Responses of the Endophytic Bacterial Communities of \textit{Juncus acutus} to Pollution With Metals, Emerging Organic Pollutants and to Bioaugmentation With Indigenous Strains

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Plants and their associated bacteria play a crucial role in constructed wetlands. In this study, the impact of different levels of pollution and bioaugmentation with indigenous strains individually or in consortia was investigated on the composition of the endophytic microbial communities of \textit{Juncus acutus}. Five treatments were examined and compared in where the wetland plant was exposed to increasing levels of metal pollution (Zn, Ni, Cd) and emerging pollutants (BPA, SMX, CIP), enriched with different combinations of single or mixed endophytic strains. High levels of mixed pollution had a negative effect on alpha diversity indices of the root communities; moreover, the diversity indices were negatively correlated with the increasing metal concentrations. It was demonstrated that the root communities were separated depending on the level of mixed pollution, while the family \textit{Sphingomonadaceae} exhibited the higher relative abundance within the root endophytic communities from high and low polluted treatments. This study highlights the effects of pollution and inoculation on phytoremediation efficiency based on a better understanding of the plant microbiome community composition.

**Keywords:** wetland plant, \textit{J. acutus}, endophytic bacterial community, metals, emerging organic contaminants

**INTRODUCTION**

Constructed wetlands (CWs) are a promising alternative for treating various chemical compounds and for preventing their dispersion in the environment (Verlicchi and Zambello, 2014). They are engineered, state of the art sustainable systems used to treat effluents rich in pharmaceutical and personal care products by exploiting plant-bacteria interactions in combination with...
physicochemical processes (Zhang et al., 2014). Recently, many studies have focused on investigating the efficiency of wetland plants to remove EOCs in hydropnic systems and the potential effects of these compounds on the plant physiological status (Dordio et al., 2011; Liu et al., 2013; Christofilopoulou et al., 2016).

They are also efficient in removing metals from various influences such as industrial wastewaters (Khan et al., 2009), landfill leachate (A et al., 2017), and acid mine drainage (Wu et al., 2015).

In such systems, the selection of both, the appropriate plant species and their associated rhizospheric and endophytic microbiota, significantly influence the performance of the CW (Guittonny-Philippe et al., 2014; Li et al., 2014). Rhizospheric microorganisms can enhance xenobiotic transformations ex planta and improve nutrient uptake, while the endophytic community is important for transforming the organic compounds in planta, thus reducing their toxicity and evapotranspiration of water-soluble and volatile compounds (Barac et al., 2004; Taghavi et al., 2005; Afzal et al., 2014). The contribution of plant-associated microorganisms to metal phytoremediation has also been highlighted through promoting plant growth in metal polluted areas, influencing metal uptake and translocation, and increasing the metal bioavailability by secretion of ligands and organic acids (Sessitsch et al., 2013; Ma et al., 2016a).

Only a few studies attempted to explore the bacterial communities associated with wetland plants and even fewer to describe the responses of such communities to mixed and highly polluted environments (Su et al., 2015; Zhao et al., 2015; Zhang D. et al., 2016; Syranidou et al., 2017b). Moreover, information relevant to the impacts of pollutants on the endophytic bacteria of wetland plants is scarce. In such complicated environments, it is unclear whether the type and level of pollution, the plant species, the application of biostimulant bacteria, or a multifactor combination influence the phytoremediation potential and underlying endophytic assemblages. However, in order to improve the performance of CWs, it is important to address these questions.

Previously, we showed that inoculation of the wetland plant J. acutus with a selected endophytic bacterial consortium removed emergent contaminants and metals faster and more efficiently compared to non-inoculated plants (Syranidou et al., 2016). Moreover, bioaugmentation with a tailored endophytic consortium enhanced phytoremediation efficacy, and the microbes seemed to alleviate the stress induced by the pollutants, especially at the high concentration treatments. The consortium consisted of strains tolerant to metals (Zn, Ni, Cd) and emerging contaminants (BPA, CIP, SMX) and they have been characterized as potential degraders of these contaminants (Syranidou et al., 2017b). Because insights in this important part was lacking completely, we decided to perform a deeper analyses and study the total endophytic community response. In this study, the effect of mixed pollutants and bioaugmentation with single indigenous endophytic strains or in a consortium was addressed on the endophytic bacterial community of J. acutus, under different levels of mixed pollutants.

