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Ph.D. Thesis

# "*Interactions between helophytes and endophytic bacteria: their synergistic effects in bioremediation of contaminants*"

# *«Αλληλεπιδράσεις μεταξύ ελόφυτων και ενδοφυτικών βακτηρίων: η συνεργιστική τους δράση στη βιοεξυγίανση των ρύπων»*

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# ABSTRACT

Emerging organic contaminants (EOCs) consist of a large and relatively new group of chemical compounds such as endocrine disrupting chemicals (EDCs) as well as pharmaceuticals and personal care products (PCPs). Despite their generally low concentrations in the environment they can cause toxic effects on biota while they remain in the environment since they are continuously released. They are mainly entering to the environment through the effluents of wastewater treatment plants because these systems have not been developed to treat this kind of compounds. Metals are considered as the main toxic and genotoxic compounds present in hydrosoluble fractions. When they are released to soil or water bodies, they remain there since they cannot be degraded. They can only be transferred.

Phytoremediation–based technologies are environmentally friendly alternatives for cleaning up soils or (ground)waters contaminated with metals and/or a variety of organic pollutants. These treatment methods exploit plants, their associated microorganisms and the developed interactions for contaminant removal or enhancement of plant stabilization and survival in such adverse environments. Constructed Wetlands (CWs) are low-cost wastewater treatment technologies that are part of phytoremediation applications. They are simplified systems but several physicochemical and biological processes take place in order to clean the water.

In this thesis, an integrated approach exploiting the wetland plant *Juncus acutus* and its indigenous endophytic community was followed in order to investigate the capability of this meta-organism to clean water contaminated with metals, bisphenol-A, ciprofloxacin and/or sulfamethoxazole.

After confirming experimentally the ability of the plant to treat efficiently bisphenol-A-contaminated water, the associated endophytic community of *Juncus acutus* was isolated and characterized. Many strains expressed plant growth promoting characteristics and were found to possess increased tolerance to metals such as Zn, Ni, Pb and Cd. Moreover, several endophytic bacterial strains tolerated and even used bisphenol-A and/or antibiotics (ciprofloxacin and sulfamethoxazole) as the sole carbon source.

Some strains combined many of the desired characteristics and they were further used in a bioaugmentation strategy in order to investigate their potential to improve the efficiency of the wetland helophyte *Juncus acutus* to deal with mixed pollution consisting of emerging organic contaminants (EOCs) and metals at two concentration levels. The beneficial effects of inoculation with tailored endophytic bacteria separately and as a mixture were more prominent in case of high contamination. Especially, the plants inoculated with an endophytic consortium removed higher percentages of organics and metals from the liquid phase in shorter times compared to the non-inoculated plants without exhibiting significant oxidative stress. Moreover, the consortium inoculated plants' phytoextraction capacity was enhanced in terms of observed metal concentrations in the plant compartments and also as total metal mass accumulated in the whole plant.

A significant shift of the root endosphere communities was observed due to increased presence of contaminants while the inoculation effort did not have a significant impact. Metal concentration decreased the root bacterial diversity but the root composition of plants inoculated with the endophytic consortium was not affected by the increased metal concentrations. At all levels of contamination, the leaf endophytic communities were not affected either by contaminants or by the inoculation effect.

Based on the experimental evidence from this work it can be inferred that bioaugmentation with indigenous endophytic bacteria is an appropriate strategy to be employed in systems such as constructed wetlands treating water with mixed contaminants. It appears that the developed synergistic relationships between plants and endophytic bacteria may point towards more efficient, resilient and robust phytoremediation applications.

# ΠΕΡΙΛΗΨΗ

Οι αναδυόμενοι οργανικοί ρύποι (Emerging organic contaminants) είναι μία μεγάλη αλλά σχετικά καινούρια κατηγορία ρύπων που περιλαμβάνουν διάφορες χημικές ουσίες όπως οι ενδοκρινικοί διαταράκτες (Endocrine disrupting chemicals), φαρμακευτικές ενώσεις (Pharmaceuticals) και προϊόντα προσωπικής φροντίδας (Personal care products). Παρόλο που η συγκέντρωσή τους στο περιβάλλον είναι μικρή, έχουν τη δυνατότητα να προκαλέσουν τοξικότητα στους οργανισμούς. Η παρουσίας τους είναι συνεχής καθώς διοχετεύονται στο περιβάλλον ασταμάτητα. Η έξοδος των βιολογικών καθαρισμών αποτελεί την κύρια πηγή εισόδου τους στο φυσικό περιβάλλον καθώς αυτά τα συστήματα δεν έχουν αναπτυχθεί για την επεξεργασία τέτοιων χημικών ενώσεων. Τα μέταλλα αποτελούν, επίσης, μία σημαντική κατηγορία ρύπων, μάλιστα θεωρούνται ως οι κύριες αιτίες πρόκλησης τοξικών και γενοτοξικών αντιδράσεων στους οργανισμούς. Μετά την εισαγωγή τους στο οικοσύστημα, εφόσον δεν είναι διασπώμενες ουσίες παραμένουν στο περιβάλλον και μπορούν μόνο να μεταφερθούν.

Οι τεχνολογίες φυτοεξυγίανσης είναι φιλικές προς το περιβάλλον εναλλακτικές για την εξυγίανση του εδάφους ή των υδάτων από μέταλλα ή/και οργανικούς ρύπους. Βασίζονται στην εκμετάλλευση των φυτών, των σχετιζόμενων μικροοργανισμών και των αναπτυσσόμενων αλληλεπιδράσεων αυτών των δύο. Με αυτό τον τρόπο, επιτυγχάνεται η απομάκρυνση των ρύπων ή η εξασφάλιση της παρουσίας και βιωσιμότητας των φυτών σε αντίξοα περιβάλλοντα. Οι τεχνητοί υγροβιότοποι είναι συστήματα επεξεργασίας αποβλήτων με χαμηλό κόστος λειτουργίας και αποτελούν μία από τις πρακτικές εκφάνσεις της φυτοεξυγίανσης. Είναι απλοποιημένα συστήματα στα οποία συντελούνται φυσικοχημικές και βιολογικές διεργασίες για τον καθαρισμό του νερού.

Στην παρούσα διατριβή, επιχειρήθηκε μία ολοκληρωμένη προσέγγιση του συστήματος του υδρόβιου φυτού *Juncus acutus* και της σχετιζόμενης με αυτό ενδοφυτικής κοινότητας, για τη μελέτη της ικανότητας αυτού του μετα-οργανισμού να καθαρίζει νερό ρυπασμένο με μέταλλα, δισφαινόλη, σιπροφλοξασίνη και σουλφαμεθοξαζόλη.

Αρχικά η ικανότητα του φυτού να επεξεργαστεί νερό ρυπασμένο με δισφαινόλη διασφαλίστηκε πειραματικά και στη συνέχεια η σχετιζόμενη με αυτό ενδοφυτική

κοινότητα απομονώθηκε και χαρακτηρίστηκε. Πολλά στελέχη βρέθηκαν ικανά να εκφράζουν ιδιότητες που προάγουν την ανάπτυξη του φυτού και ταυτόχρονα να μπορούν να αντέξουν αυξημένες συγκεντρώσεις μετάλλων (ψευδάργυρο, νικέλιο και κάδμιο). Επίσης, κάποια στελέχη μπορούσαν να ανεκτούν υψηλές συγκεντρώσεις δισφαινόλης ή/και αντιβιοτικών και να τα χρησιμοποιούν ως μοναδική πηγή άνθρακα.

Τα ενδοφυτικά βακτήρια που βρέθηκαν να συνδυάζουν την πλειονότητα των επιθυμητών χαρακτηριστικών, επιλέχθηκαν για να χρησιμοποιηθούν σε πείραμα βιοενίσχυσης (bioaugmentation). Σκοπός του πειράματος ήταν να μελετηθεί η πιθανότητα αύξησης της αποδοτικότητας του υδρόβιου φυτού *Juncus acutus* να επεξεργαστεί νερό ρυπασμένο με μέταλλα και οργανικούς ρύπους σε δύο διαβαθμισμένες συγκεντρώσεις. Τα οφέλη του εμβολιασμού με ένα επιλεγμένο μείγμα ενδοφυτικών βακτηρίων ήταν εμφανή όταν η συγκέντρωση των ρύπων ήταν η υψηλότερη. Συγκεκριμένα, τα φυτά που εμβολιάστηκαν με αυτό το μείγμα βακτηρίων απομάκρυναν το μεγαλύτερο ποσοστό μετάλλων και οργανικών ρύπων από το διάλυμα σε συντομότερο χρόνο, σε σύγκριση με τα φυτά που δεν εμβολιάστηκαν. Ταυτόχρονα, τα εμβολιασμένα φυτά δεν έδειξαν σημάδια σημαντικού οξειδωτικού στρες. Ο εμβολιασμός με το επιλεγμένο μείγμα ενδοφυτικών βακτηρίων ωφέλησε και την εγγενή ικανότητα του φυτού να αποθηκεύει μέταλλα στο εσωτερικό του τόσο σε επίπεδο συγκέντρωσης όσο και στο επίπεδο συνολικής μάζας.

Τα αυξημένα επίπεδα ρύπων προκάλεσαν σημαντική αλλαγή της ενδοφυτικής κοινότητας που εδρεύει στη ρίζα, ωστόσο η επίδραση των διαφορετικών προσπαθειών εμβολιασμού δεν επέφερε σημαντικές αλλαγές σε αυτήν. Οι συγκεντρώσεις των μετάλλων μείωσαν επίσης τη βακτηριακή ποικιλότητα αλλά η σύνθεση της κοινότητας της ρίζας των φυτών που εμβολιάστηκαν με το επιλεγμένο μείγμα βακτηρίων δεν επηρεάστηκε. Ακόμη, η κοινότητα των βακτηρίων που απομονώθηκε από τα φύλλα δε μεταβλήθηκε ως απόκριση στα αυξημένα επίπεδα των μετάλλων ή των εμβολιασμένων βακτηρίων.

Τα πειραματικά αποτελέσματα συνηγορούν στο ότι η βιοενίσχυση με συγκεκριμένα αυτόχθονα ενδοφυτικά βακτήρια θεωρείται ως μία κατάλληλη στρατηγική για την αντιμετώπιση ρύπανσης από διαφορετικές πηγές και μπορεί να αξιοποιηθεί στους τεχνητούς υγροβιότοπους που καλούνται να επεξεργαστούν παρόμοια λύματα. Η εκμετάλλευση της συνεργιστικής δράσης των φυτών με τα ενδοφυτικά τους βακτήρια

δείχνει ικανή να αυξήσει την αποδοτικότητα, την αντοχή και την ευρωστία των εφαρμογών φυτοεξυγίανσης.

# Chapter 1

# Introduction-Literature Review

Phytotechnologies refers to the application of science and engineering in order to provide solutions for treatment of soil and (ground)water contamination by exploiting the synergistic relationship between plants and their associated microorganisms. In this introduction, the characteristics and the potential impact of selected contaminants to the environment are described. Next, the extent of contaminants uptake by plants and their contribution in phytoremediation are investigated together with the role of endophytic bacteria. Constructed Wetlands is one of the applications of phytotechnologies and the important role of macrophytes along with their associated microorganisms in such systems is highlighted.

## 1. Contaminants

### 1.1 Emerging organic contaminants (EOCs)

Emerging organic contaminants (EOCs) consist of a large and relatively new group of chemical compounds. Endocrine disrupting chemicals (EDCs) as well as pharmaceuticals and personal care products (PCPs) belong to this category and a potential risk of their presence in the environment arises since their impact, fate and their degradation products have not been well described (Garcia-Rodríguez *et al.*, 2014).

Despite their generally low concentrations in nature, they behave as persistent pollutants since they are continuously released to the environment. EDCs are natural or synthetic compounds that can alter the physiological endocrine functions, thus inducing problems in the reproduction and in metabolism in humans and animals (Campbell *et al.*, 2006). PCPs are widely adopted compounds for external use, they have been found to cause toxicity to marine wildlife, bacteria and mammalian cells and a potential risk

for bioaccumulation exists since the majority of them are hydrophobic compounds (Hopkins and Blaney, 2016). Concerning pharmaceuticals, the spreading of antibiotic resistance genes in the environment along with the risk of human exposure to them raises concern to public (Benotti and Brownawell, 2009).

These chemical compounds are released to the environment through point or diffuse sources, but mainly through the effluents of wastewater treatment plants (Rizzo *et al.*, 2013). Such systems have not been designed to treat these compounds and the promising advanced technologies for successful degradation are avoided due to the their high cost (Ávila *et al.*, 2013). As a consequence, they have been detected at potentially environmentally significant concentrations in many media such as surface waters, groundwater, seas, compost and manure. Generally, their concentration is lower in surface waters in comparison to the WWTP effluents, however, they have been detected in freshwater rivers in North America, Europe, Asia and Australia (Pal *et al.*, 2010). In these reservoirs, the xenobiotics may be eliminated due to biotransformation, photolysis, sorption, volatilization and dispersion, or a combination of them. The concentrations of some EOCs are higher in surface waters than groundwater while exactly the opposite is the case for others (Stuart *et al.*, 2012). For example, several EOCs have been found in concentrations higher that 100 ng $L^{-1}$ in groundwater in Spain (Jurado *et al.*, 2012). The specific properties of the compound together with the environmental characteristics and many processes such as dilution, adsorption, degradation and initial concentration influence the fate of EOCs in aquifers (Jurado *et al.*, 2012; Lapworth *et al.*, 2012).

### 1.1.1 Bisphenol-A

Bisphenol A (BPA) (4,4-isopropylidenediphenol; 2,2-bis(4- hydroxyphenyl)-propane) is a chemical compound with molecular weight of 228.29 g $cm^{-3}$. The compound is a white, crystalline solid substance with a melting-point of 156◦C and boiling-point of 220◦C (at pressure of 5 hPa). The water-octanol partition coefficient of BPA expressed in logarithmic form is 3.32, which shows its good solubility in fats and low solubility in water (about 200 mg $dm^{-3}$ at 25◦C). BPA belongs to a group of phenols, which have hydroxyl residues directly bound to the aromatic rings. The presence of hydroxyl groups in BPA determines its good reactivity. BPA may be converted to ethers, esters

and salts. Moreover, it undergoes electrophilic substitution like nitration, sulphonation or alkylation.

It is the most important synthetic xenoestrogen and among the most detected EDCs in groundwater samples (Lapworth *et al.*, 2012; Peng *et al.*, 2014). Moreover, its presence in humans may be associated with the development of chronic diseases like diabetes and obesity, cardiovascular disease, birth defects and reproductive disorders (Rezg *et al.*, 2014). Even at low concentration, the xenobiotic can interfere with the activity of endogenous estrogens by altering the physiological activity of the hormone receptors (Wetherill *et al.*, 2007b). It can also affect the androgen system, the development, differentiation, and function of the central nervous system, disrupt the thyroid hormone function and influences the immune system. In plants, increased BPA concentrations in the surrounding aqueous have also shown to alter the ratios of growth and stress hormones in the roots, resulting in growth inhibition at higher concentrations (Wang et al., 2015).

Bioremediation is one of the approaches used for cleaning the environment from BPA since many plant species have been found able to remove BPA from aqueous media (Loffredo *et al.*, 2010; Christofilopoulos *et al.*, 2016; Saiyood *et al.*, 2010). Their ability can be attributed to the plant uptake along with the microbial co-metabolism in the rhizosphere; indeed many microorganisms have been isolated from various sources that are able to degrade BPA (Husain and Qayyum, 2012). Among them, there are many gram-negative strains belonging mainly to the genus *Sphingomonas* and *Pseudomonas* and many gram-positive bacteria mainly associated with *Bacillus* (Zhang *et al.*, 2013). The key genes, enzymes and degradation pathways of BPA degradation has been described in specific bacteria (Sasaki *et al.*, 2008; Kolvenbach *et al.*, 2007; Sasaki *et al.*, 2005). Besides bacteria and plants, many other organisms like fungi, fish and mammals can degrade BPA through various mechanisms (Kang *et al.*, 2006).

### 1.1.2 Ciprofloxacin

Ciprofloxacin is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid with molecular weight of 331.3 g $mol^{-1}$. It is a faintly yellowish to light yellow crystalline substance. The log $K_{ow}$ of ciprofloxacin at pH 7.04 is calculated to be about 0.28, and its water solubility is 30000 mg $L^{-1}$ (at 20 ◦C). The

melting point of the compound is 257◦C and at a pH of 7.04, the molecule carries both a negative and a positive charge.

