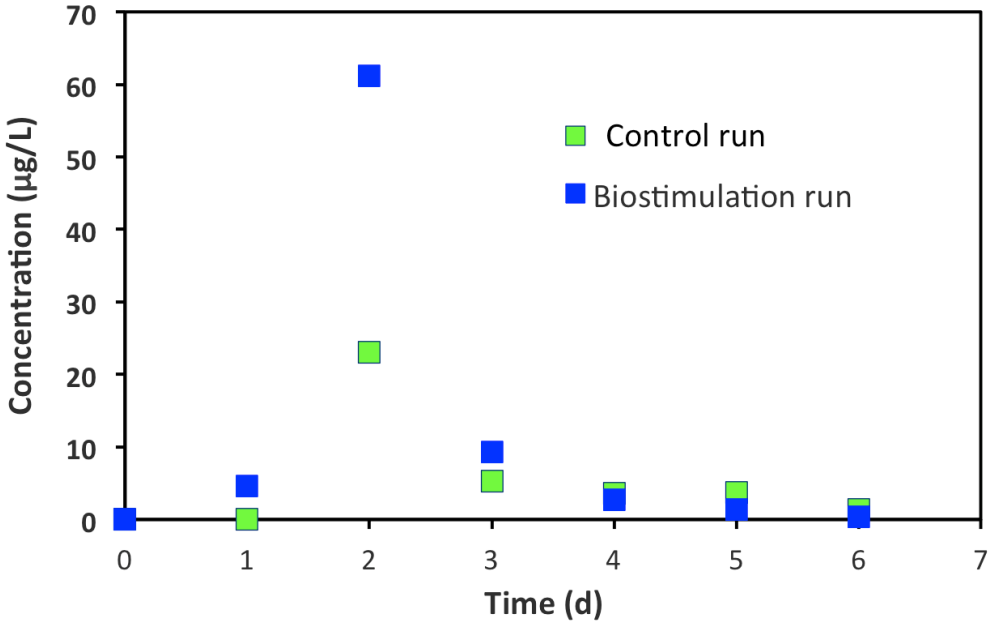

Figure 2. BPA concentration (µg L^{-1}) measured in the effluent in the control run (with BPA but no stimulants) and in the biostimulation run where organic acids (oxalic, tartaric, formic, malonic and lactic) were added.

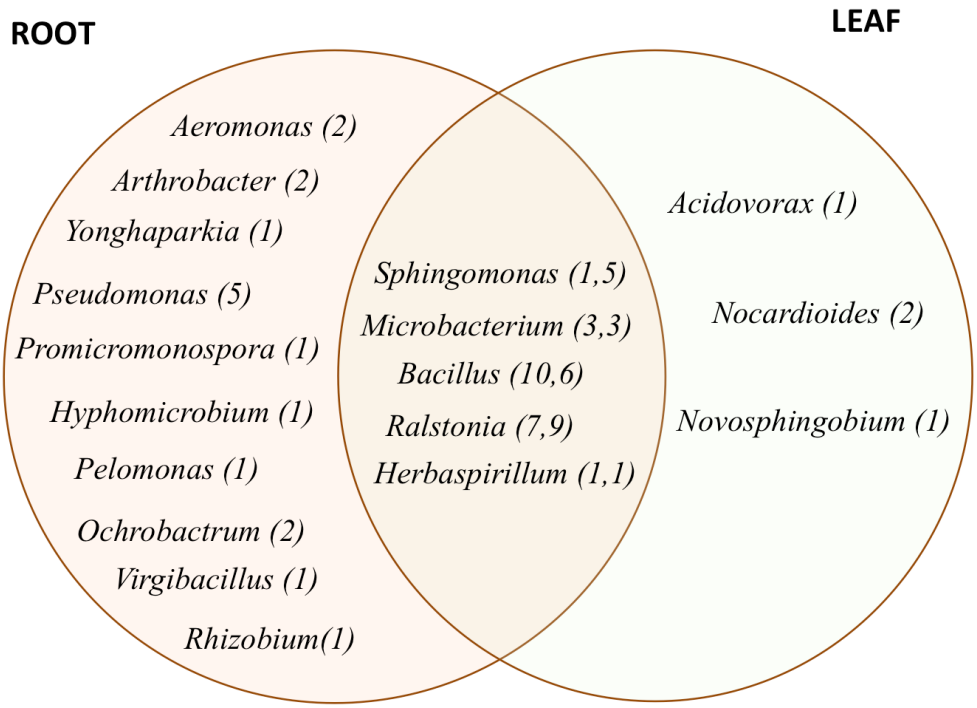

Figure 3. Distribution of endophytic isolates in plant compartments (the first number between brackets represents the number of isolates in roots and the second number between brackets represents the number of isolates in leaves).