### MATERIALS AND METHODS

#### Experimental Design

Three endophytic strains (leaf B1- Sphingomonas sp. U33, root B2- Bacillus sp. R12, root B3- Ochrobactrum sp. R24) (Syranidou et al., 2016) were inoculated separately and as a consortium to beakers (n = 10 for every treatment) with J. acutus plants. One week later, two different concentrations of metals (Zn, Ni, Cd), bisphenol-A (BPA) and two antibiotics [ciprofloxacin (CIP) and sulfamethoxazole (SMX)] were added. More specifically, 50 μg L\(^{-1}\) CIP, 250 μg L\(^{-1}\) SMX, 5 mg L\(^{-1}\) BPA, 200 mg L\(^{-1}\) Zn, 20 mg L\(^{-1}\) Ni, and 1 mg L\(^{-1}\) Cd were added to the low concentration treatments and 100 μg L\(^{-1}\) CIP, 500 μg L\(^{-1}\) SMX, 10 mg L\(^{-1}\) BPA, 400 mg L\(^{-1}\) Zn, 40 mg L\(^{-1}\) Ni, and 2 mg L\(^{-1}\) Cd were used in the high concentration treatments. Further, in four treatments no pollutants were added in order to investigate the potential effects of inoculation on the endophytic community in the absence of pollutants. A two-factor design study was followed with factor 1 pollutant concentration (three levels, zero, low, high), and factor 2 bioaugmentation treatments (five levels, no inoculation, strain 1, strain 2, strain 3, consortium). In total, there were five different treatments (one non-inoculated control and four bioaugmented treatments) concerning the inoculation effect and three different concentrations of the mixture of pollutants (one without pollutants-NO, one low concentration-LC and one high concentration-HC) concerning the pollution effect. The experiment lasted for 21 days and was irrigated with 50 mL tap water every week. A schematic representation of the experimental design in provided in Supplementary Figure S1.

#### Sample Collection

Fresh root (0.3 g) and leaf (1 g) tissue samples (n = 3 for every treatment) were collected for DNA extraction at the end of the experiment. In order to sterilize the outer surface, the plant plants were immersed in 70% ethanol for 30 s and subsequently in 2% NaClO solution supplemented with one droplet Tween 80 per 100 mL solution for 10 min. Subsequently, they were rinsed three times with sterile distilled water for 1 min and 100 μL of the last rinsing solution were streaked on 869 plates (Mergeay et al., 1985) and incubated for 7 days at 30°C to verify the surface sterility. The maceration of the plant samples was performed with liquid nitrogen and total DNA was extracted using the Invisorb® Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin, Germany).

#### 16S rRNA Gene Amplicon Libraries Preparation

The forward 799F primer (AACMGGATTAGATACCCCGK) and the reverse primer 1193R (ACGTCATCCCCCCACCTTCC) were used for the amplification of the V5–V7 hypervariable region of the bacterial 16S rRNA gene, producing a ~400 bp fragment (Schlaepf et al., 2014). The primer pair was selected based on the 2 bp mismatch at the 3’-end of the 799F primer with the chloroplastidal DNA. After the first PCR, the bacterial DNA was selected over the not always present mitochondrial DNA (approximately 800 bp). The bacterial amplicons were collected and purified from agarose with the QIAquick gel extraction kit.
Every PCR reaction contained 1 × FastStart High Fidelity Reaction buffer (Roche) with 1.8 mM MgCl₂ (Roche), 200 μM of each dNTP (Roche), 250 nM forward primer, 250 nM reverse primer, 1.25 U FastStart High Fidelity Taq DNA polymerase (Roche), 1 μL DNA template and RNase free water until a total volume of 25 μL. The PCR conditions were: an initial denaturation step of 2.5 min at 95°C, 35 (1st PCR) or 20 (2nd PCR) cycles of denaturation of 1 min at 94°C, annealing for 40 s at 53°C and extension for 40 s at 72°C, and a final extension step of 7 min at 72°C. The bacterial amplicons produced by the second PCR were purified and the concentration of purified DNA was determined with the Quant-iT Picogreen dsDNA assay (Life Technologies Europe, Ghent, Belgium) according to the manufacturer’s protocol. Equimolar mixtures of different samples were prepared. For checking the amplicons, 1 μL of the library was loaded on a DNA-chip (DNA 1000 kit, Agilent Technologies, Diegem, Belgium) and analyzed on a 2100 Bioanalyzer (Agilent Technologies, Diegem, Belgium). The libraries were clonally amplified using the emPCR Lib-L kit and then sequenced using the Roche 454 GS-FLX Plus Life Sciences Genome Sequencer at Macrogen, Seoul, South Korea.