The first quinolone, nalidixic acid, was introduced into clinical use in 1962 and in the mid-1980s ciprofloxacin, a fluoroquinolone with a wide spectrum of *in vitro* antibacterial activity, became clinically available (Colomer-Lluch *et al.*, 2014). This antibiotic inhibits DNA synthesis by blocking type II topoisomerases (DNA gyrase in gram-negative bacteria and topoisomerase IV in gram-positive organisms), enzymes that control DNA supercoiling. It is prescribed as an antibiotic for several infections such as bone and joint infections, certain types of infectious diarrhea, respiratory tract infections and skin infections. In bacterial communities, many resistant isolates have been found carrying plasmid-mediated genes and their chromosomal homologues that code a pentapeptide repeat protein protecting type II topoisomerases from quinolones (Rodríguez-Martínez *et al.*, 2011).

Ciprofloxacin is a commonly used veterinary and clinical antibiotic and it is among the compounds with the highest frequency in WWTP effluents in Europe (Loos *et al.*, 2013) and in Australia (Watkinson *et al.*, 2007). Its concentration is usually higher in hospital effluents than in influents of WWTP treating municipal wastewater (Varela *et al.*, 2014); indeed the number of resistance genes to fluoroquinolones seemed to increase in the effluents of WWTP leading to the spread of antibiotic resistant genes into surface waters (Rodriguez-Mozaz *et al.*, 2015). It can be characterized as a recalcitrant compound since it is not affected by natural attenuation in surface waters or by UV (photolysis) in engineered systems (Pal *et al.*, 2010). However, it is susceptible to ozonation but the ozone does not react with the part of the compound that is responsible for the pharmacological effect. It has been demonstrated that ciprofloxacin is not a readily biodegradable compound; indeed information of ciprofloxacin degradation by bacteria is scarce (Amorim *et al.*, 2013). Moreover, in a mesocosm experiment, decreases in diversity and functionality of bacterial communities were observed after ciprofloxacin addition (Weber *et al.*, 2011). The removal mechanism in WWTP is rather adsorption to sludge than biodegradation (Le-Minh *et al.*, 2010); as a result, this antibiotic is released in the environment mainly through the application of biosolids to the agricultural lands.

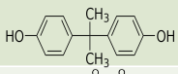
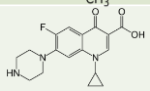
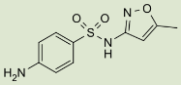
### 1.1.3 Sulfamethoxazole

Sulfamethoxazole (4-Amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide) is a sulfonamide bacteriostatic antibiotic, used for bacterial infections such as urinary tract infections, bronchitis and prostatitis. It has a molecular weight of 253.3 g $mol^{-1}$ and the melting point is 167 ◦C. The log $K_{ow}$ of SMX is 0.89 and the water solubility is 2800 mg $L^{-1}$ at 20 ◦C.

The compound inhibits the two pathway steps in bacterial folic acid synthesis. Folate derivatives are essential cofactors in the biosynthesis of purines, pyrimidines and bacterial DNA in all living cells. It is mainly utilized by humans and as a result it is among the most detected pharmaceutical in groundwater aquifers (Lapworth *et al.*, 2012). It was demonstrated at the highest concentrations in groundwater from urban areas in Spain (Jurado *et al.*, 2012) and was also detected with high frequency in groundwater samples due to municipal landfilling in China (Peng *et al.*, 2014) and in WWTP effluents in Greece (Thomaidi *et al.*, 2015). This compound was the most prevalent antibiotic compound with stable concentrations in two different reservoirs in Spain (Huerta *et al.*, 2013).

Several rates of SMX removal were observed by different systems. For example, 52-70% removal was demonstrated in pilot scale MBRs independently of the initial concentration while 90% overall removal was achieved in a conventional WW treatment (Larcher and Yargeau, 2012a). However, other studies showed limited degradation in WWTPs (Benotti and Brownawell, 2009). Due to the low log $k_{ow}$, SMX sorption to sludge is not significant while the primary removal mechanism can be attributed to biodegradation. In order to optimize these technologies, researchers tried to focus on the degradation capacity of individual strains with promising results. When the SMX-degrading abilities of seven individual strains were investigated in the presence and absence of glucose (6 mg $L^{-1}$ SMX± 0.5 g L-1 glucose), the results showed that SMX was successfully degraded by *Rhodococcus equi* (15 % up to 29 % removal with glucose) while the other six bacterial strains only realized marginal SMX degradation even with the addition of glucose (< 6 % removal) (Larcher and Yargeau, 2011). In another study, 24 to 44 % SMX mineralization was achieved by an inoculum from an established lab-scale MBR after 16 days of cultivation at 127 mg $L^{-1}$ initial SMX concentration (Bouju *et al.*, 2012).

Table 1. List of the EOCs used.

| Compounds | Category | Chemical structure | Log $k_{ow}$ | Molecular weight | pKa |
|---|---|---|---|---|---|
| Bisphenol A | Endocrine disruptor |  | 3.32 | 228.29 g/mol | 9.6 |
| Ciprofloxacin | Antibiotic-Fluoroquinolones |  | 0.28 | 331.34 g/mol | 6.09 |
| Sulfamethoxazole | Antibiotic-Sulfonamides |  | 0.89 | 253.28 g/mol | 5.7 |

## 1.2 Metals

Release of metals from various industrial, agro-chemical, atmospheric and domestic sources represents a major threat to the environment. Their presence can have serious effects on soil and water quality, plant and animal nutrition, as well as human health. With the term "metals", the elements that demonstrate metallic properties (transition metals, metalloids, lanthanides, and actinides), with atomic density greater than 4 g/cm$^3$, or 5 times or more, greater than water, and are toxic, even at low concentrations, are defined (Colin *et al.*, 2012). They are considered as the main toxic and genotoxic compounds present in hydrosoluble fractions and since they cannot be degraded, they may enter the trophic webs or spread into the groundwater and sediments and remain there (Guittonny-Philippe *et al.*, 2014a).

It is necessary to clean up the contaminated sites in order to drastically reduce the potential impacts of metal contamination. Immobilization, soil washing, and phytoremediation are some of the usually proposed techniques/technologies for remediation of metal contaminated soils (Wuana and Okieimen, 2011). For polluted waters, physicochemical strategies such as filtration, chemical precipitation, electrochemical treatment, oxidation/reduction, ion exchange and membrane technologies and biological strategies such as bioremediation and phytoremediation have been implemented (Guo *et al.*, 2010).

Plants have many mechanisms to recruit in order to cope with metals, such as changing the soil properties in the rhizosphere, the glutathione–phytochelatin-mediated defence mechanism, chelation, while reduction of membrane permeability to metals, efflux

pumps to remove metals from the cell interior, EPS sequestration and metal complexation are some of the bacterial metal resistance mechanisms (Ullah *et al.*, 2015). In high concentrations, metals have been found to have adverse effects on microbial communities in terms of functionality and diversity (Epelde *et al.*, 2015) and on plants in terms of growth and survival (Christofilopoulos *et al.*, 2016). For example, metals such as Cu and Pb can alter the microbial community structure, thus influencing the function of this community in salt marshes (Mucha *et al.*, 2013).

### 1.2.1 Zinc

Zinc is a transition metal with the following characteristics: period 4, group IIB, atomic number 30, atomic mass 65.4, density 7.14 g $cm^{-3}$, melting point 419.5◦C, and boiling point 906◦C. It is considered as an essential microelement with many roles in cellular metabolism, since several enzymes contain zinc, it participates in the formation of carbohydrates and has a structural role in several transcription factors (Nagajyoti *et al.*, 2010). However, high Zn concentrations can lead to toxic effects in plants. Excess Zn inhibits many plant metabolic functions, plant growth and causes chlorosis in the younger leaves (Nagajyoti *et al.*, 2010). For example, Zn excess in hydroponic cultures significantly reduced the growth of *Juncus acutus* plants, the mean height of tillers and the photosynthetic pigments (Mateos-Naranjo *et al.*, 2014). In another experiment, at high Zn concentrations germination of *J. acutus* seedlings was decreased, total absence of photosynthetic activity (a) and impairment of the onset of light saturation (Ek) were observed as well as increase in the activities of specific antioxidant enzymes (Santos *et al.*, 2014).

This metal is also an essential trace element for microorganisms, yet in high concentrations it may have adverse effects on bacteria. However, many Zn-tolerant bacterial strains that have been isolated from different environments such as soil and sewage sludge (Misra *et al.*, 2012; He *et al.*, 2010). Limcharoensuk *et al.* (2015) demonstrated that Zn-tolerant bacteria can accumulate zinc through adsorption/precipitation on the cell walls rather than accumulation in the cell interior.

### 1.2.2 Nickel

Nickel is a transition element with atomic number 28 and atomic weight 58.69. The ion of this metal changes depending on the pH (Wuana and Okieimen, 2011). At low pH, the nickelous ion Ni(II) is formed, while in neutral to slightly alkaline solutions, it precipitates as nickelous hydroxide, $Ni(OH)_2$, which is a stable compound. In acid conditions nickelous hydroxide dissolves and forms Ni(III) and in very alkaline conditions it forms the nickelite ion, $HNiO_2$.

Nickel is recognized as another essential micronutrient for living organisms and is a component of the enzyme urease, which is essential in animals. Exposure to nickel excess can cause allergic dermatitis known as nickelitch, and hair loss while inhalation can cause cancer of the lungs, nose, throats and stomach (Ali *et al.*, 2013). Increased Ni concentration causes various physiological alterations, toxicity symptoms, impairment of nutrient balance and changes in water balance in plants (Nagajyoti *et al.*, 2010). By consequence, high Ni concentrations inhibit the shoot growth, reduce the water and chlorophyll content as well as the proline accumulation in wheat (Gajewska *et al.*, 2006). Many plants have been characterized as nickel-hyperaccumulators such as *Alyssum bertolonii* and an increased number of Ni-resistant bacteria colonize their rhizosphere or harbor the endopshere (Mengoni *et al.*, 2009; Barzanti *et al.*, 2007).

### 1.2.3 Cadmium

Cadmium is located at the end of the second row of transition elements with atomic number 48, atomic weight 112.4, density 8.65 g $cm^{-3}$, melting point 320.9◦C, and boiling point 765◦C. Cadmium (Cd) can originate from metal mining and smelting industry, commercial fertilizers, batteries and automobile emissions. This metal is carcinogenic, mutagenic, and teratogenic, it acts as an endocrine disruptor, it interferes with calcium metabolism in biological systems and it causes renal failure and chronic anemia (Ali *et al.*, 2013). In plants, it inhibits photosynthesis, and diminishes water and nutrient uptake, resulting in chlorosis, growth retardation, browning of root tips, ultra-structural damage and ultimately to death (Najeeb *et al.*, 2011).

It also affects microbial communities (Lorenz *et al.*, 2006), however, many cadmium-resistant strains have been isolated from various environments such as the plant

endosphere (Luo *et al.*, 2011), rhizosphere (Prapagdee *et al.*, 2013), soil (Ansari and Malik, 2007) or sewage sludge (Limcharoensuk *et al.*, 2015). Along with bacteria, many plants can tolerate high cadmium concentrations and even some of them can accumulate it in their interior tissues, being characterized as cadmium hyperaccumulators (Qiu *et al.*, 2008; Boominathan and Doran, 2003).

## 2. Plant uptake

Many studies have demonstrated that plants can accumulate various metals in the plant compartments (Lu *et al.*, 2013; Anjum *et al.*, 2014) and according to the accumulated amount they are categorized into different groups. For example, metal hyperaccumulators are plants which accumulate extreme amounts of trace metals in their aboveground biomass when growing in metal enriched habitats (mg $kg^{-1}$; >10,000 (Mn or Zn), >1,000 (Cu, Co,Cr, Ni, Pb) or >100 (Cd). Several plant species (e.g., *Alyssum bertolonii*, *Arabidopsis halleri*, *Solanum nigrum*, *Eichhornia crassipes*, and *Thlaspi caerulescens*) have been proposed for phytoextraction of Ni, Cd, Zn and Pb (McGrath *et al.*, 2006). Metal uptake by plants can be influenced by many important factors related to the plant species, sediment characteristics, chemical speciation of metals in the rhizosphere, the overlying and interstitial water chemistry, and also the behavior of the microorganisms (Almeida *et al.*, 2004).

Organic contaminants can be taken up by plants depending on their concentration, the physicochemical properties and the plant species (Garcia-Rodríguez *et al.*, 2014). Many predictive relationships between the organic compound uptake by plant compartments as a function of physical-chemical characteristics of the organic compound have been addressed (Burken and Schnoor, 1998). It assumes that the uptake of an organic compound can be estimated as the transpiration stream concentration factor (TSCF) multiplied by the volume of water transpired and linear average of the bulk solution concentration from time t1 to time t2. Generally, the compounds with a log $k_{ow}$ between 0.5-3.5 are considered to move fast into the plants, thus the contact time with the rhizosphere microorganisms is short (Weyens *et al.*, 2009e). Recently, a new approach has been proposed, it is based on Hildebrand solubility parameters and it considers both the affinity of the solute to its amorphous host, and the solute's “incompatibility” with

water promoting binding of the solute to the polymer sorbent phase (Poerschmann and Schultze-Nobre, 2014).

Initially, the compound is absorbed by the matrix, then is moved into the root xylem and is transported to the shoot and to the leaves. Many compounds may even follow the transpiration rate, they are taken up by plants, transferred to leaves and then evapotranspired to the atmosphere (Burken and Schnoor, 1998). The rate of translocation to aerial parts differs among the different organic compounds. Especially helophytes may transfer volatile organic compounds to the atmosphere via their aerenchymatous tissues (Imfeld *et al.*, 2009), thus causing serious environmental problems.

Several studies highlight the ability of various plants to take up and translocate emerging organic compounds in different experimental set-ups, *e.g.* hydroponical (Bouldin *et al.*, 2006; Dodgen *et al.*, 2013) and soil experiments (Hawker *et al.*, 2013) under controlled conditions and also in field conditions (Wu *et al.*, 2014). The information about the ability of species of the genus *Juncus* to take up these compounds is scarce. The ability of *Typha* spp. to remove carbamezine from water and accumulate it in the plant tissues was demonstrated (Dordio *et al.*, 2011). In another study, the fate of 20 frequently used pharmaceuticals and personal care products was investigated concerning potential plant uptake and translocation (Wu *et al.*, 2013). It was demonstrated that the neutral compounds were adsorbed on the root surfaces while a hydrophilicity-regulated transport determines the translocation to the aboveground tissues. Hydrophilic compounds were susceptible to transport to the leaves in the direction of the transpiration stream. Treated wastewater used for agricultural irrigation and biosolids and manure amendments, pose a risk since these compounds are taken up by the vegetables or other crop plants mainly through the transpiration stream (Hurtado *et al.*, 2016; Malchi *et al.*, 2014; Clarke and Smith, 2011). However, several studies suggest that the concentration of these compounds in the edible parts is negligible (Prosser and Sibley, 2015) or that the impact of human exposure to them needs further investigation (Wu *et al.*, 2015b).

# 3. Microbial-assisted phytoremediation

## 3.1 Phytoremediation

Development, selection and application of suitable methods for cleaning up contaminated environments continues to be an important topic in terms of environmental restoration and protection. Plant (phytoremediation)-based technologies are considered as environmentally friendly alternative methods for cleaning up soils or (ground)waters contaminated with heavy metals and/or a variety of organic pollutants from the environment (Lu and Zhang, 2014; Vangronsveld *et al.*, 2009). Among those processes are: (a) phytoextraction (plants are used to adsorb/take up contaminants from the sediment/soil and to transport and concentrate them in harvestable above-ground biomass/tissues), (b) phytovolatilization (plants take up water soluble metals and release them as they transpire the water), (c) phytostabilization (the plants completely immobilize the contaminants through accumulation by roots or precipitation within the rhizosphere and/or by changing their chemistry), (d) rhizofiltration (based on root activities, aquatic plants and/or hydroponically cultivated plants are used to remediate contaminated water through absorption, concentration, and precipitation of pollutants), (e) phytoaccumulation (pollutants are accumulated in plants' biomass), (f) rhizo- and phytodegradation (pollutants are being degraded into insoluble or non-toxic compounds, through plant metabolic processes and/or interaction with microorganisms) and (g) phytoexcretion (salt tolerant species capable of exuding salt also possess the ability to exude other toxic ions) (Manousaki and Kalogerakis, 2011; Anjum *et al.*, 2014).

It is considered as more cost-effective than the engineering-based remediation technologies such as soil excavation, soil washing or burning, or pump-and-treat systems (Becerra-Castro *et al.*, 2011). Moreover, this technology contributes to soil stabilization, production of biomass with economic value, maintain or even enhance the local diversity and demands simple monitoring methods (Vangronsveld *et al.*, 2009). Of course, there are many limitations in this technology that need to be overcome towards implementation on a wide scale. It can be applied to low or moderate contaminated environments, due to potential phytotoxicity to the plants (Ali *et al.*, 2013). High concentrations of contaminants can inhibit the plant metabolism, affecting the establishment and growth and they can even cause senescence. Furthmore, the zone

of activity is restricted to the root zone of proliferation, which is plant species dependent (Chirakkara *et al.*, 2016). The bioavailability of the metal or organic xenobiotic is a critical factor, significantly influencing the phytoremediation outcome (Gerhardt *et al.*, 2009). Generally, some metals tend to be accumulated in the plant parts whereas others show slower uptake rates. Concerning organics, highly hydrophobic compounds cannot move into the plants while only the hydrophilic ones can be transferred to the aboveground tissues.