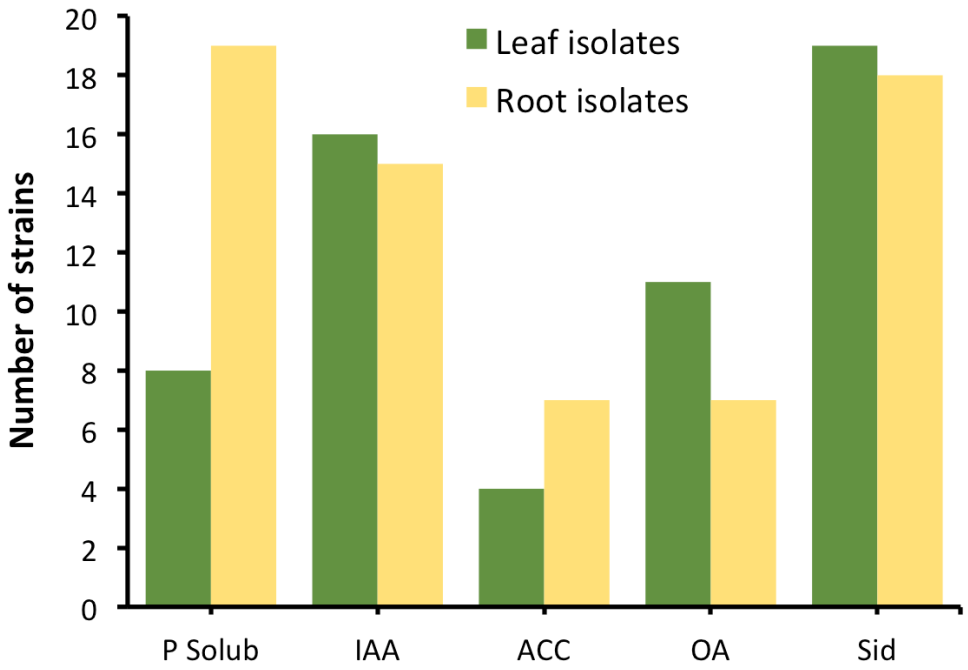

Figure 4. Percentage of endophytic strains isolated from *Juncus acutus* that have the potential to carry plant growth promoting characteristics (P Solub: Phosphate solubilizers, IAA: Indolacetic acid producers, ACC: ACC deaminase producers, OA: Organic acid producers, Sid: Siderophores producers).

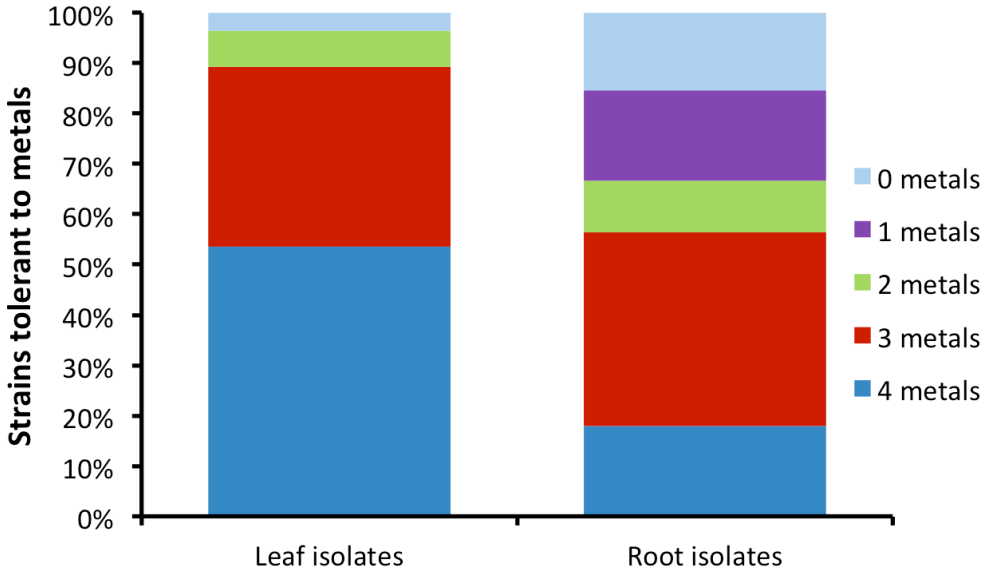

Figure 5. The number of *Juncus acutus* isolates that were tolerant to 0, 1, 2, 3 or 4 metals when they grew on 284 agar medium supplemented with trace metals (1 mM Ni, Cd, Pb or 4 mM Zn).