16s rRNA Gene Sequences Analysis
Bacterial amplicons were quality filtered and trimmed using the DADA2 v1.8 pipeline in R v3.4 (Callahan et al., 2016) with the adapted following read settings: filtering criteria (max N = 0, Max EE = 2, TruncQ = 2, trimming of the first 15 bp, and fixed trunclength of 300 bp). Prediction of Absolute sequence variants (ASVs) was performed with DADA2, homopolymer gap penalty was set to 1 and band size was equal to 32, followed by de novo chimera removal. The high quality reads were classified against the SILVA v132 training dataset (Quast et al., 2013; Yilmaz et al., 2014; Glöckner et al., 2017) using the naive Bayesian classifier method in DADA2. The standard workflow format (SFF) files were deposited in the NCBI Sequence Read Archive (SRA) under the accession number SRP158657.

Metal Analysis in Plant Parts
Juncus acutus roots and leaves (n = 10 for every treatment) were washed with tap water (3×) followed by washing with distilled water in order to remove any adhered particles. Next, they were dried at 50°C for 4 days. Dried plant samples (0.2 g) were digested with 9 mL HNO₃ (>69%, Sigma-Aldrich) and diluted with ultrapure water and centrifuged. Supernatants were subsequently filtered (0.45 μm, Whatman), diluted at 1:10 (v/v) with ultrapure water and analyzed by ICP-MS (ICP-MS 7500cx coupled with Autosampler Series 3000, both from Agilent Technologies).

Colonization and Distribution of Endophytes Within Host Plants
Strains were tagged with fluorescent proteins as previously described (Sánchez-López et al., 2018) in order to monitor their colonization to the host plant under similar experimental conditions. The labeled strains were inoculated (10⁹ cells mL⁻¹) to plants with and without addition of pollutants. Their colonization efficiency was investigated with a confocal laser microscope Ultra VIEW VoX, PerkinElmer (Zaventem, Belgium) using an excitation wavelength of 561 nm (red) for mCherry, and 405 (Dapi) for plant cell walls while the confocal pictures were analyzed using ImageJ software and Amira 3D visualization software version 6.1.0 (FEI Visualization Sciences Group, Hillsboro, OR, United States) as previously described (Sánchez-López et al., 2018).