Interactions among contaminants, microbes and plants have attracted attention because of the biotechnological potential of microorganisms for metal or organics removal directly from polluted media or their possible plant growth promotion effects in contaminated environments. Therefore, research in phytoremediation is interdisciplinary and requires the synergism among different fields of science such as chemistry, biology, ecology as well as environmental engineering. Despite the challenges, several methods have been developed towards improving the efficiency of this technology and a large number of them concerns the enhancement through the use of plant-associated bacteria.

Box 1. Role of plants and their associated bacteria in phytoremediation (Bell *et al.*, 2014).

The possible fates of soil pollutants following plant introduction are described clearly in a review by Pilon-Smits [69]. Here we show the major contributions of both introduced plants and partner microorganisms in the phytoremediation of mixed contaminant soils (Figure I). Soil microorganisms are the primary agents of organic mineralization in soil, and may also convert contaminants such as heavy metals to stable and/or less toxic forms. Although such microbial activity can occur in bulk soil, introduced plants have the potential to augment microbial contributions to bioremediation in the soil via stimulation of microbial biomass and/or activity in the rhizosphere, although this activity is dependent on which specific microorganisms and activities are promoted. Plant–microbe interactions are complex, and plants may favor microorganisms that promote their growth or provide protection from pathogens if pollutant stress is not sufficiently elevated, whereas opportunistic microorganisms that do not contribute to phytoremediation may also capitalize on plant-produced compounds.

Microorganisms can also facilitate the uptake of pollutants such as heavy metals by plant roots, which are then translocated (absorbed and relocated) to other components of the plant. Plants can store many contaminants in biomass that can later be harvested, but some compounds are volatilized from the aerial portions of the plant. However, volatilization without prior transformation may simply release toxic compounds into the air. Microorganisms that reside on or within aerial plant tissue can help to stabilize and/or transform contaminants that have been translocated, which may limit the extent of volatilization. Microorganisms that form direct associations with either the above-ground or below-ground portions of plants can positively (for a review on plant-growth-promoting bacteria, see [70]) or negatively influence plant growth and fitness, which alters the ability of a plant to directly remediate and/or stimulate associated organisms.

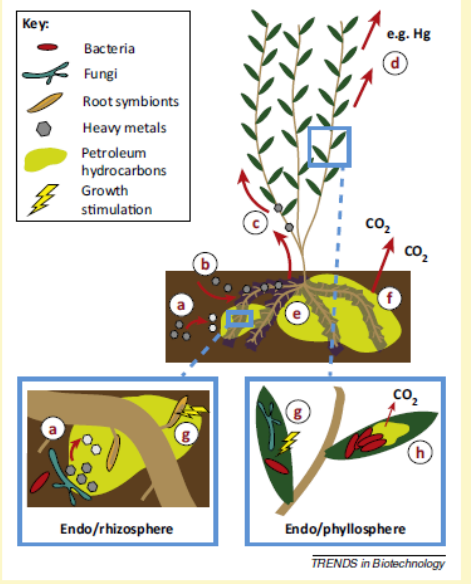



**Figure I.** Primary contributions of plants and associated microorganisms to phytoremediation in mixed contaminant soils. Metals may be (a) transformed by microorganisms in the rhizosphere, (b) taken up by the plant, (c) translocated to plant tissue, and/or (d) volatilized. Microbial activity can be stimulated directly by plants through (e) root exudate release, which may be particularly important for promoting (f) microbial degradation of hydrocarbons. Through a variety of mechanisms, microorganisms also influence (g) plant growth and can be involved in (h) pollutant transformation in the aerial parts of plants.

## 3.2 Endophytic bacteria

A recent, promising strategy to improve phytoremediation and detoxification of contaminants is the exploitation of endophytic bacteria (Weyens *et al.*, 2009c). Endophytic bacteria have been isolated from a wide variety of plants and the terminology refers to those bacteria that reside in the plant interior without causing any negative effects on their host (Doty, 2008). Considering the vast majority of plants of which the endophytes have not been studied yet, there is high chance to find new, beneficial microorganisms (Ryan *et al.*, 2008a). They are classified as obligate or facultative according to their life strategies. Obligate endophytes are transferred from one generation to another through seeds and are strictly dependent on the host plant for their growth and survival while facultative colonize the root interior mainly via the roots (Weyens *et al.*, 2009f). Once they are inside, they can inhabit a specific organ or they can be spread throughout the plant since they can reside within cells, in the intercellular spaces, or in the vascular system (Reinhold-Hurek and Hurek, 2011). A two step-selection progress has been proposed to explain how the root microbiota differentiates from the rhizosphere (Bulgarelli *et al.*, 2013). Soil parameters shape the soil microbiota while in the first step, the rhizodeposits alter the soil assemblages. Further, host-dependent interactions determine the differentiation in endophytic community in the second.

Bacterial endophytes may offer several advantages to the host plant, as they can promote plant growth through several mechanisms or increase their host's resistance to the stress. Their contribution in remediation of soil/water contaminated with metals or organics has been illustrated and reviewed in several studies (Ma *et al.*, 2016a; Afzal *et al.*, 2014) (Fig.1). For example, endophytic strains equipped with the appropriate degradation gene decreased the evapotranspiration of toluene when they were inoculated to their host plants (Barac *et al.*, 2004; Weyens *et al.*, 2009a). With regard to metals, many endophytic strains were isolated from *J. acutus* that were found able to grow at high Cr(VI) concentrations and even reduce the Cr(VI) to the less toxic Cr(III) in a short period of time (Dimitroula *et al.*, 2015).

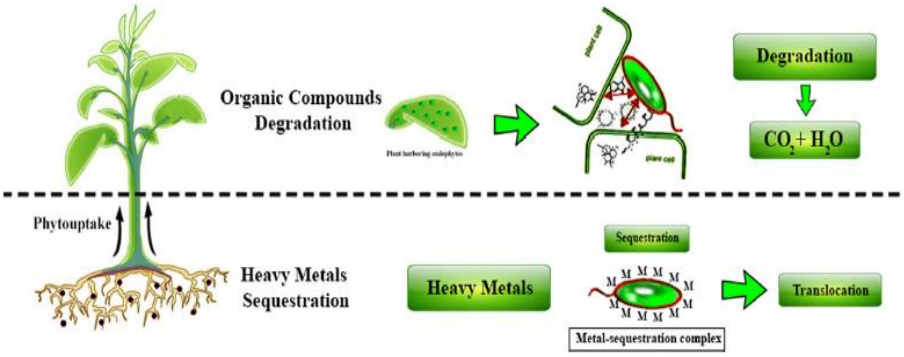



Figure 1. The mechanisms of endophytic bacteria in order to tackle with organic compounds and metals (Ijaz *et al.*, 2015a).

## 3.3 Beneficial effects of bacteria to the plant

The exploitation of plant-associated bacteria can improve the efficiency of phytoremediation since bacteria can have direct or indirect beneficial effects on the plant (Ullah *et al.*, 2015). There are three approaches available for this technology: (i) bioattenuation, which is the method of monitoring the natural progress of degradation to ensure that the contaminant concentration decreases with time; (ii) biostimulation, where natural biodegradation and/or biotransformation are stimulated with nutrients, electron acceptors, or substrates; and (iii) bioaugmentation, which is a way to enhance the bio- degradative or biotransforming capacities of contaminated sites by inoculation with microorganisms that possess the desired characteristics (Colin *et al.*, 2012).

The plant growth promoting bacteria (rhizospheric or endophytic) may facilitate the plant growth through several direct or indirect mechanisms. Nitrogen fixation, nutrient solubilization (*e.g.* iron or phosphorus), production of phytohormones and enzymes synthesis are classified as direct mechanisms (Glick, 2010). The indirect mechanisms involve the production of antibiotics against pathogens, depletion of iron in the rhizosphere, production of lysing enzymes, competition for binding sites or increasing the resistance of the host (Jha *et al.*, 2012).

Nitrogen is one of the principal plant nutrients, becoming a limiting factor for plant growth especially in contaminated ecosystems. Many nitrogen fixing bacteria were identified to develop a mutualistic relationship with plants, alleviating the N

deficiencies in N-poor soils (Rosenblueth and Martínez-Romero, 2006; Ikeda *et al.*, 2013). Many bacteria can reduce gaseous dinitrogen to ammonia by the nitrogenase enzyme complex or reduce nitrate to nitrite, and nitrite can be further converted to nitrogen oxides ($N_2O$ and NO) or ammonia. NO is a potent signaling molecule in plants, influencing the root growth and proliferation in an auxin-dependent manner. Inoculation with a bacterium isolated from nodules increased the nitrogen in roots and shoots of the host plant (Wani *et al.*, 2008).

Phosphorus is the second most important macronutrient for plant growth and is considered as a limiting nutrient in many soils since an important fraction of this element is often present in unavailable forms (insoluble phosphates and organic P compounds). Many rhizospheric and endophytic bacteria have been found able to solubilize inorganic phosphorus (Oteino *et al.*, 2015; Gagne-Bourgue *et al.*, 2013), thus assisting the plant to get access to the appropriate amount of phosphorus.

Microorganisms produce and secrete siderophores mainly to sequester iron. This element is not available to plants because of the low solubility of $Fe^{3+}$-oxides. Siderophores are low-molecular chelating agents and are classified as catecholates, hydroxamates, and α-carboxylates, depending on the chemical nature of their coordination sites with iron (Pérez-Miranda *et al.*, 2007). Metal-resistant siderophore producing bacteria significantly contribute in the plant growth and survival in metal contaminated areas by ameliorating the plant stress induced by metals and supplying the plant with nutrients, particularly iron (Rajkumar *et al.*, 2010). Along with iron, siderophore secretion may be used for improving metal bioavailability, however, it is still unclear how metals stimulate the production of these molecules (Schalk *et al.*, 2011).

The phytohormone ethylene is known to have an important role in various processes, such as senescence, leaf abscission, fruit ripening and pathogen-defence signaling (Glick, 2014). When a plant is exposed to various unfavorable conditions, its ethylene biosynthesis is induced, hence root elongation may be inhibited (Santoyo *et al.*, 2016). Bacteria possessing the 1- aminocyclopropane-1-carboxylate (ACC) deaminase may protect the plant since this enzyme reduces plant ethylene levels by catalyzing the conversion of the ethylene precursor ACC exuded by plants into ammonia and α-ketobutyrate (Sgroy *et al.*, 2009). As a result, the plant exudes more ACC in an attempt

to maintain the equilibrium between the internal and external ACC levels, and reduces the synthesis of ethylene inside the plant cell.

Plant hormones are involved in virtually all stages of plant growth and development such as cell elongation, cell division and tissue differentiation. Among them, indole-3-acetic acid (IAA) is very important and the main precursor for its biosynthesis is tryptophan. A relation has been demonstrated between the levels of IAA produced by bacteria and root elongation. Low levels of IAA stimulate the growth of the primary root while increased levels promote lateral and adventitious root formation but inhibit primary root growth (Rajkumar *et al.*, 2009). In turn, an extended root system is available for colonization. Moreover, IAA bacterial production protects the plants from adverse effects after exposure to high environmental stress (Glick, 2010).

Biological control, or biocontrol, is the process of suppressing deleterious/pathogenic living organisms by using other living organisms. Several mechanisms may be involved, including the production of antimicrobial compounds since rhizosphere or endophytic bacteria can synthesize a wide range of compounds with antimicrobial activity. For example, the *Agrobacterium radiobacter* K84 produces an antibiotic agrocin 84 (Hibbing *et al.*, 2010). This compound is highly specific for a subset of plant-pathogenic *A. tumefaciens* and mimics the opines agrocinopine A and agrocinopine B, which are customized nutrient sources. When the plant is infected by *A. tumefaciens*, the toxin is imported using the same route as the nutrients while the *A. radiobacter* K84 encodes a factor that confers self-immunity to the toxin. The production of 2,4-diacetylphloroglucinol (DAPG) has a wide range of properties by which it suppresses the diseases in plants (Gaiero *et al.*, 2013). Moreover, beneficial and pathogenic bacteria are competing for the same nutrient sources and space. Bacterial endophytes colonize the same niches as pathogens, thus reducing the possibility of plant infection (Ryan *et al.*, 2008b). Other bacteria can activate/stimulate the plant defence mechanisms without causing visible symptoms of stress on the host. This induced systemic resistance (ISR) depends mainly on jasmonate and ethylene signaling.

# 4. Constructed Wetlands (CWs) and Wetland plants

Constructed Wetlands (CWs) are low-cost wastewater treatment technologies that are part of phytoremediation applications. In such systems, physicochemical (evaporation, photodegradation, oxidation, hydrolysis, retention or root sorption into the gravel bed) and biological (microbiological degradation, biofilm, root and plant uptake) processes are exploited in order to treat different kinds of wastewater (Wu *et al.*, 2015a) (Fig.1). They are engineered wetlands, designed and operated to mimic natural wetland systems and they are classified into different categories: the free water surface flow (SF-CWs), horizontal subsurface flow (HSSF-CWs) systems, vertical subsurface flow constructed wetlands (VSSF- CWs) and hybrid constructed wetlands (hybrid CWs) (Li *et al.*, 2014a).

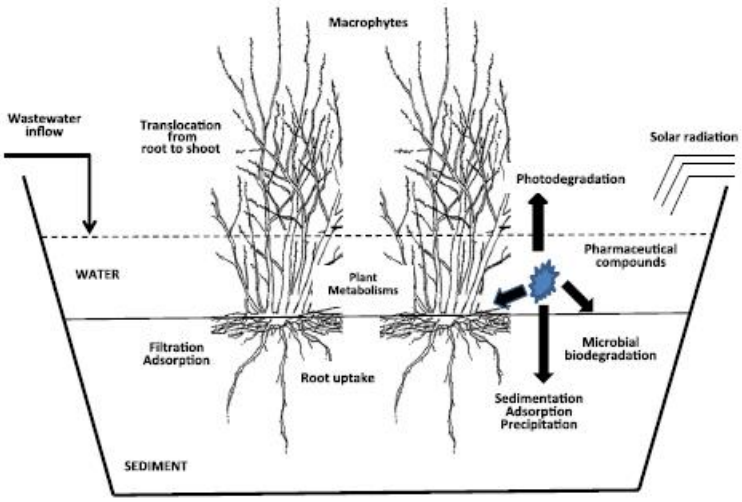



Figure 2. Mechanisms of pharmaceutical removal in CWs (*Zhang et al., 2014)*.

In a comparative study, the removal efficiencies of emerging contaminants of five major sewage treatment technologies were investigated (Melvin and Leusch, 2016). It was concluded that constructed wetlands were able to remove the highest proportion of studied contaminants while membrane bioreactors (MBR) showed the greatest overall removal. Constructed wetlands are also efficient for metal removal from industrial wastewater (Khan *et al.*, 2009; Guittonny-Philippe *et al.*, 2014b).

Appropriate plant for application in phytoremediation strategies should possess the following characteristics: (i) High growth rate, (ii) Production of high above-ground

biomass, (iii) Widely distributed and highly branched root system, (iv) Higher take up of the target heavy metals from soil, (v) Translocation of the assimilated metals from roots to shoots, (vi) Tolerance to the toxic effects of the target heavy metals, (vii) Good adaptation to the prevailing environmental and climatic conditions, (viii) Resistance to pathogens and pests, (ix) Easy cultivation and harvest, (x) Repulsion to herbivores to avoid food chain contamination (Ali *et al.*, 2013).

The role of macrophytes in CWs systems is of of high importance since these plants provide niches for growth of functional microbial taxa, decrease the water current velocity, stabilize the surface of the bed and protect the surface against frost in winter (Brisson and Chazarenc, 2009). Moreover, they transfer oxygen from aerial tissues to the rhizosphere (Stottmeister *et al.*, 2003a) and they excrete a range of exudates (Bais *et al.*, 2006), thus stimulating the growth of the specific microbial taxa with the desired characteristics. Therefore, synergistic biodegradation between plants and rhizosphere microorganisms or accumulation of the contaminants have been proposed as the main mechanism of removal in several CWs (Hijosa-Valsero *et al.*, 2016; Yan *et al.*, 2016). Combination of different plant species led to greater microbial functional diversity (Button *et al.*, 2016) which may enhance the microbial ability to treat wastewater. However, interspecies competition should be taken into account in multi-species wetlands (Zheng *et al.*, 2016).