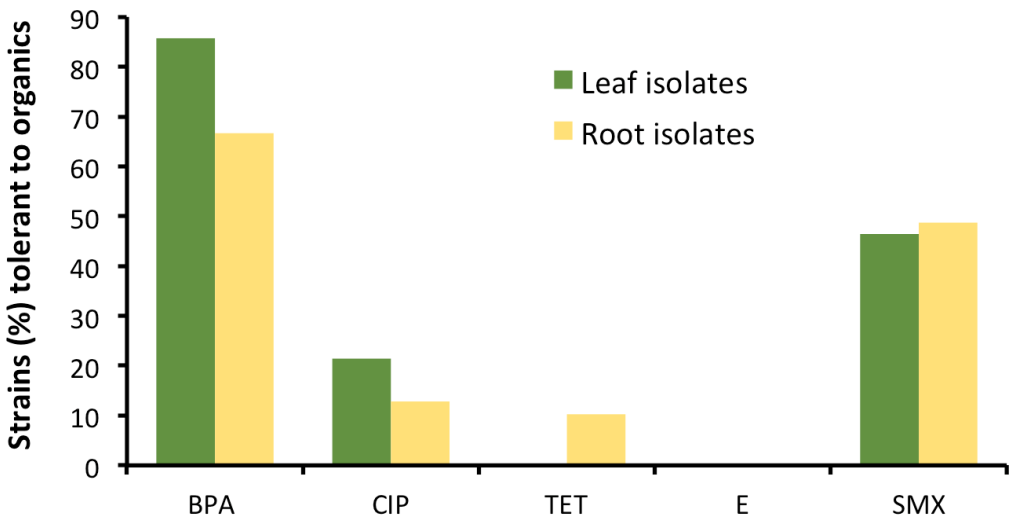

Figure 6. Percentage of leaf and root isolates that were able to grow in presence of organic pollutants (BPA: Bisphenol-A, CIP: Ciprofloxacin, TET: Tetracycline, E: Erythromycin, SMX: Sulfamethoxazole).

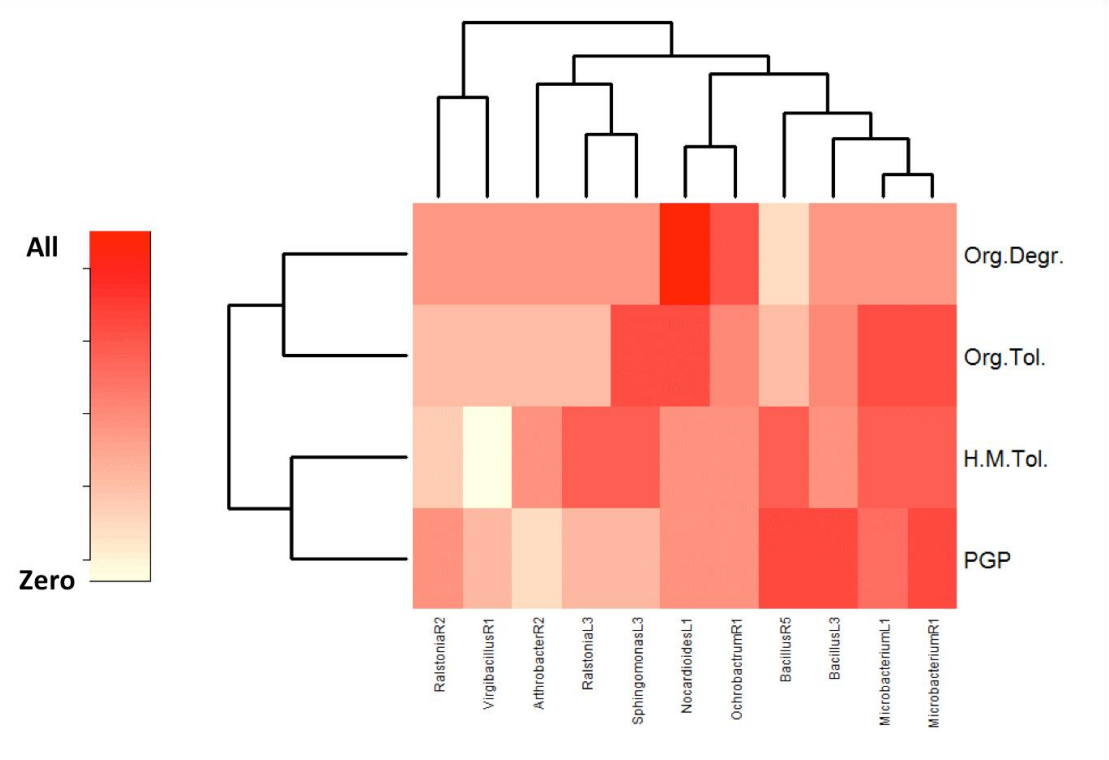

Figure 7. Heatmap with selected *J. acutus* endophytic strains and their score in the *in vitro* tested characteristics (PGP: plant growth promoting traits, H.M. Tol.: Heavy metal tolerance, Org. Tol.: Resistance to organic pollutants, Org. Degr.: Potential ability for organic pollutant degradation).