**PCR and qPCR**
The occurrence of various ARGs such as the sulfamethoxazole (sulI) and ciprofloxacin resistance genes [qnrA, qnrS and aac(6')-Ib] was screened via polymerase chain reaction (PCR) detection assays. The primers sulI-F (5'-TTT GGA GC-3') and sulI-R (5'-CCA AGG CGG AAA CCC GCG CC-3') (Jacobs and Chenia, 2007), aac-F (5'-TTGCGA TGCTC TATGAGTGGC-3') and aac-R (5'-CTCGAATGCCTGGC GTGTTT-3') (Park et al., 2006), qnrA-F (5'-GAT AAA GTT TTT CAG CAA GAG G-3') and qnrA-R (5'-ATC CAG ATC GGC AAA GGT TA-3') and qnrS-F (5'-GTA TAG AGT CCC GTG CGT GTG A-3') and qnrS-R (5'-GGT TCG TTC CTA TCA AGC GAT T-3') (Mao et al., 2015) were used for the detection of sulI, aac(6')-Ib-cr, qnrA, and qnrS genes, respectively. PCR assays were performed as previously described (Park et al., 2006; Jacobs and Chenia, 2007; Mao et al., 2015). Since only the sulI was detected in the endophytic communities, the abundance of this gene was estimated using a StepOne Plus System (Applied Biosystems Inc., Foster City, CA, United States). qPCR reaction was performed in a 20 μL volume mixture and conducted in 96 well plates containing 10 μL of SYBR Green Dye (Applied Biosystems), 0.2 μM of each primer and 2 μL of template DNA. The detailed protocol was as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 55°C for 60 s and 72°C for 2 min. All samples and standards were amplified in triplicates. For the standard curve, six-fold serial dilution of the sulI gene isolated from the B3 endophytic strain was performed. The amplification efficiency and coefficient (r²) was 110% and 0.98, respectively. Melting-curve and a 1.5% agarose gel were used for assuring the specificity of the products.

**Statistical Analysis**
Statistical analysis was performed with R v3.3.2 (R Development Core Team, 2016). Differences in the metal concentrations and alpha diversity indices in different plant parts among the treatments were estimated with an analysis of variance (two-way ANOVA). After detecting significant differences, a multiple Tukey comparison test was performed. Correlation analysis between the diversity indices and the different metal concentrations was performed in R [package: Hmisc and corplot (Wei and Simko, 2017), based on Pearson’s product moment correlation coefficient. Processed amplicon sequencing data were analyzed using the Bioconductor package phyloseq v.1.19.1 (McMurdie and Holmes, 2013). The raw data was used prior to alpha-diversity analyses. For beta-diversity analyses, an inclusion
threshold of 2% prevalence was used, data was normalized by total sum scaling and expressed in relative abundance %, followed by a $\log(1+x)$ transformation. Community dissimilarities were represented by non-metric multidimensional scaling (NMDS) using the Bray–Curtis distance and by Principal coordinate analysis (PCoA) using weighted and unweighted UniFrac distance.

To evaluate the similarity of community assemblages among the samples, PERMANOVA (R-vegan function Adonis) was performed onto the Bray–Curtis dissimilarity matrix with $n = 999$ permutations. Mantel test analysis was performed in order to detect any significant correlation between the presence of metals under different levels of mixed pollution and endophytic bacteria composition using the Bray–Curtis dissimilarity matrix. LEfSe [Linear Discriminant Analysis (LDA) Effect Size] was used to detect the bacterial taxonomic biomarkers across the different treatments (Segata et al., 2011). In addition, differentially abundant taxa were identified using DESeq2 v1.14.1 analyses in R (Love et al., 2014). The Venn diagrams were generated according to Wang et al. (2016).

**RESULTS**

The effects of bioaugmentation with endophytic bacteria on the efficiency of phytoremediation of mixed pollutants was investigated with promising results (Syranidou et al., 2016). In this study, the effects of mixed pollutants and bioaugmentation on the root and leaf endophytic communities were investigated in depth to obtain a better understanding of the interactions between microbes and their host plants during wastewater treatment.

**Metal Uptake by Plants**

The metal concentrations in *J. acutus* roots and leaves tended to increase in the treatments with high concentrations of mixed pollution (Supplementary Figures S2–S4) while roots accumulated higher concentrations of metals compared to the leaves in every treatment. Bioaugmentation through inoculation with indigenous endophytic bacteria increased Zn concentrations in roots and leaves and two-way ANOVA revealed significant effects of the level of pollution as well as of inoculation on the metal concentrations in plants. When exposed to 200 mg L$^{-1}$ Zn, all the inoculants significantly enhanced the phytoextraction capacity of the plants compared to the non-inoculated ones. At elevated Zn concentrations, plants inoculated with strain B1, B3 and the consortium accumulated significantly higher amounts of Zn in the roots while B1, B2, and B3 inoculated plants accumulated significantly more Zn in the leaves in comparison to the non-inoculated plants, indicating an increase in the translocation factor. With respect to nickel, the beneficial effects of bioaugmentation on the phytoextraction capacity of *J. acutus* were less pronounced. Nevertheless, significant effects on plant Zn concentration were observed depending on the metal concentrations they were exposed to. At low Ni concentrations, the B1 inoculated plants accumulated significantly more Ni in the roots in comparison to the non-inoculated plants while a significantly higher Ni concentration was detected in the leaves of the plants inoculated with the consortium. When exposed to 40 mg L$^{-1}$ Ni, a significant increase in the Ni concentration in the roots of B1 inoculated plant was observed in comparison to the non-inoculated plants. The B1, B2 and B3 inoculated plants contained significantly higher Ni concentrations in their leaves in comparison to the non-inoculated plants.