*Phragmites australis*, *Typha* spp. or *Juncus* spp. are the most frequently used helophytes in CWs (Vymazal, 2013). It is recommended that autochthonous species should be preferred over exotic species or cultivars in order to avoid the spreading of their genes and disturbance of the local flora (Schröder and Prasse, 2013; Guittonny-Philippe *et al.*, 2014b). The genus *Juncus* comprises helophytes, mainly mash species with wide distribution (Vymazal, 2013). Species of this genus have been found to successfully contribute to remediation of metals and organics in contaminated areas and constructed wetlands.

# Chapter 2

## Objectives of PhD thesis

Constructed Wetlands are considered as an efficient green technology for treating wastewater from industrial or domestic effluents contaminated with various compounds such as metals and emerging organic compounds. The selection of the appropriate helophyte(s) for assuring high efficiency is still a controversial issue since the ideal macrophyte should combine a lot of desired characteristics. However, the most common used plant species are exotic to the regions where they are applied, thus threatening the indigenous fauna and flora. Therefore, researchers suggest that the knowledge is too limited and more experiments are needed in order to evaluate the performance of indigenous species, especially with those that naturally colonize the contaminated areas (Guittonny-Philippe *et al.*, 2015).

In this context, an autochthonous wetland species, *Juncus acutus* was proposed as a suitable candidate for being exploited in CWs across the Mediterranean region after assuring its capability to treat BPA-contaminated groundwater in a phytoremediation pilot and water co-contaminated with metals and EOCs. The isolation and genotypic and phenotypic characterization of its endophytic community was performed in order to identify the promising strains with high potential to improve the plant's phytoremediation performance (Chapter 3). The root and leaf endophytes were discriminated into separate groups according to their genotypic profiles and were further tested for their ability to promote plant growth. Next, their tolerance to different metals and resistance and potential degradation to different emerging organic contaminants was investigated.

The potential beneficial effects on the helophyte's efficiency were evaluated in a bioaugmentation experiment with tailored bacterial strains (Chapter 4). The three most promising strains according to their score in plant growth promoting tests, metal

tolerance, resistance to and potential degradation of emerging organic contaminants were inoculated separately and as a consortium to *J. acutus* plants. The eventual increases of the removal efficiency were evaluated together with the effects of the contaminants on the plant's physiology.

The potential effects on the root and leaf bacterial communities due to contaminant exposure or inoculation were investigated in order to determine the driving forces that shape the endosphere communities (Chapter 5). Total DNA was analyzed through next-generation sequencing and the diversity and structure of the different endophytic assemblages were compared. The qPCR of the antibiotic resistant genes to ciprofloxacin and sulfamethoxazole together with the 16S rRNA gene is still on progress. The results will provide information concerning potential gene transfer in this experiment.

In CWs, where the optimization strategies in design and operation parameters are extensively reviewed, selection of appropriate plants with their tailored associated bacteria seems the only promising strategy for increasing the performance. Exploring the indigenous flora will reveal the best performing species of each area and taking advantage of their endophytic inhabitants will lead to a more robust and efficient green technology.

# Chapter 3

# Bisphenol-A removal by the halophyte *Juncus acutus* in a phytoremediation pilot: Characterization and potential role of the endophytic community [1]

**Scope**

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.

## 3.1 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

[1] The material of this chapter was published in : Journal of Hazardous Materials, May 2016. doi: 10.1016/j.jhazmat.2016.05.034

cost, solar powered technology with wide public acceptance (Glick, 2010). 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 (Fester *et al.*, 2014). Verlicchi & Zambello (Verlicchi and Zambello, 2014) 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 (Michael *et al.*, 2013).

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 (Michałowicz, 2014). 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 (Rezg *et al.*, 2014). 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 (Zhang *et al.*, 2013) next to the herbaceous plant species that showed an ability to decrease BPA concentrations from aqueous media (Loffredo *et al.*, 2010).

The exploitation of plant-associated microorganisms may support a strategy towards enhanced degradation rates and improved performance of plants in CWs (Abhilash *et al.*, 2012). 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 (Li *et al.*, 2014b). 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 (Weyens *et al.*, 2009b). 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 (Rajkumar *et al.*, 2009; Ma *et al.*, 2011a). In the case of organic contaminants, endophytic bacteria equipped with appropriate catabolic genes enhance the *in planta* degradation of xenobiotics (Afzal *et al.*, 2014). 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 (Shehzadi *et al.*, 2015; Chen *et al.*, 2012; Dimitroula *et al.*, 2015; Sauvêtre and Schröder, 2015; Egamberdieva and Kucharova, 2009; Ho *et al.*, 2012).

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 (Sauvêtre and Schröder, 2015). Moreover, Dimitroula et al. (Dimitroula *et al.*, 2015) 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 (Bais *et al.*, 2006; Phillips *et al.*, 2012).

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.

## 3.2 Materials and Methods

### 3.2.1 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.3). 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 (Christofilopoulos *et al.*).

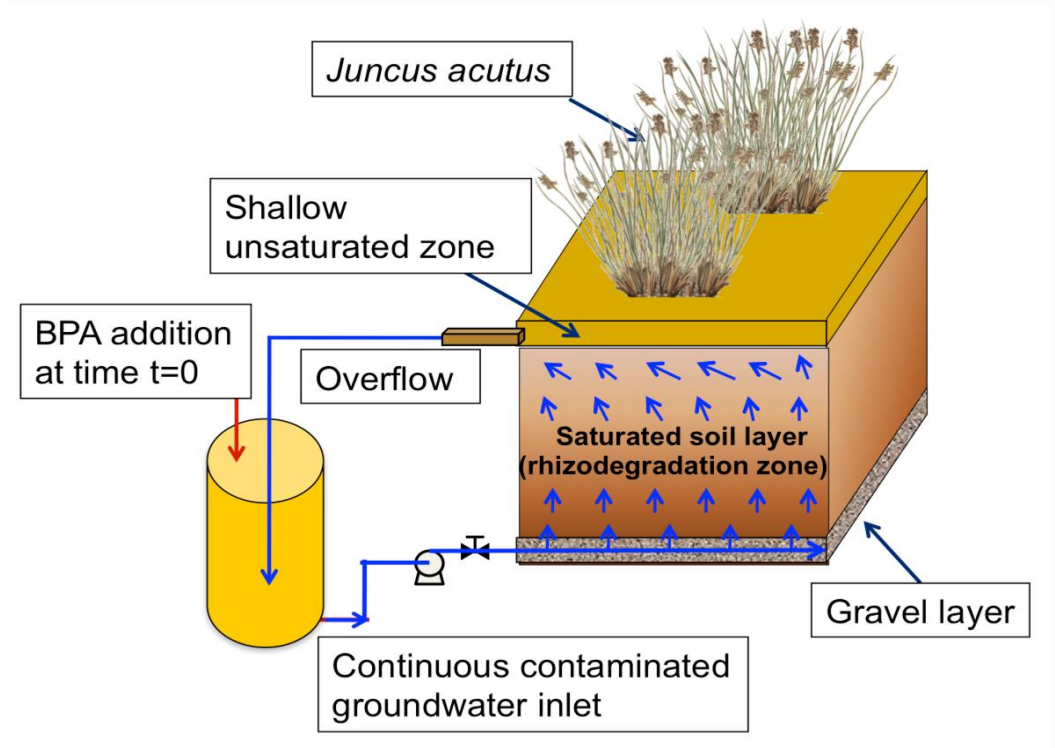



Figure 3. Schematic representation of the Shallow Aquifer Phytoremediation Pilot, planted with two *Juncus acutus* plants.

### 3.2.2 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.

### 3.2.3 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 (Cawthray, 2003) 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).

### 3.2.4 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 (Mergeay *et al.*, 1985) 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.

### 3.2.5 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 (Becerra-Castro *et al.*, 2011). 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 (Weyens *et al.*, 2009f). 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 (Tamura *et al.*, 2011). The sequences of the strains are now available in the GenBank database under accession numbers KU598698- KU598764.

### 3.2.6 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 (Nautiyal, 1999). 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 (Sheng *et al.*, 2008). The 1-aminocyclopropane-1-carboxylate (ACC) deaminase capacity of the strains was determined as previously described by Belimov et al. (Belimov *et al.*, 2005). Siderophore production was qualitatively estimated by the Chrome Azurol S assay (Schwyn and Neilands, 1987); 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 (Cunningham and Kuiack, 1992).

### 3.2.7 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.

### 3.2.8 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.

### 3.2.9 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.

### 3.2.10 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.

## 3.3 Results and Discussion

### 3.3.1 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 (Bais *et al.*, 2006). 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 (Wenzel, 2009). 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 (Zeng *et al.*, 2008). Similarly, the exudation pattern of two metallophytes was shown to be highly variable depending on the Cu concentration they were exposed to (Meier *et al.*, 2012); 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 (Mucha *et al.*, 2005; Phillips *et al.*, 2012). 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 (Mucha *et al.*, 2005). 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 (Mucha *et al.*, 2010). 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. 4, 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 (Ribeiro *et al.*, 2014). 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.

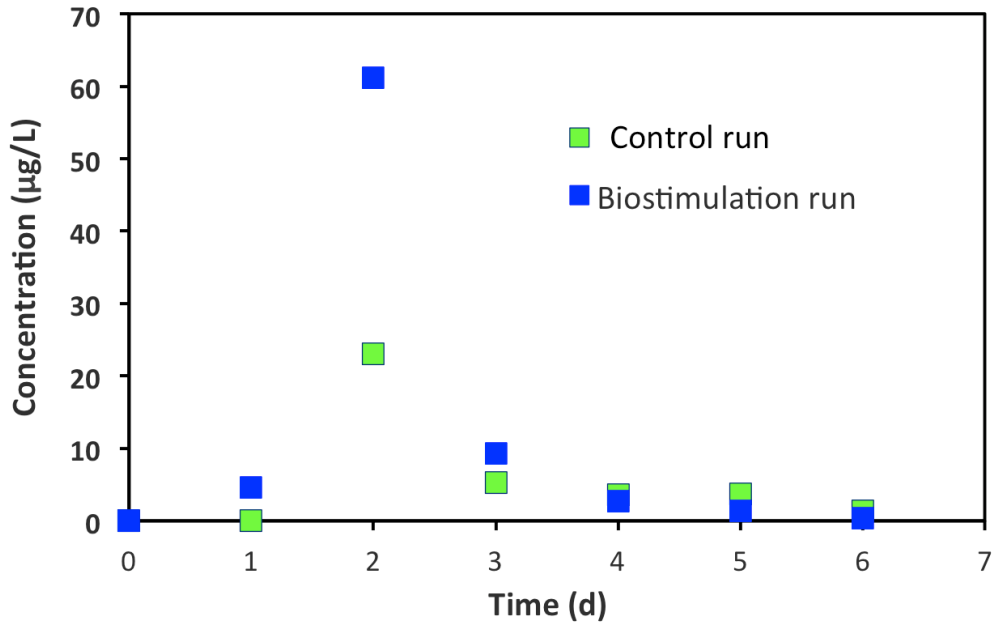



Figure 4. BPA concentration (µg $L^{-1}$) measured in the effluent in the control run (with BPA but no stimulants) and in the biostimulation run where organic acids (oxalic, tartaric, formic, malonic and lactic) were added.

### 3.3.2 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.5). 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.

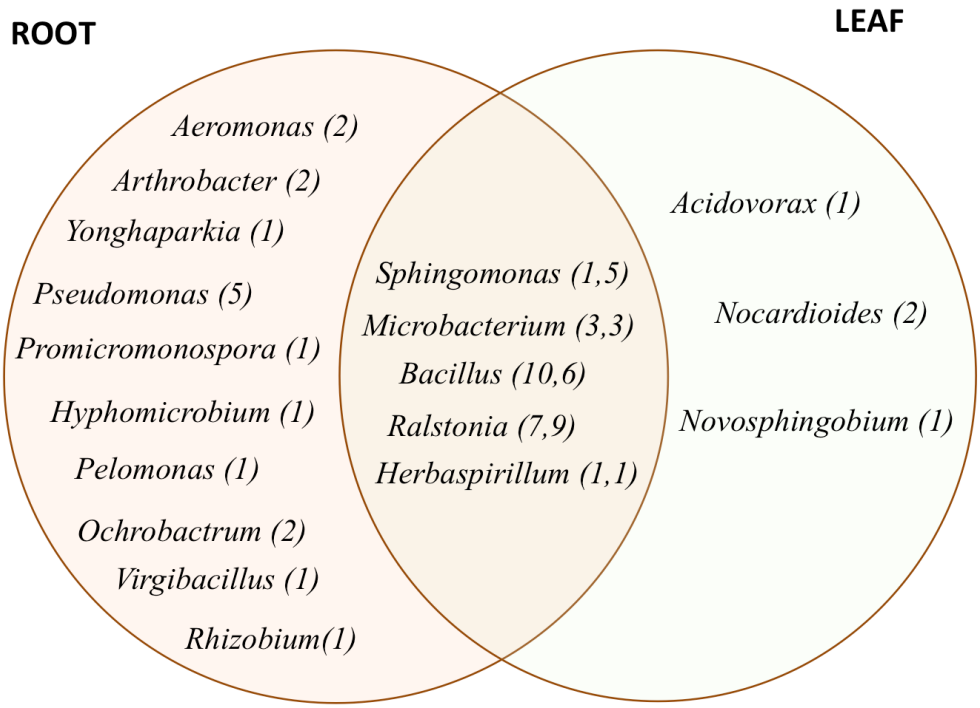



Figure 5. Distribution of endophytic isolates in plant compartments (the first number between brackets represents the number of isolates in roots and the second number between brackets represents the number of isolates in leaves).

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 (Truyens *et al.*, 2015b; Afzal *et al.*, 2014). The *J. acutus*

endophytic community contains many common genera that were also found in other plant species growing on contaminated sites (Moore *et al.*, 2006; Barzanti *et al.*, 2007; Shehzadi *et al.*, 2015; Thijs *et al.*, 2014a).

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 (Ryan *et al.*, 2007) and during the years this genus became a model genus for the study of metal tolerance mechanisms (Mergeay *et al.*, 2003). Moreover, bioaugmentation with *Ralstonia* strains enhanced the uptake of Cd and Zn by *Helianthus annuus* (Marques *et al.*, 2013) and Cr and Pb translocation to maize shoots (Braud *et al.*, 2009). 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) (Guo *et al.*, 2010). Shin et al. (Shin *et al.*, 2012) 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.

### 3.3.3 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 (Weyens *et al.*, 2009a; de-Bashan *et al.*, 2012; Weyens *et al.*, 2009b). 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.6). 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*.

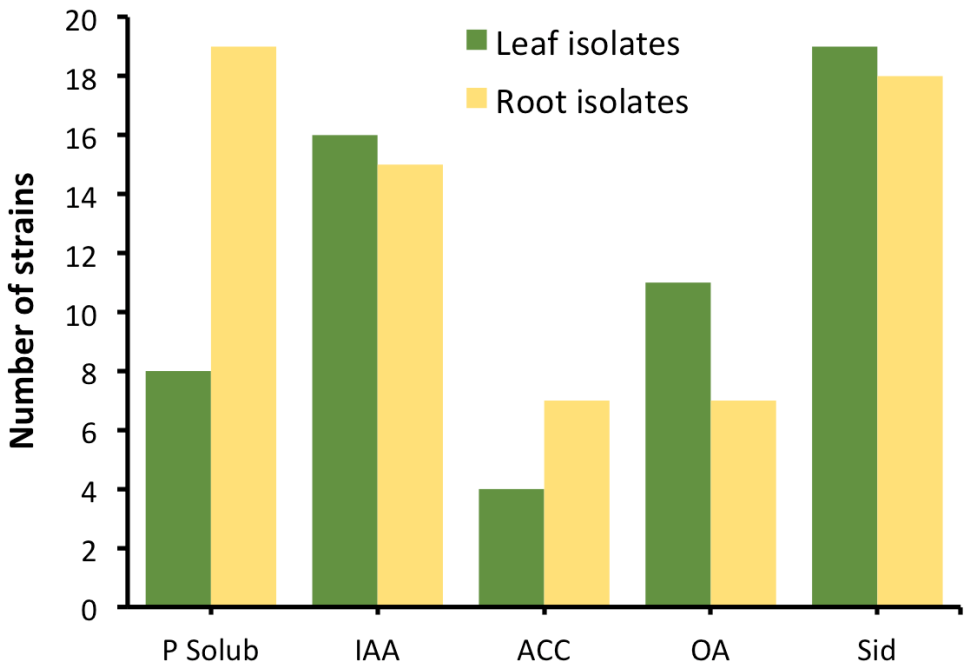



Figure 6. Percentage of endophytic strains isolated from *Juncus acutus* that have the potential to carry plant growth promoting characteristics (P Solub: Phosphate solubilizers, IAA: Indolacetic acid producers, ACC: ACC deaminase producers, OA: Organic acid producers, Sid: Siderophores producers).

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.

### 3.3.4 Tolerance to metals

Since *J. acutus* is a commonly used macrophyte in CWs worldwide (Wu *et al.*, 2015a), 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 (Almeida *et al.*, 2006), 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.7). 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%).

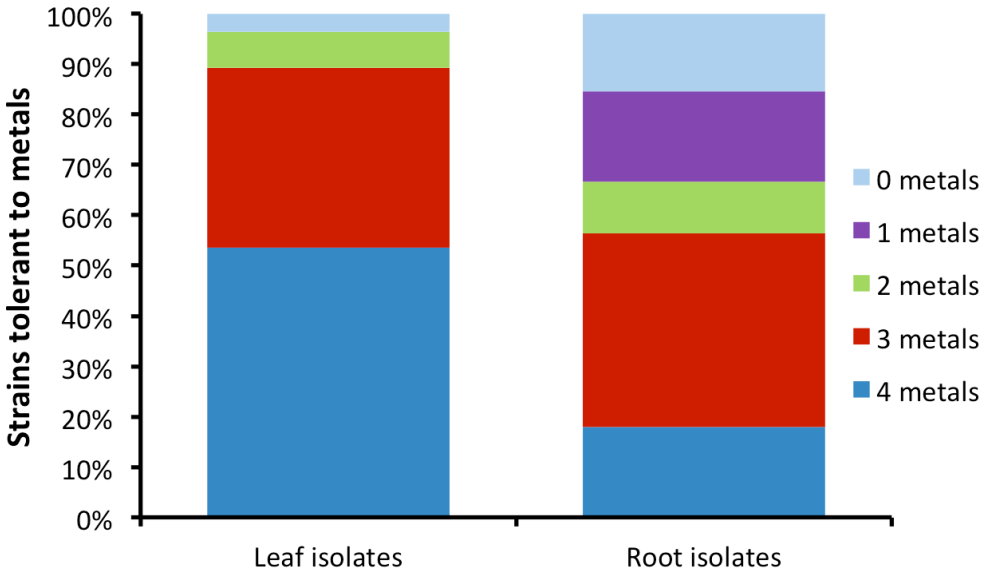



Figure 7. The number of *Juncus acutus* isolates that were tolerant to 0, 1, 2, 3 or 4 metals when they grew on 284 agar medium supplemented with trace metals (1 mM Ni, Cd, Pb or 4 mM Zn).

### 3.3.5 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 (Siciliano *et al.*, 2001). In this context, many studies (Kukla *et al.*, 2014; Sura-de Jong *et al.*, 2015; Weyens *et al.*, 2009f; Truyens *et al.*, 2015b) 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 (Toyama *et al.*, 2009; Saiyood *et al.*, 2010). 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 (Saiyood *et al.*, 2010). 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.8), 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.

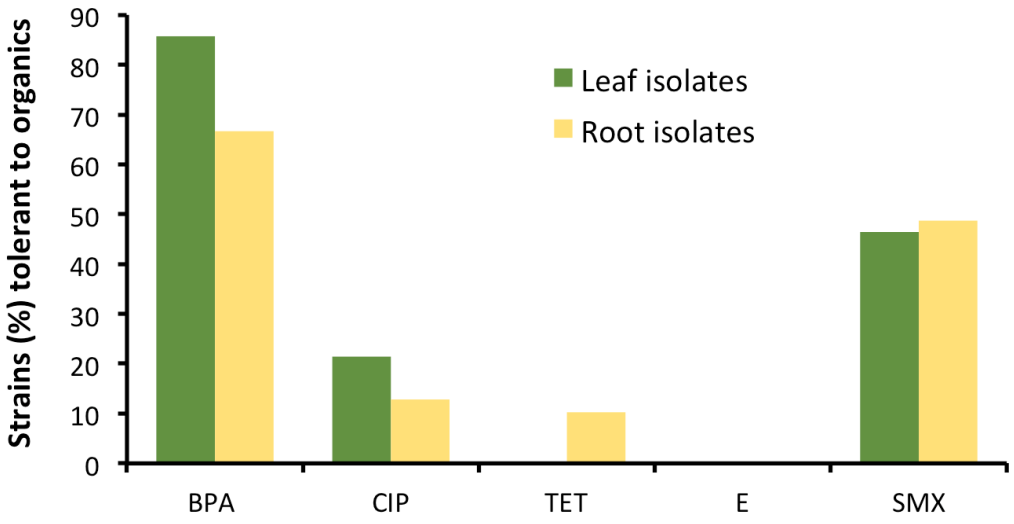



Figure 8. Percentage of leaf and root isolates that were able to grow in presence of organic pollutants (BPA: Bisphenol-A, CIP: Ciprofloxacin, TET: Tetracycline, E: Erythromycin, SMX: Sulfamethoxazole).

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 (Zhang *et al.*, 2013). 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 (Toyama *et al.*, 2009). Moreover, two *Enterobacter* sp.

strains and one *Bacillus* sp. strain associated with *Dracaena sanderiana*, enhanced BPA removal from hydroponic systems and mixed cultures (Saiyood *et al.*, 2010).

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 (Hu *et al.*, 2010), while in the wetland plant *Phragmites australis*, higher levels of antibiotics were found in roots and concentrations decreased from leaves to stems (Liu *et al.*, 2013).

About 50% of the isolates from roots and leaves of *J. acutus* showed resistance to sulfamethoxazole (Fig.8). 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 (Larcher and Yargeau, 2012b). 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 (Herzog *et al.*, 2013). 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 (Ricken *et al.*, 2011). 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. (Amorim *et al.*, 2013) 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. 9).

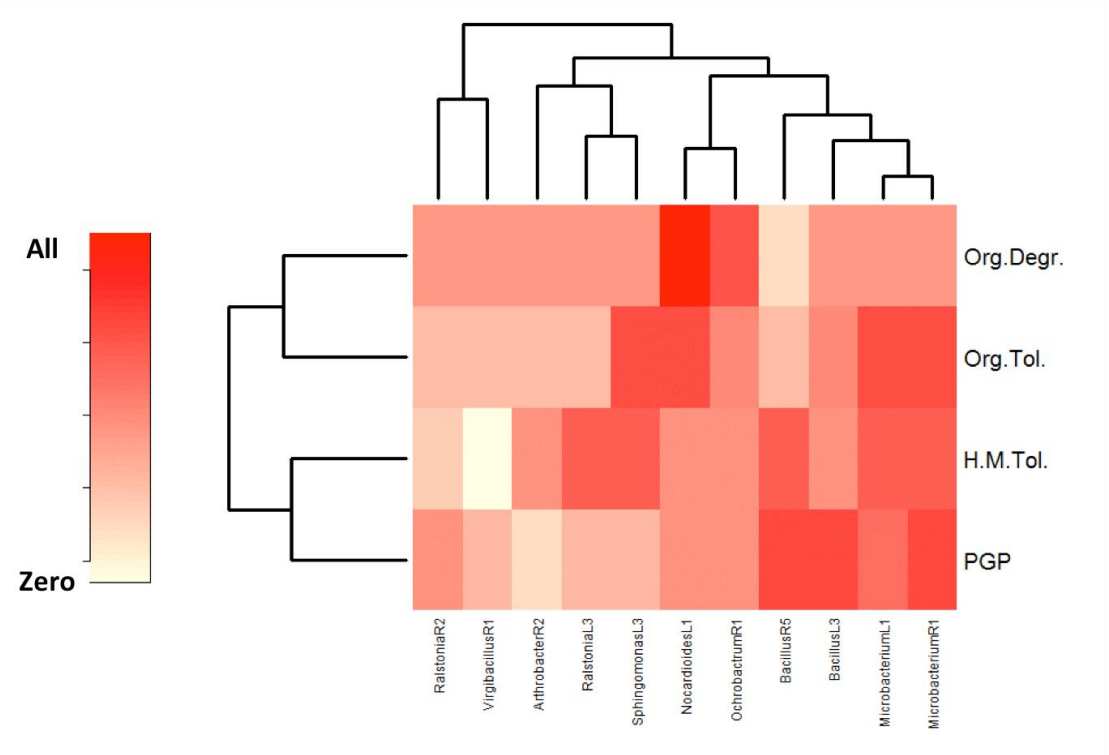



Figure 9. Heatmap with selected *J. acutus* endophytic strains and their score in the *in vitro* tested characteristics (PGP: plant growth promoting traits, H.M. Tol.: Heavy metal tolerance, Org. Tol.: Resistance to organic pollutants, Org. Degr.: Potential ability for organic pollutant degradation).

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 (Peng *et al.*, 2014; Saiyood *et al.*, 2010), the appropriate endophytic strains equipped with the desired characteristics may be employed.

## 3.4 Overall Remarks

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.

# Chapter 4

# Exploitation of endophytic bacteria to enhance the phyto-remediation potential of the wetland helophyte *Juncus acutus*[2]

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## SCOPE

This study investigated the potential of indigenous endophytic bacteria to improve the efficiency of the wetland helophyte *Juncus acutus* to deal with a mixed pollution consisting of emerging organic contaminants (EOCs) and metals. The beneficial effect of bioaugmentation with selected endophytic bacteria was more prominent in case of high contamination: most of the inoculated plants (especially those inoculated with the mixed culture) removed higher percentages of organics and metals from the liquid phase in shorter times compared to the non-inoculated plants without exhibiting significant oxidative stress. When exposed to the lower concentrations, the tailored mixed culture enhanced the performance of the plants to decrease the organics and metals from the water. The composition of the root endophytic community changed in response to increased levels of contaminants while the inoculated bacteria did not modify the community structure. Our results indicate that the synergistic relationships between endophytes and the macrophyte enhance plants' performance and may be exploited in constructed wetlands treating water with mixed contaminations. Taking into account that the concentrations of EOCs used in this study are much higher than the average contents of typical wastewaters, we can conclude that the macrophyte *J.*

[2] The contents of this chapter were published in Frontiers in Microbiology

*acutus* with the aid of a mixed culture of tailored endophytic bacteria represents a suitable environmentally friendly alternative for treating pharmaceuticals and metals.

## 4.1 Introduction

Emerging organic contaminants (EOCs) consist of a wide range of synthesized and natural compounds, such as pharmaceuticals, personal care products, surfactants and industrial chemicals (Luo *et al.*, 2014). Conventional wastewater treatment plants are the main point sources of their release into the environment since these systems have not been designed to treat these kinds of chemical products (Jiang *et al.*, 2013). EOCs are discharged into surface waters, wherein they can cause toxicity already at low concentrations (Matamoros *et al.*, 2016) and change the microbial ecosystems (Baquero *et al.*, 2008). Huang et al. (2012) identified multiple antibiotic resistant bacteria in secondary effluents of a municipal wastewater treatment plant in China, posing the risk of dispersion of antibiotic genes. In another study, Hu et al. (2010) demonstrated that several antibiotics are spread in the environment and food-chains through water or even absorption in vegetables that are fertilized by manure.

Constructed wetlands (CWs) are reliable green engineered systems that are employed for treating different kinds of effluents such as domestic, industrial or agro-industrial wastewater and acid mine drainage (Wu *et al.*, 2015a). These systems take advantage of the ability of plants together with their associated microorganisms to remove organic xenobiotics and metals from the water. At the same time with the biological processes, complex physical and chemical processes contribute to the degradation/detoxification/elimination of contaminants (Zhang *et al.*, 2014). Several studies have revealed the effective role of aquatic plant-and microbe-based systems in the removal of emerging contaminants (Verlicchi and Zambello, 2014; Vymazal and Březinová, 2015; Ávila *et al.*, 2013).

Selection of the appropriate macrophyte(s) in CWs is of high importance because plants play a significant role in pollutant removal by direct mechanisms or by enhancing the degradation activity of the microorganisms in the rhizosphere (Li *et al.*, 2014b). Species like *Typha* spp., *Cyperus alternifolius* and *Phragmites australis* were shown to efficiently remediate EOCs contaminated water (Dordio *et al.*, 2011; Yan *et al.*, 2016;

Liu *et al.*, 2013). However, these EOCs may cause toxicity to the plants thus decreasing their performance. A potential strategy to overcome this problem is the exploitation of endophytic bacteria. These microorganisms reside in the internal plant tissues without negatively affecting the host plant and often are involved in mutualistic relationships. Endophytes are known to possess plant growth promoting traits and degradation genes that assist their host plant to cope with various environmental stresses. Once they are inside, they can contribute *in planta* to the detoxification of organic contaminants by degrading them and/or enhance the metal translocation providing a potential towards sustainable treatment of mixed contaminations (Ho *et al.*, 2013; Visioli *et al.*, 2015; Babu *et al.*, 2013). There exist a limited number of studies that highlight the use of endophytic bacteria in terms of enhancing the performance of wetlands treating sewage effluent (Ijaz *et al.*, 2015b) or textile effluent (Shehzadi *et al.*, 2014).

*Juncus acutus* is a helophyte widely used in CWs in Europe and North America (Vymazal, 2013). The aim of this study is to investigate the potential of indigenous endophytic bacteria to improve the efficiency of the wetland plant *Juncus acutus* in dealing with emerging organic contaminants, one endocrine disruptor (Bisphenol-A) and two antibiotics (Ciprofloxacin, Sulfamethoxazole) and metals (zinc, nickel, cadmium). The oxidative stress that is induced in plants was assessed measuring the activities of enzymes involved in cellular defence against oxidative stress (SPOD, GR, GPOD and SOD), among the different treatments in order to evaluate the impact of the contaminants on the plant. The potential consequences of inoculation on the root microbial community were also determined.

## 4.2 Materials and Methods

### 4.2.1 Selection of most promising bacterial strains

#### 4.2.1.1. Bacterial strains

The bacterial strains used in this study were isolated from roots and leaves of the wetland plant *J. acutus* growing on a BPA-contaminated pilot (Syranidou *et al.*, 2016b). The strains (shown in Table 2) were selected based on their *in vitro* plant growth

promoting (PGP) abilities [phosphate solubilization, production of indolacetic acid (IAA), ACC-deaminase (ACC), organic acids and siderophores], tolerance to metals (Zn, Ni, Cd) and emerging contaminants (BPA, CIP, SMX) and potential for degradation of these contaminants. All the tests were described and performed previously (Syranidou *et al.*, 2016b).

Table 2. Isolated endophytic bacteria based on promoting traits and corresponding treatments (P Solub: Phosphate solubilizers, IAA: Indolacetic acid producers, ACC: ACC deaminase producers, OA: Organic acid producers, Sid: Siderophores producers, Zn: Zinc, Ni: Nickel, Cd: Cadmium, BPA: Bisphenol A, CIP: Ciprofloxacin, SMX: Sulfamethoxazole TET: Tetracycline, E: Erythromycin, x: positive in the tested characteristic, -: not possessing the tested characteristic) from (Syranidou et al., 2016).

| ID | Description of treatment (inoculations) | Isolated from | Promoting Traits | | | | | Metal Tolerance | | | EOCs tolerance | | | | | EOCs potential degradation | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | P solub | ACC | IAA | OA | Sid | 4mM Zn | 1mM Ni | 1mM Cd | BPA | CIP | SMX | TET | E | BPA | CIP | SMX | TET | E |
| NIN | Non-inoculated | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| IN1 | *Microbacterium* sp. U50/ *Microbacterium* sp. R31 | Leaves/ Roots | x/x | x/- | x/x | x/x | -/x | x/x | x/x | x/x | x/x | x/x | x/x | -/- | -/- | x/x | -/- | -/- | -/- | -/- |
| IN2 | *Herbaspirillum* sp. L32 | Leaves | - | - | x | - | x | x | x | x | x | - | - | - | - | - | - | - | - | - |
| IN3 | *Yonghaparkia* sp. R2b | Roots | - | x | x | x | x | x | x | - | - | - | - | - | - | - | - | - | - | - |
| IN4 | *Sphingomonas* sp. U33 (also denoted as B1) | Leaves | x | x | x | - | - | x | x | x | x | x | x | - | - | - | x | - | - | - |
| IN5 | *Bacillus* sp. R12 (also denoted as B2) | Roots | x | - | - | - | x | x | x | - | x | - | - | - | - | x | - | - | - | - |
| IN6 | *Ochrobactrum* sp. R24 (also denoted as B3) | Roots | - | x | x | - | - | x | x | x | x | - | x | - | - | x | - | x | - | - |
| IN7 | *Acidovorax* sp. U3 | Leaves | - | - | x | - | - | x | x | x | x | - | x | - | - | - | - | x | - | - |
| IN8 | *Ralstonia* sp. U42/ *Ralstonia* sp. R52 | Leaves/ Roots | x/x | x/- | x/x | -/x | x/x | x/x | x/x | x/x | x/x | -/- | -/x | -/- | -/- | x/- | -/- | -/- | -/- | -/- |
| IN9 | *Pseudomonas* sp. R15 | Roots | x | - | - | - | x | x | x | - | x | - | x | - | - | - | - | - | - | - |

#### 4.2.1.2. *In vivo* plant growth promotion

In a 1- month greenhouse experiment, the 11 most promising strains, based on their *in vitro* traits, were inoculated (concentration: $10^8$ cfu $mL^{-1}$) to glass beakers with *J. acutus* plants (n=10) and vermiculate as substrate. Young *J. acutus* plants were collected from Suda Bay (Chania, Greece). The control treatment consisted of plants without inoculation. The system was irrigated with 30 mL tap water every week and at the end of the experiment the root and shoot fresh and dry weights, and the root length were determined. Based on the results, the three strains with the highest *in vivo* plant growth promotion were selected for an inoculation experiment upon exposure to mixed contamination.