Cadmium was not detected in leaves of all plants regardless of the initial exposure concentration and inoculation effort. In roots, there was no significant difference in Cd accumulation capacity of inoculated and non-inoculated plants at low and high Cd exposure.

**Responses of Endophytic Bacterial Communities**

In order to investigate the interactions between the host plant and its associated endophytic microbial community during phytoremediation of mixed polluted water as well as in case of bioaugmentation with indigenous endophytic strains, the total DNA was extracted from *J. acutus* leaves and roots and was analyzed using high-throughput sequencing. A total of 358035 16S rRNA gene sequences were obtained from 66 samples, with average read length of 353 bp. After the sequence quality filtering, denoising and removing of all chimeric sequences 63 samples remained with 231202 assembled high-quality sequences and 2680 ASVs recorded.

High levels of mixed pollution had a negative effect on alpha diversity indices of the root communities (Figure 1A). In particular, pollution significantly lowered the Shannon diversity ($F: 8.8, p = 0.001$) as well as all the indices. Significant differences were detected between the indices of the communities from highly polluted with the communities of plants exposed to low pollution and no pollution, while no significant differences were noticed between the low and non-polluted treatments. The Simpson diversity index significantly differed in communities exposed to low pollution and no pollution. The effect of various inoculants on Shannon diversity was minimal ($F: 1.1, p = 0.37$), as well as on all the indices.

Correlation analysis revealed that the number of observed species, phylodenericity, and Shannon and Simpson diversity of the root communities negatively correlated with increased concentrations of metals in roots (Figure 1B). The number of observed species was negatively affected by nickel, zinc and cadmium concentrations in the root compartment (Ni: $p < 10^{-4}$, $r = -0.53$, Zn: $p < 10^{-4}$, $r = -0.56$, Cd: $p < 10^{-4}$, $r = -0.53$), as well as the Simpson diversity index (Ni: $p < 10^{-4}$, $r = -0.73$, Zn: $p < 10^{-4}$, $r = -0.77$, Cd: $p < 10^{-4}$, $r = -0.77$). Similarly, the increased metal concentrations decreased significantly the Shannon diversity (Ni: $p < 10^{-4}$, $r = -0.71$, Zn: $p < 10^{-4}$, $r = -0.76$, Cd: $p < 10^{-4}$, $r = -0.74$).

The *J. acutus* root endophytic communities seemed to alter in function of the level of pollution. As seen in Figure 2A, separate groups are formed, while this separation is statistically significant ($p = 0.001$). Each group involved samples of plants exposed to the same concentration of the mixture of metals.
and emerging organic pollutants (High, Low, NO), indicating an induced shift in the root community composition. Root endophytic communities from the non-polluted treatments were clearly different from the others, whereas a slight overlap was noticed between communities of plants exposed to low and high pollution. With respect to the composition of the leaf endophytic community, no clear pattern was detected (Supplementary Figure S5). It appears that almost all samples are grouped together regardless of the level of pollution or inoculation effort.

Mantel correlation coefficients indicated that significant relationships existed between root communities of plants exposed to different concentrations of metals (Figure 2B). When the root communities from the different inoculation treatments were
investigated separately, different correlations between endophytic assemblages and the exposure concentration of pollutants were revealed. The root endosphere of B2 inoculated plants was significantly correlated with the increasing metal concentrations in this plant compartment, while no association was observed between the other endophytic communities and each metal, indicating treatment-specific responses.