### 4.2.2 Effect of inoculation in the presence of mixed contamination

#### 4.2.2.1. Experimental set-up

*J. acutus* plants were collected from Suda Bay at Chania (Greece) and were acclimatized for 2 months in a greenhouse. Subsequently, plants (~ 20 g fresh weight) were transferred to glass beakers with small-size gravel (0.2 - 0.5cm) as substrate and the system was irrigated with 50 mL tap water every week. After 2 weeks, the three best growth promoting endophytic strains with different degradation capacities (B1- *Sphingomonas* sp. U33, B2- *Bacillus* sp. R12, B3- *Ochrobactrum* sp. R24) were inoculated (concentration: $10^8$ cfu $mL^{-1}$) separately and as a consortium to the beakers (n=10 for every treatment). The endophytic strains were cultured in 869 medium until the late log-phase, washed three times in 10 mM $MgSO_4$ and resuspended in sterile water to reach an inoculum concentration of approximately $10^9$ cfu $mL^{-1}$. One week later, two different concentrations of metals (Zn, Ni, Cd), BPA and two antibiotics: CIP and 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 LC 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 added to the high concentration (HC) treatments. A two-factorial study design was followed with factor 1 contaminant concentration (two levels, LC, HC), 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 two different concentrations of the mixture of contaminants (one LC and one HC) concerning the contamination effect (Table 3). The experiment lasted for 21 days and was irrigated with 50 mL tap water every week.

Table 3. Experimental Design - Treatments Examined (Leaf-Endo: leaf endophytic isolate, Root-Endo: root endophytic isolate).

| ID | Description of Contaminants | Controls (non-inoculated) | Leaf-Endo | Root-Endo | Root-Endo | Mixed culture |
|---|---|---|---|---|---|---|
| | | C | B1 | B2 | B3 | MIX |
| NC | No contamination | C_NC | | | | |
| LC | 50µg $L^{-1}$ Ciprofloxacin _ 250µg $L^{-1}$ Sulfamethoxazole _ 5mg $L^{-1}$ BPA _ 20mg $L^{-1}$ Ni _ 1mg $L^{-1}$ Cd _ 200mg $L^{-1}$ Zn | C_LC | B1_LC | B2_LC | B3_LC | MIX_LC |
| HC | 100µg $L^{-1}$ CIP _ 500 µg $L^{-1}$ SMX _ 10mg $L^{-1}$ BPA _ 40mg $L^{-1}$ Ni _ 2mg $L^{-1}$ Cd _ 400mg $L^{-1}$ Zn | C_HC | B1_HC | B2_HC | B3_HC | MIX_HC |

#### 4.2.2.2. Sampling

Water samples were taken at days 0, 14 and 21 after addition of the contaminants and were analyzed for their concentrations of metals and organic contaminants. The soluble metals were determined by inductively coupled mass spectrometry (ICP-MS 7500cx coupled with Autosampler Series 3000, both from Agilent Technologies) while BPA, CIP and SMX concentrations were measured by High-performance liquid chromatography (HPLC) (Shimadzu Corp., Kyoto, Japan), equipped with LC-10 ADVP solvent delivery module, SPD-M10 AVP Diode Array Detector, RF- 10AXL Fluorescence Detector, and SIL-10 ADVP autosampler. Separation of BPA was accomplished on a Nucleosil 100–5 C- 18 column and separation of CIP and SMX was performed on an Alltech PrevailTM Organic Acid 5u as previously described by Christofilopoulos *et al.* (2016).

At the end of the experiment, the plants were harvested and washed first with water and then with distilled water. The fresh weights of roots and leaves were determined, plant parts were cut in small pieces and 0.4 g of each plant compartment was sampled for enzymatic analysis while 0.3 g of roots was taken for DNA extraction. The plant samples for further analysis were immediately snap-frozen in liquid nitrogen and stored at -80 °C; the remaining material was dried at 45 °C and weighed.

#### 4.2.2.3. Enzyme assays

The activities of the antioxidative enzymes were assessed in order to estimate the oxidative stress induced in the plants (n=10). Fresh leaves and roots were macerated in liquid nitrogen and then homogenized in 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM 1,4-Dithiothreitol and 1 mM Ethylenediaminetetraacetic acid. The homogenate was centrifuged at 13.500 rpm and 4 °C for 10 min and the supernatant was used for

the enzyme analysis. The activity of GR that catalyze the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH) by oxidizing NADPH was measured at 340 nm and the activity of GPOD was estimated at 436 nm by the appearance of tetra-guaiacol (Bergmeyer *et al.*, 1974). The SPOD involved in $H_2O_2$ scavenging, were determined by the oxidation of syringaldazine at 530 nm (Imberty *et al.*, 1985). SOD deal with superoxide anions and were determined by following the cytochrome c-inhibition at 550 nm (McCord and Fridovich, 1969). All enzyme activities were expressed as units per gram fresh weight.

#### 4.2.2.4. DNA extraction and endophytic bacterial community profile

The fresh plant roots were immersed in 70% ethanol solution for 30 sec and subsequently in 2% NaClO solution supplemented with one droplet Tween 80 per 100 ml solution for 10 min. The surface-sterilized plant parts were washed three times with 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. The absence of colonies on the plates confirmed the successful disinfection. Next, the samples were macerated with liquid nitrogen and total DNA was extracted using the Invisorb® Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin, Germany).

Automated Ribosomal Intergenic Spacer Analysis (ARISA) PCR was performed in order to estimate the root bacterial diversity and community structure among the different treatments. The primers ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) were used for the amplification of the intragenic transcribed region ITS1 in the rRNA operon plus ca. 282 bases of the 16S and 23S rRNA (Cardinale *et al.*, 2004). A mixture of 0.2 mM of each of the four deoxynucleoside triphosphates, 2 mM $MgCl_2$, 0.2 µM each of the forward and reverse primers and 1 U of High Fidelity Platinum Taq DNA polymerase per 25 µL was used to perform the PCR. The cycling conditions of the PCR were: one denaturation cycle at 94°C for 3 min, followed by 30 cycles at 94°C for 45 sec, 56°C for 45 sec, 72°C for 2 min, and a final extension at 72°C for 7 min.

The gel-dye mix, marker, PCR products and ladder were loaded to the DNA chip according to the manufacture's protocol (Agilent DNA 1000 Assay Protocol), next the

chip was inserted to the 2100 Bioanalyzer (Agilent Technologies, Diegem, Belgium) and the chip run was executed.

### 4.2.3 Data analysis

The statistical analysis was performed with the automatic R (R Development Core Team, 2009); ANOVA and non-parametric tests were applied to data that follow and did not follow normal distribution respectively. Concerning the contaminants, a two-way ANOVA was performed and the results indicated an interaction effect between the level of contamination and inoculation, so the main effects of the independent variable were investigated separately. Next, analysis of ARISA fragments was performed with Bioanalyzer software. Only peaks with sizes between 100 and 1500 bp and a minimum peak height of 150 fluorescence units were considered for analysis. The binning of ARISA fragments was performed according to Ramette (2009). Briefly, the automatic R binning script was applied to replicates of the same treatment in order to find the window size (WS) and the shift value (Sh) and a WS of 1bp was selected for the OTU binning algorithm for ARISA profiles of endophytic bacteria. The analysis of the OTU table was performed by Primer6 software. A multidimensional scaling (MDS) plot was used to describe the root community structure while the degree of similarity was explored with the permutation-based hypothesis statistical test analysis of similarities (ANOSIM).

## 4.3 Results

### 4.3.1 Selection of most promising bacterial strains

The endophytic strains with the best results for *in vitro* plant growth promoting traits (Table 2) were tested *in vivo* for their capability to enhance the plant biomass production. Eleven strains were inoculated in pots with young *J. acutus* plants (11.3cm leaf height, 0.44g fresh biomass) in a 1-month greenhouse experiment. The effect of inoculation on plant weight varied among the treatments (Figure 10). Overall, some endophytic strains increased the plant weight in comparison to the non-inoculated controls. More specifically, the dry weight of the plants inoculated with *Sphingomonas* sp. U33 (IN4), *Bacillus* sp. R12 (IN5), *Ochrobactrum* sp. R24 (IN6) and *Pseudomonas*

sp. R15 (IN9) was statistically ($p<0.05$) higher in comparison to the dry weight of the non-inoculated plants (Figure 1). The root lengths of the inoculated plants with IN1, IN4, IN5 and IN8 were significantly higher (25.51, 27.87, 30.00 and 25.61 cm respectively) in comparison to non-inoculated control plants (22.48 cm).

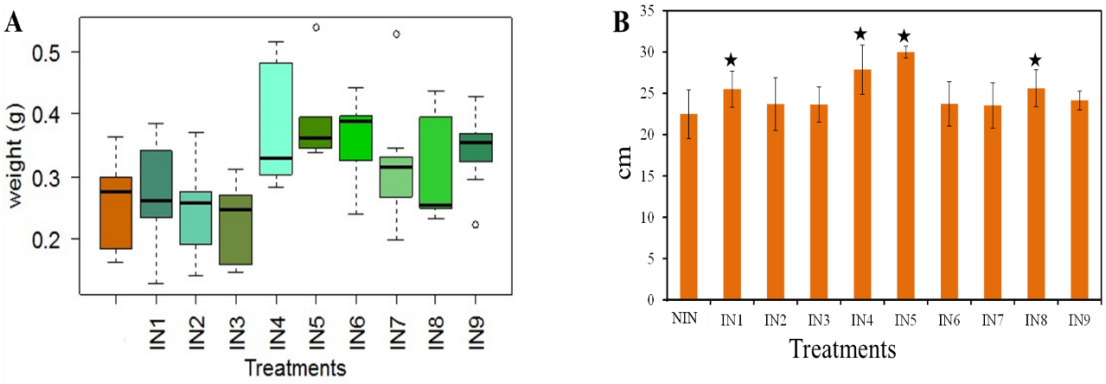



Figure 10. Box-plot with the dry weight of the plantlets (A), the root length of the plantlets (B), NIN: non-inoculated, IN1: inoculated with *Microbacterium* sp. U50/ *Microbacterium* sp. R31, IN2: inoculated with *Herbaspirillum* sp. L32, IN3: inoculated with *Yonghaparkia* sp. R2b, IN4: inoculated with *Sphingomonas* sp. U33, IN5: inoculated with *Bacillus* sp. R12, IN6: inoculated with *Ochrobactrum* sp. R24, IN7: inoculated with *Acidovorax* sp. U3, IN8: inoculated with *Ralstonia* sp. U42/ *Ralstonia* sp. R52, IN9: inoculated with *Pseudomonas* sp. R15 (Error bars are ±standard error (n=10), data with asterisk are significantly different ($p<0.05$) compared to the non-inoculated plants).

Considering the performance of endophytic strains in demonstrating plant growth promoting traits *in vivo*, together with their results concerning metal tolerance, BPA/CIP/SMX resistance and potential degradation (see Table 1), three strains (B1- *Sphingomonas* sp. U33, B2- *Bacillus* sp. R12, B3- *Ochrobactrum* sp. R24) were selected for further investigation of their effects on *Juncus acutus* under stress conditions. These three strains were tested separately and as a consortium.

### 4.3.2 Removal of organic contaminants in bioaugmented microcosms

In order to investigate the removal efficiencies of the different microcosms, mixtures of organic contaminants were added to the pots.

In LC treatments (low concentration of contaminants were added to the pots), 5 mg $L^{-1}$ BPA was supplemented in each pot and after 14 days more than 70% BPA removal from the water was observed in all cases, independent of inoculation (Figure 11A). The highest percentage of removal was realized by the B2 inoculated plants (90.7%) while

the B3 inoculated plants removed 70.1% BPA from the water. At the same sampling day, the non-inoculated plants realized a removal of 78.1% BPA from the surrounding water. After 21 days, BPA concentration was lower than 0.5 mg $L^{-1}$ for the B3 and consortium inoculated plants and less than 0.1 mg $L^{-1}$ for the B1 and B2 inoculated and the non-inoculated plants. The non-inoculated plants removed the highest percentage (98.4%) of BPA in comparison with all the inoculated.

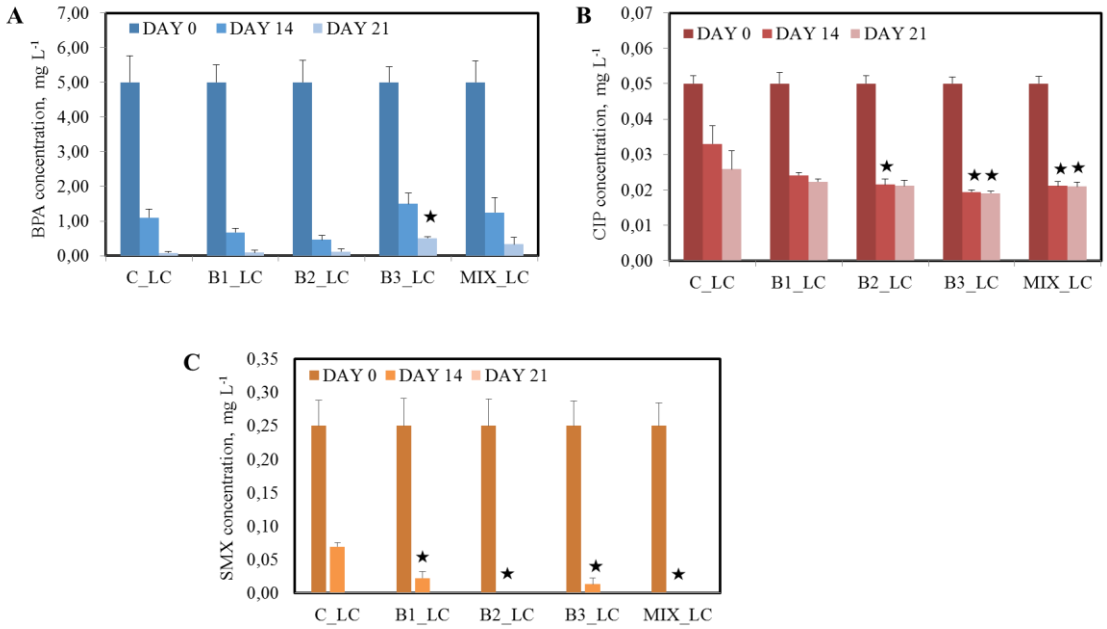



Figure 11. Concentration of BPA (A), CIP (B) and SMX(C) of the different treatments after 0, 14 and 21 days when the low concentration (LC) of contaminants were added (data with asterisk are significantly different ($p<0,05$) compared to the non-inoculated plants).

When 10 mg $L^{-1}$ BPA was added to the pots, all plants showed a comparable capacity to remove BPA; thus, no statistically significant differences were observed among the treatments (Figure 12A). At day 14, BPA concentration was approximately 2.7 mg $L^{-1}$ in the surrounding water of non-inoculated and B2-inoculated plants and less than 2.5 mg $L^{-1}$ in the other inoculated plants. Similarly, 1.7 mg $L^{-1}$ BPA remained in the water of plants with only their indigenous community, approximately 1.6 mg $L^{-1}$ in the water of B1 and B2 inoculated pots, 1.48 mg $L^{-1}$ in the water of plants inoculated with the consortium and 1.38 mg $L^{-1}$ in the water of B3 inoculated plants after 21 days of inoculation. At that day, the lowest removal was observed for the non-inoculated plants (83%) while the highest was recorded for the B3-inoculated (86.2%) demonstrating that the differences in removal are minor.

The pots were also contaminated with two different concentrations of the antibiotic CIP, 0.05 mg $L^{-1}$ in the LC and 0.1 mg $L^{-1}$ in the HC treatments. After 14 days of incubation, significant decreases in antibiotic concentrations were detected in all water samples. Moreover, significant differences were observed between the samples of inoculated plants and the non-inoculated plants at the same time point (Figure 11B) in the LC treatments. For example, the CIP concentrations were decreased with 34% and 48.3% after 14 and 21 days respectively by the non-inoculated plants while all the inoculated plants realized decreases of more than 50% in CIP concentration after 14 days. In the HC treatments, the capacity of plants inoculated with endophytes to remove CIP was significantly higher than the non-inoculated plants since they removed more than 70% CIP in comparison with 63% after 14 days. At the end of the experiment, more than 79% removal of CIP was established by all inoculated plants and 73.5% by the non-inoculated plants (Figure 12B).

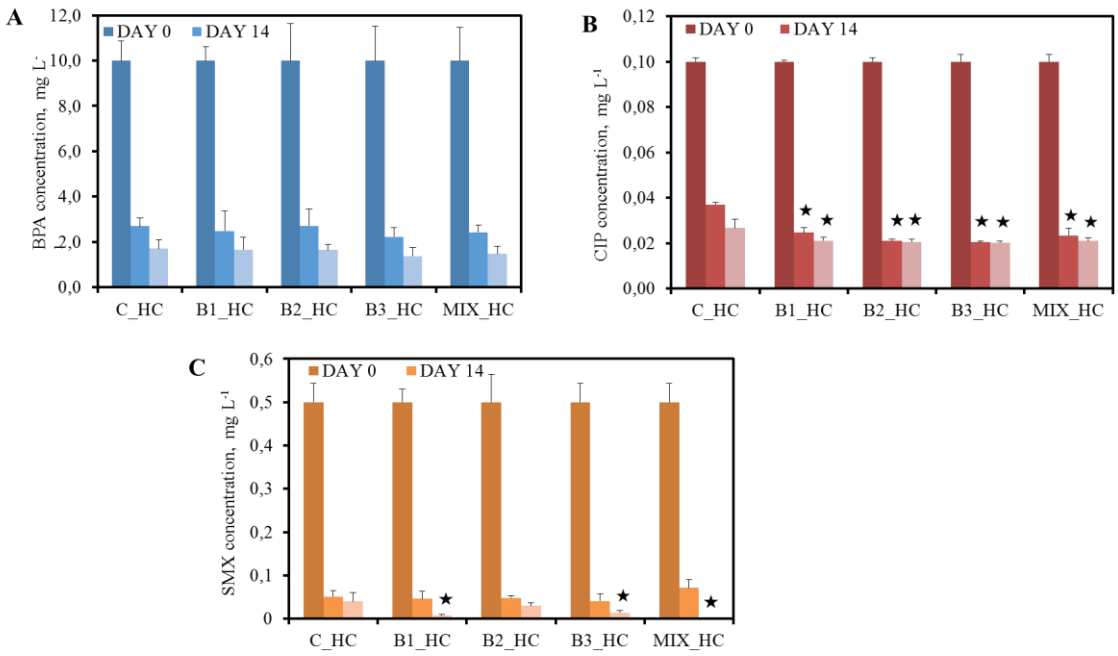



Figure 12. Concentration of BPA (A), CIP (B) and SMX(C) of the different treatments after 0, 14 and 21 days when the high concentration (HC) of contaminants were added (data with asterisk are significantly different ($p<0,05$) compared to the non-inoculated plants). Bacteria names are described at table 2.

The ability of plants to remove SMX from the water seemed to be correlated to the inoculation effect in the LC treatment. At an initial concentration of 0.25 mg $L^{-1}$, the non-inoculated plants removed 72.4% SMX from the surrounding water after 14 days while all the inoculated plants removed more than 90% (Figure 11C). Moreover, after 14 days SMX could not be detected anymore in samples inoculated with B2 and the

consortium while after 21 days, SMX was below detection limit in all water samples. In the HC treatment the initial SMX concentration was the double (0.5 mg $L^{-1}$); at day 14 all the plants showed similar removal efficiency (approximately 90%) but after 21 days the inoculated plants demonstrated a higher removal (Figure 12C), except in case of B2. It is worth noticing that at that sampling day, SMX was below detection limit in the water samples of consortium-inoculated plants.

### 4.3.3 Removal of metals by the several treatments

Besides the organic contaminants, the water in the pots was contaminated with zinc, nickel and cadmium, like for the other contaminants at two different concentrations. In the LC treatments, 200 mg $L^{-1}$ Zn was added and an approximately 45% decrease in concentration was recorded for most of the different treatments after 14 days (Figure 13A). The B3-inoculated plants showed the lowest efficiency to remove zinc from the water (about 35%). At day 21, the performance of all inoculated plants was significantly higher in comparison to the non-inoculated plants: the B1 and B2-inoculated plants removed more than 90% zinc, the consortium-inoculated 81% and B3-inoculated plants 79% while the non-inoculated plants reduced the zinc concentration in the water with only 64%. When the pots were supplemented with 400 mg $L^{-1}$ Zn, the efficiency of the plants to decrease the Zn concentration in water was lower (Figure 13B). On day 14, the Zn concentrations in the waters of the non-inoculated and consortium-inoculated plants were significantly different, being respectively 277 mg $L^{-1}$ (32% removal) and 172 mg $L^{-1}$ (66% removal). This difference was even more pronounced at the second sampling point: 207 mg $L^{-1}$ Zn in water samples of non-inoculated plants and 65 mg $L^{-1}$ Zn in case of consortium-inoculated plants. This corresponded to 48% removal by non-inoculated plants and 84% removal by the plants inoculated with the consortium. The other treatments differed less from the non-inoculated plants.

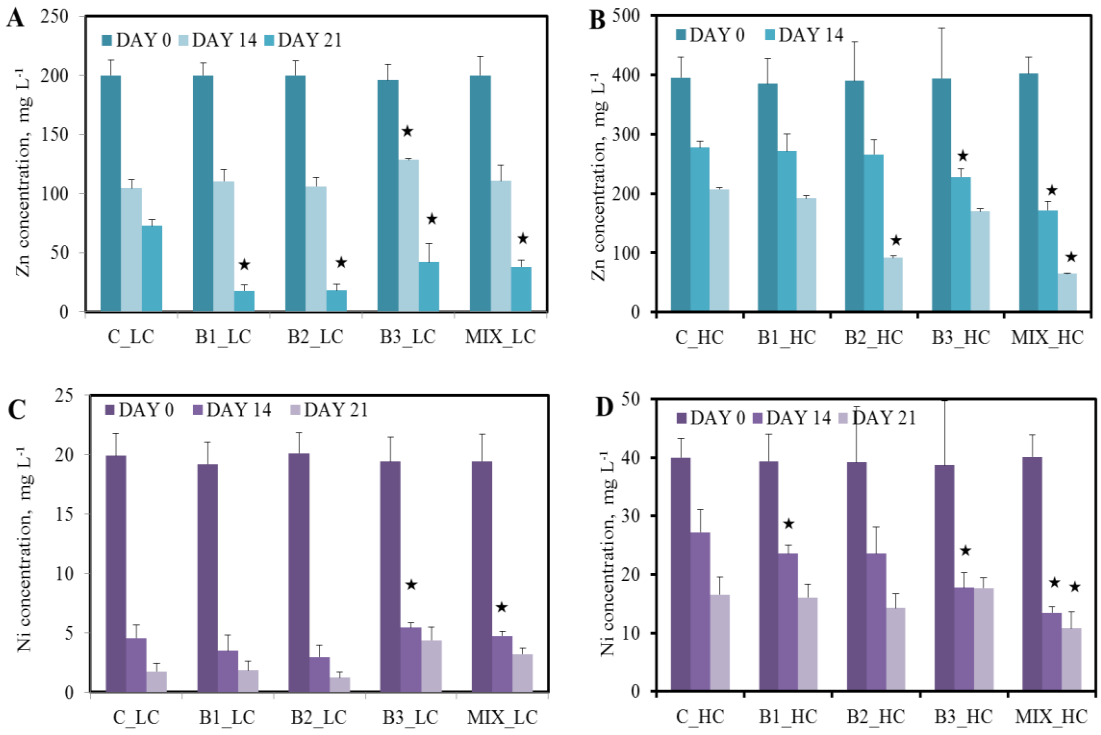



Figure 13. Concentration of Zn (A,B) and Ni (C, D) of the different treatments after 0, 14 and 21 days when the low (A,C) and high (B,D) concentration of contaminants were added (data with asterisk are significantly different ($p<0,05$) compared to the non-inoculated plants).

Nickel was added at 20 mg $L^{-1}$ in the LC treatments and the plants seemed efficient in removing it from water. Significant differences were not observed between the inoculated and non-inoculated plants; the recorded Ni reductions fluctuated between 73% to 85% for all treatments (Figure 13C). At the second sampling point, the nickel removal for untreated, B1 and B2 inoculated plants increased to, respectively, 91%, 91% and 94% while the B3-inoculated plants decreased the Ni concentration with 78% Ni in total. In the HC treatments (40 mg Ni $L^{-1}$) the capacity of plants to remove Ni from the water was lower (Figure 13D). Consortium-inoculated plants showed the highest efficiency: 66% and 73% decreases were recorded after respectively 14 and 21 days. B1 and B2 inoculated plants followed while B3 inoculated and non-inoculated plants demonstrated the lowest ability to remove Ni from water.

It is important to mention that although Cd could be measured in the samples from day 0, it could not be detected any more in all water samples taken after 14 days of incubation, independently of the initial concentration.

### 4.3.4 Effect of contaminants on biomass and leaf stress enzymes

At the end of experiment, the plants were harvested and their dry weight was determined and compared among the treatments. When the LC of contaminants was added to the pots, the weight of the non-inoculated and the B1- and B2- inoculated plants was significantly affected compared to the weight of the unexposed control plants growing in absence of contaminants (Figure 14A). However, the dry weights of B3 and consortium-inoculated plants were not affected by the presence of the contamination mixture: the leaf biomass of those treatments were 2.8 g and 2.7 g respectively and the leaf biomass of unexposed control plants was 2.9 g (data not shown). The weights of the inoculated plants were also compared with the weights of the non-inoculated plants under low level of exposure. Only the weight of the B2-inoculated plants was significantly lower in comparison with the non-inoculated plants while no significant differences were detected among the other treatments. A significant increase in root weight of B3-inoculated plants was observed in comparison to the weight of the non-inoculated ones (data not shown). Increasing the concentration of contaminants had a significant negative impact on the weight of all plants (Figure 14B) in comparison to the unexposed control plants while no significant differences were detected among the differences treatments of exposed plants. It is important to mention that the leaf weight of all plants was significantly affected compared to the unexposed control plants (data not shown). Only the root dry weight of non-inoculated plants (0.89 g) was significantly impacted by the presence of HCs of contaminants in comparison to the unexposed control plants (1.39 g).

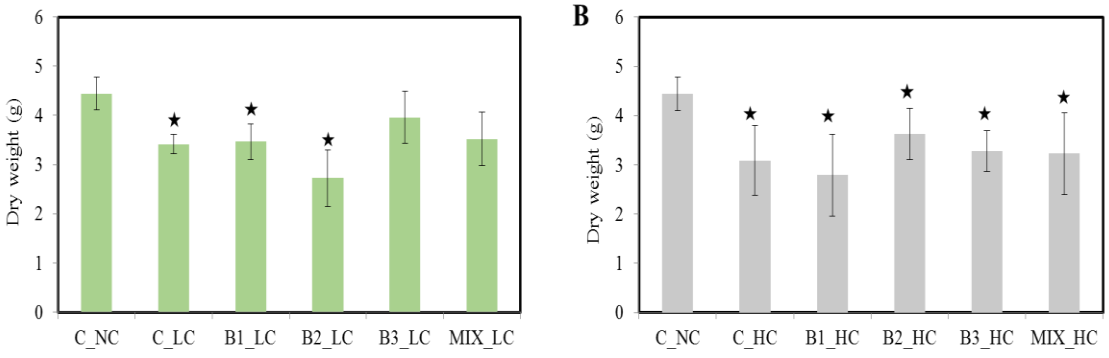
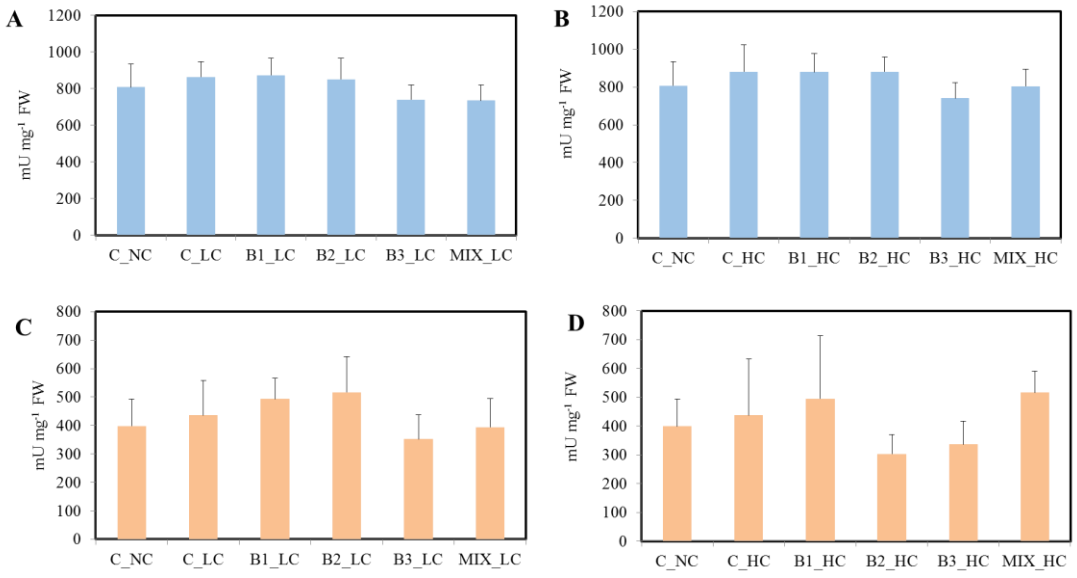



Figure 14. The dry weight of the different treatments after 21 days when the low (A) and high (B) concentration of contaminants were added (data with asterisk are significantly different ($p<0,05$) compared to the non-inoculated plants without any supplement of contaminants).

The effects of the different treatments on the activities of enzymes involved in anti-oxidative defense were determined in the leaves of the plants (Figure 15). In all treatments, the SOD and GR were not significantly affected by exposure to either LC or HC concentrations of contaminants. The activities of GPOD were significantly different between unexposed control plants and B2- and B3-inoculated plants at LCs of contaminants and between non-inoculated plants and the B2- and B3- inoculated plants. In HC treatments, the activity of this enzyme in leaves of all plants was significantly higher compared to the activity of the enzyme in leaves of unexposed plants. Significant increased activities of SPOD were recorded in leaves of non-inoculated and B3-inoculated helophytes in comparison to the unexposed control at LC of contaminants. Significant differences were also observed between the non-inoculated plants and B1, B2 and consortium inoculated plants. The activity of these SPODs was significantly higher in leaves of non-inoculated, B2-, B3- and consortium-inoculated plants in the HC treatments and only the activity in B1-inoculated plants was significantly lower than in the non-inoculated ones.



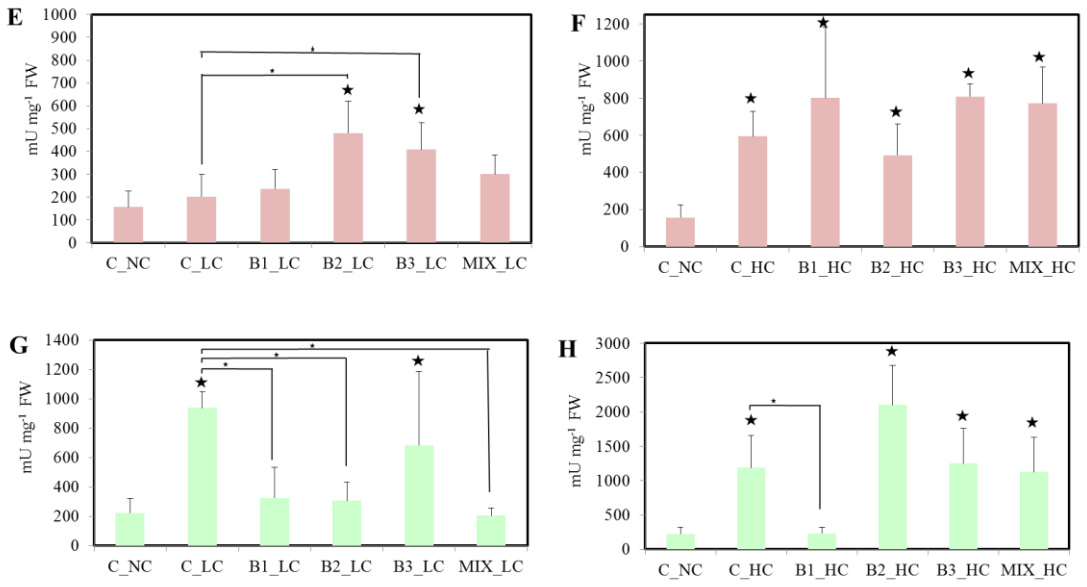



Figure 15. Leaf stress enzymes of *J. acutus* of various treatments, A) and B) correspond to SOD, C) and D) correspond to GR, E) and F) correspond to GPOD, G) and H) correspond to SPOD (data with big asterisk are significantly different ($p<0,05$) compared to the non-inoculated plants without any supplement of contaminants, small are significantly different ($p<0,05$) compared to the non-inoculated plants with supplement of contaminants ).