Endophytic Community Composition

In this study, 16 phyla were detected in the endosphere of *J. acutus*; overall the phylum Proteobacteria dominated the endophytic bacterial community. The root community consisted mainly of Alphaproteobacteria followed by Gamma-proteobacteria, and members of Bacteroidia, Fibrobacteria, and Actinobacteria (Figure 3).

The abundance of Proteobacteria was significantly increased in root communities exposed to elevated concentrations of mixed pollution, while the phyla Bacteroidetes and Fibrobacteres significantly decreased. In accordance, the abundance of Alphaproteobacteria was significantly increased in roots of plants grown in high polluted treatments in comparison to unpolluted ones, while the abundance of Fibrobacteria, Bacteroidia, and Deltaproteobacteria was significantly decreased. Within the root endophytes, those affiliated with the order Sphingomonadales were enriched in the *J. acutus* roots exposed to high pollution whereas those affiliated with Flavobacteriales were enriched in roots exposed to low pollution. The family *Sphingomonadaceae* exhibits the higher relative abundance within the root endophytic communities from high and low polluted treatments while the abundance of the families *Pseudomonadaceae* and *Rhizobiaceae* is higher in roots of plants grown in absence of pollutants.

The phylum Proteobacteria dominated almost all leaf communities; in general endophytic leaf community abundance profiles showed more variable patterns compared to the root communities. The most abundant classes were Alphaproteobacteria followed by Gamma-proteobacteria, and members of Bacteroidia, Fibrobacteria and Actinobacteria, while Bacilli and Saccharimonadidae exhibited high relative abundance (Figure 4). It is important to notice that the class
Bacilli dominated the leaf community of B2 inoculated plants that were not exposed to pollution which is in accordance with the inoculant. Similarly, the relative abundance of the B1 strain was higher in the leaf endophytic communities of unexposed plants in comparison to exposed ones. As seen in Figure 5, the endophytic strain B3 could efficiently colonize the roots surface of *J. acutus* in the presence of mixed pollution.

Changes in the community members were observed in response to increasing levels of pollution (Figure 6). The root communities from plants exposed to different concentrations of mixed pollution were compared in order to identify the potential indicator taxa. Only 85 root ASVs (LEfSE, $p < 0.05$, log$_{10}$ LDA score >3.5) were enriched or depleted across the pollution gradient. More specifically, members of Proteobacteria (affiliated to *Burkholderiaceae*, *Micromonosporaceae*, *Xanthobacteraceae*, and *Rhodocyclaceae*) and Bacteroidetes (affiliated to *Flavobacteriaceae*) were enriched at the low level of pollution. ASVs assigned to the families such as *Xanthobacteraceae*, *Dongiaceae*, *Xanthomonadaceae*, *Rhizobiaceae* and *Caulobacteraceae*, *Devosiacae*, *Fibrobacteraceae*, *Pseudomonadaceae* were discriminative for roots of plants grown in the absence of pollution. In the roots of plants exposed to high pollution, the genera *Herminiimonas*, *Methylophilus*, *Cupriavidus*, *Novosphingobium*, and *Oligotropha* were enriched. According to Venn diagrams, a high number of ASVs is shared among the root communities exposed to different levels of pollution (Figure 7). Similarly, the high percentage of ASVs can...
be found in both plant compartments along the pollution gradient.

**qPCR**

One sulfonamide gene (*sulI*) was detected in the endosphere of roots of plants exposed to mixed pollution, while it was not found in the roots of non-exposed plants (*Figure 8*). Fluoroquinolone resistant genes [*qnrA, qnrS, and aac(6′)-Ib-cr*] were not found in roots. At low concentrations of pollutants, the abundance of *sulI* gene showed significantly higher values in non-inoculated roots while it was not detected in plants inoculated with the consortium. When elevated concentration of pollutants was added to the aqueous phase, the abundance of SMX resistance genes increased in B1 and B3 inoculated plants as well as plants inoculated with the consortium after 21 days of incubation. In non-inoculated and B2 inoculated plants, the *sulI* abundance remained stable at the two levels of pollution.

**DISCUSSION**

Applying phytotechnologies to sites with mixed pollution is a complex issue, since possible interactions between organic xenobiotics and metals may occur as well as with the associated...
FIGURE 6 | Biomarkers of the endophytic community of roots exposed to high and low concentration of pollutants and of the roots in unpolluted treatments. LEfSe was used to validate the statistical significance and the size effect of the differential abundances of taxa (Kruskal–Wallis and Wilcoxon rank-sum $p < 0.05$ and LDA score $>3.5$). In the cladogram, the class, order, and family are presented and the genus is represented using letters.

FIGURE 7 | Venn diagrams of root endophytic communities exposed to a mixed pollution gradient (A), and endophytic communities of J. acutus exposed to high (B), low (C) pollution as well as no pollution (D).
were inoculated to J. acutus plant growth properties (ACC deaminase, siderophores, IAA) studies (Mesa et al., 2015). Metal tolerant bacteria expressing no effect of metal accumulation was demonstrated by other endophytic bacteria stimulates the host to uptake metals (Ma et al., 2016a). Often, the inoculation of bacterial strains possessing plant growth promoting traits along with tolerance to the target compound has a positive phytoremediation outcome (Rajkumar et al., 2009; Afzal et al., 2014). Our results corroborate the before-mentioned observations. After considering the total amount of metals accumulated in the whole plant biomass, it was demonstrated that (in almost all cases) significantly higher amounts of Ni and Zn were accumulated inside inoculated plants in comparison to non-inoculated ones. Utilizing a variety of metal tolerant strains ensured that many PGP characteristics will be expressed since the single inoculants are able to produce a limited number of traits.

The bacterial community is an important factor in determining the removal rates in CWs (Meng et al., 2014), and is strongly affected in terms of functionality and diversity by the presence of helophytes (Fernandes et al., 2015; Button et al., 2016). The type and the concentration of pollutants are also significant factors that shape the plant associated communities; negative trends in diversity of endophytic bacteria have been described in response to increased levels of pollutants (Peng et al., 2013; Su et al., 2015). High metal concentrations were reported to have negative or adverse effects on the microbial community (Khan et al., 2010; Mucha et al., 2013). In the more sensitive environment such as CW in comparison to soils, multi-metal pollution caused changes in the bacterial community (Zhang C. et al., 2016). A significant negative correlation between the alpha indices of the root endophytic community and the metal concentration was observed (Figure 1). Moreover, the root communities were separated in response to the level of pollution; communities from the non-polluted treatments were clearly different from the others, whereas a slight overlap was observed between communities of low and high-polluted treatments (Figure 2). Similarly, significant differences were detected in the cultivable endophytic communities isolated from Halimione portulacoides across a gradient of metal(loid) pollution (Fidalgo et al., 2016). A significant dissimilarity has also been revealed between the DDE-exposed and non-exposed root endospheres of Cucurbita pepo (Eevers et al., 2016).

For the leaf endosphere, a convergence among the leaf composition has been demonstrated and it may be attributed to the lower metal concentrations in this plant compartment (Figure 4). This suggests that this compartment is affected to a lesser extent by the mixed pollution. However, the cultivable leaf endophytic communities of Spartina alterniflora were reported to vary among plants harvested from oil polluted sites (Kandalepas et al., 2015). Most likely, the limited dispersal possibilities of seeds along with the oil presence and stochastic phenomena contribute
to this dissimilarity. Host plant, sampling site and time have been identified to be significant factors that influence the leaf community at non-polluted areas (Ding et al., 2013).

Proteobacteria dominated the cultured endophytic community of several wetland plants growing in CWs (Calheiros et al., 2016). In our study, the family Sphingomonadaceae exhibits the higher relative abundance within the root endophytic communities from high and low pollution treatments (Figure 3). This is not surprising since this family comprises of well-known genera for their ability to degrade a variety of organic pollutants (Acosta-González et al., 2015; Waigi et al., 2017). This family together with the Methylophilaceae, Burkholderiaceae, and Xanthobacteraceae are the marker families in the root microbiome at high pollution treatments (Figure 6). Members of the family Methylophilaceae has been found to exhibit higher abundances in metal polluted soils (Kou et al., 2018). Increase in the abundance of the families Burkholderiaceae and Xanthobacteraceae has been demonstrated in the enrofloxacin and cefitiofur acclimated communities from rhizosphere sediments of CWs (Alexandino et al., 2017). It is important to mention that the abundance of ASVs affiliated with the family Pseudomonadaceae decreased significantly in the roots exposed to low and high pollution although this family is frequently isolated from plants at polluted sites (Aafzal et al., 2014).

The plant itself in combination with the environmental parameters such as the type and concentration of pollutants can selectively enrich specific genotypes of endophytic bacteria (Siciliano et al., 2001). For example, the concentration of genes encoding for PAH-ring hydroxylating dioxygenases was stimulated in endophytic communities isolated from plants growing in more polluted areas (Oliveira et al., 2014). Moreover, the abundance of these genes was significantly higher in a root endosphere of plants exposed to phenanthrene in comparison to the control community (Hong et al., 2015). In our study, an antibiotic resistance gene to sulfamethoxazole was identified in the root endosphere of plants exposed to phenanthrene in comparison to the control community (Hong et al., 2015). The increased concentration of the mixture of pollutants seemed a crucial factor that decreased the diversity and shaped the root endosphere communities while the inoculation effort had minor impact. Moreover, the diversity of the root communities showed a significant correlation with the metal concentrations in this plant part as well as the community composition, while the extent of this correlation varied among the inoculated plants. In contrast, the leaf communities seemed to remain unchanged across the pollution gradient the plants were exposed to. Specific ASVs were enriched in the root compartment in response to high levels of mixed pollution. However, it has not yet been examined whether plants growing in multi-polluted soils/water alter the survival potential of specific resistant and/or beneficial microbes. Thus, it is crucial to explore the diversity, distribution, and activity of endophytic microbial communities associated with various plant species in phytoremediation studies and monitor the changes induced due to pollution. The best of our knowledge, this is the first study that uses high throughput analysis in order to elucidate the responses of endophytic communities to different levels of mixed pollution, including metals and emerging organic contaminants. More studies are needed to reveal the underlying mechanisms that drive the

CONCLUSION

Engineering the plant endosphere towards enhancing the efficiency of the host is expected to expand CW applications. Bioaugmentation with indigenous endophytic bacterial strains was shown to improve the metal phytoextraction potential of the wetland plant *J. acutus*. The increased concentration of the mixture of pollutants seemed a crucial factor that decreased the diversity and shaped the root endosphere communities while the inoculation effort had minor impact. Moreover, the diversity of the root communities showed a significant correlation with the metal concentrations in this plant part as well as the community composition, while the extent of this correlation varied among the inoculated plants. In contrast, the leaf communities seemed to remain unchanged across the pollution gradient the plants were exposed to. Specific ASVs were enriched in the root compartment in response to high levels of mixed pollution. However, it has not yet been examined whether plants growing in multi-polluted soils/water alter the survival potential of specific resistant and/or beneficial microbes. Thus, it is crucial to explore the diversity, distribution, and activity of endophytic microbial communities associated with various plant species in phytoremediation studies and monitor the changes induced due to pollution. To the best of our knowledge, this is the first study that uses high throughput analysis in order to elucidate the responses of endophytic communities to different levels of mixed pollution, including metals and emerging organic contaminants. More studies are needed to reveal the underlying mechanisms that drive the
synergistic relationships between plants and their endophytic bacteria in order to exploit this symbiosis towards more robust and resilient phytoremediation technologies.

**AUTHOR CONTRIBUTIONS**

ES performed most of the experimental work and wrote the first draft manuscript. ST helped with the amplicon libraries preparation, the analysis of the metagenomic data, and with the discussion of the statistics. ES, ST, and IP monitored the preparation, the analysis of the metagenomic data, and with the proofreading, and revision of the manuscript.

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**SUPPLEMENTARY MATERIAL**

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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