In the roots, significant differences in the activities of SOD were detected between the non-inoculated and B1- and B2- inoculated plants when the concentration of contaminants was low (Figure 16). At the higher contaminants concentration significant differences were not observed among the treatments. GR activity of plants inoculated with the consortium was significantly lower in comparison to unexposed plants in LC treatments. In HC treatments, the activity of GR was significantly affected in roots of B2- and MIX- inoculated plants in comparison with the unexposed control. The activities of GPOD and SPOD were significantly lower in the roots of consortium-inoculated plants compared to the non-inoculated plants at both levels of the contamination.

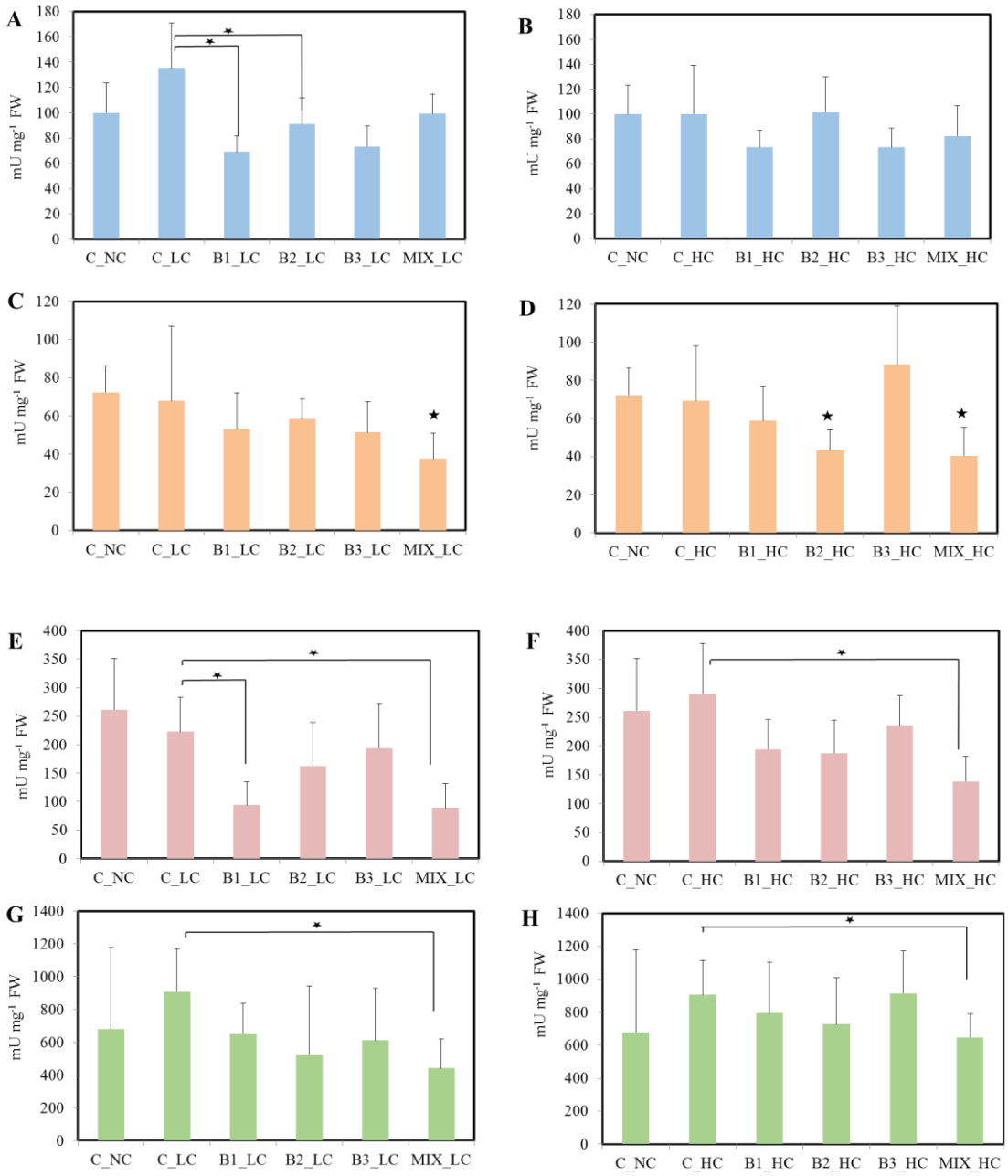



Figure 16. Root stress enzymes of *J. acutus* of various treatments, A) and B) correspond to SOD, C) and D) correspond to GR, E) and F) correspond to GPOD, G) and H) correspond to SPOD (data with big asterisk are significantly different ($p<0,05$) compared to the non-inoculated plants without any supplement of contaminants, small are significantly different ($p<0,05$) compared to the non-inoculated plants with supplement of contaminants ).

### 4.3.5 Principal Component Analysis (PCA) with the root stress enzymes and dry weight towards the contaminants

Since the plant roots were in direct contact with all the contaminants applied in this experiments, the potential relationships between a contaminant and a specific root stress

enzyme responses or root dry weight were investigated. The HC of organic contaminants and metals differently affected the various antioxidants (Figure 17), leading to the hypothesis that this effect may be treatment-specific. The same phenomenon was observed when the concentration of contaminants was low (Figure 18).

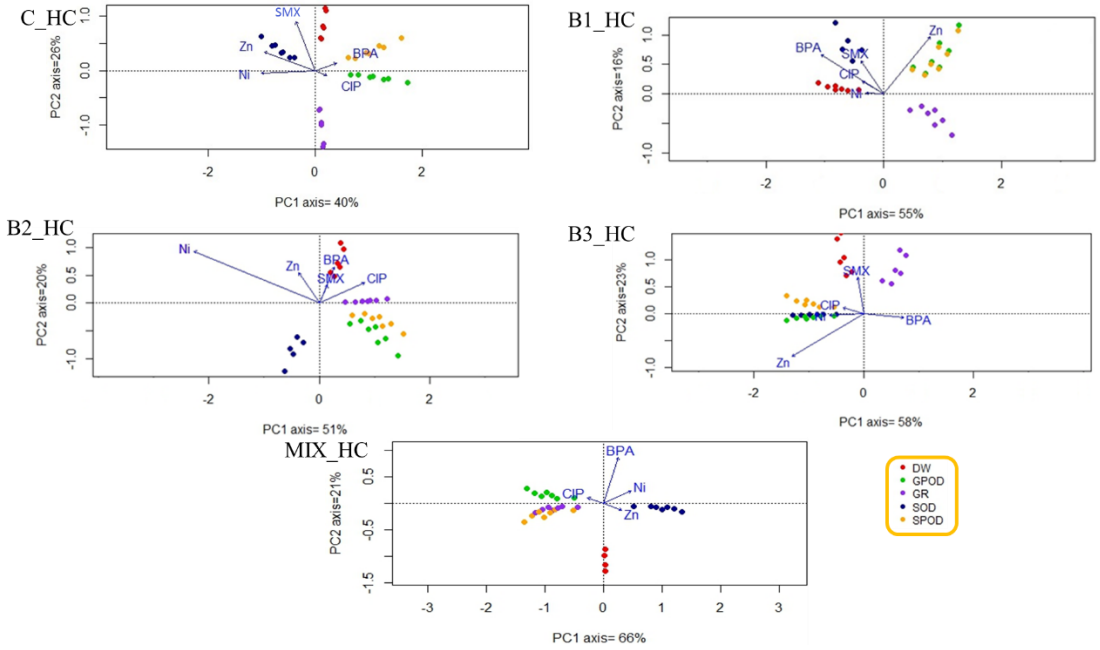



Figure 17. PCA with the root stress enzymes and root dry weight and the fitted environmental variables in high contamination treatments

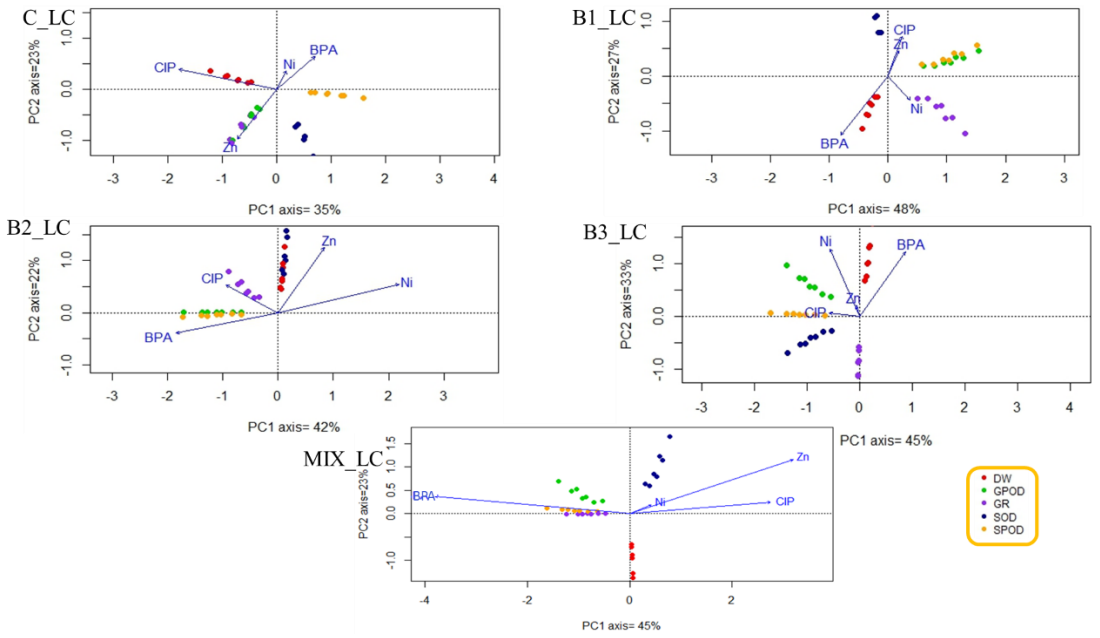


Figure 18. PCA with the root stress enzymes and root dry weight and the fitted environmental variables in low contamination treatments.

### 4.3.6 Root endophytic bacterial community and diversity in response to contamination

ARISA fingerprints were analyzed in order to estimate the potential impact of the contamination and the inoculation effect on the bacterial diversities and community structures in roots at the end of the experiment. The MDS plot (Figure 19A) presents the distribution of the communities with the level of contamination as criterion and indicates that a separation occurred among the communities. The highest dissimilarity was observed between the bacterial communities of roots of plants growing in the highly contaminated pots and the roots of unexposed control plants (ANOSIM R= 0.334, $p<0.05$). However, no significant differences were found among the root communities when the selecting factor was the inoculation effect. These results imply that the high level of contamination had a strong impact on the bacterial communities while the inoculated endophytes did not significantly alter the root communities.

Root bacterial diversity did not significantly differ among the treatments, although the diversity of the roots of non-inoculated, B1- and B2- inoculated plants seemed to be favored (not significantly) by the presence of LC of contaminants in comparison to the endophytic community of unexposed plants (Figure 19B). When the plants were exposed to the HC of contaminants, decreases of the diversity were noticed for all root communities. However, only the non-inoculated, B2- and B3-inoculated roots harbored a significantly less diverse community compared to the unexposed control roots (Figure 19C).

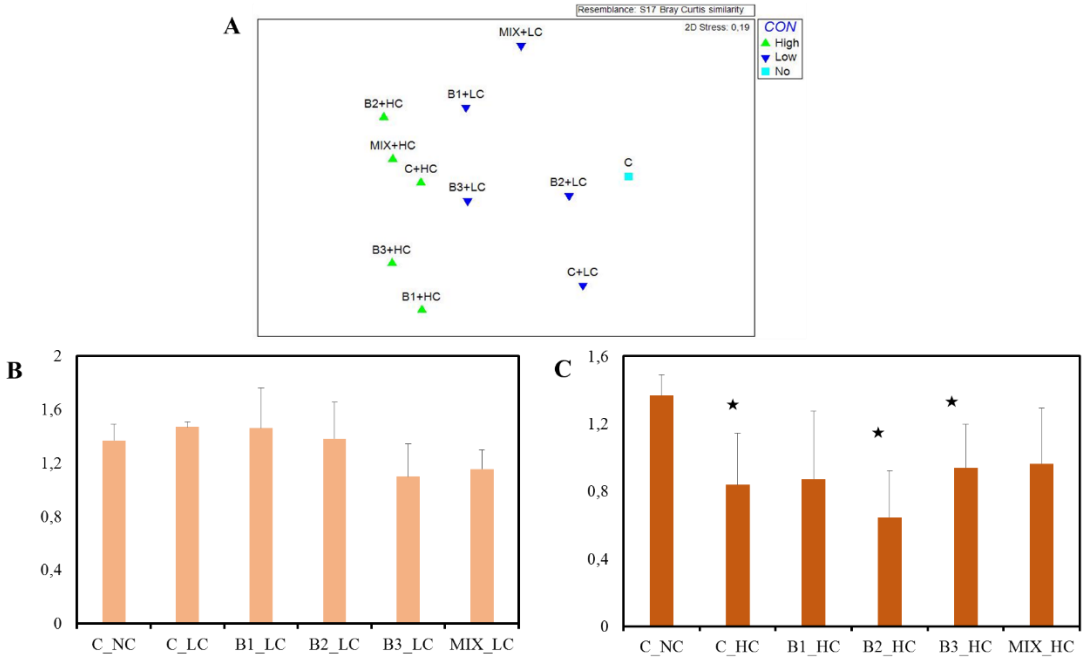



Figure 19. A) Multidimensional scaling (MDS) ordination based on Bray–Curtis similarities from ARISA fingerprints of *J. acutus* root communities under different levels of contaminants exposure. Shannon–Wiener diversity index among the different *J. acutus* root communities under low (B) and high (C) level of contaminants exposure (data with asterisk are significantly different ($p<0,05$) compared to the non-inoculated plants without any supplement of contaminants).

## 4.4 Discussion

Species of genus *Juncus* have been widely used in constructed wetlands treating various types of influents (Wu *et al.*, 2015a) such as industrial wastewater (Khan *et al.*, 2009) and urban stormwater runoff (Ladislas *et al.*, 2013). The species *J. acutus* is a common halophytic plant in Mediterranean ecosystems and has been studied for its high zinc tolerance and accumulation (Santos *et al.*, 2014; Mateos-Naranjo *et al.*, 2014). Moreover, this species has been found able to rhizofiltrate groundwater polluted with Cr(VI) and to accumulate it in the internal tissues (Dimitroula *et al.*, 2015). Christofilopoulos et al. (2016) revealed the efficiency of *J. acutus* to treat contaminated water with organics (CIP, SMX, BPA) and metals (zinc, nickel, chromium and cadmium), especially when the concentrations of these contaminants were environmentally relevant.

In this study, the concentrations of the mixtures of contaminants that were used simulated hospital effluents with high loadings and/or industrial effluents. The ability

of *J. acutus* to treat a variety of EOCs and metals was investigated. At such HCs (especially the HC treatments), it may become difficult for the plant to cope with the contaminants and its performance might decrease. As a possible solution for this, the potential increase of phytoremediation efficiency by inoculating endophytic bacteria with the appropriate traits was investigated. It has been demonstrated that inoculation of endophytic bacteria can positively influence the outcome of phytoremediation processes in areas contaminated with organics and/or metals (Weyens *et al.*, 2010, 2013; Ma *et al.*, 2015b; Babu *et al.*, 2015). It is important to mention that the *J. acutus* plants in this experiment were grown on gravels and only irrigated with tap water in order to inhibit the growth of rhizospheric bacteria; due to this experimental set-up, the only carbon sources were the organic contaminants and the root exudates.

The effect of inoculation with rhizospheric or endophytic bacteria on plants growing on contaminated soils has been investigated by several studies (Rajkumar *et al.*, 2009; Ma *et al.*, 2011a; Abhilash *et al.*, 2012). In most of the these studies the beneficial effects of endophytic inoculants were highlighted, accomplished through promotion of plant growth and protection from stress factors (Becerra-Castro *et al.*, 2013; Mesa *et al.*, 2015a) and even through enhancement of metal translocation in plant tissues (Babu *et al.*, 2013; Ma *et al.*, 2015a). With respect to organic xenobiotics, the role of endophytic bacteria in detoxification processes is of high importance since plants are photoautotrophic organisms lacking C metabolization pathways suitable for contaminant degradation, and only exhibiting contaminant transformation or immobilization mechanisms (Weyens *et al.*, 2009d). For example, reductions of toluene or TCE evapotranspiration were demonstrated after inoculation with a natural endophyte equipped with the pTOM toluene-degradation plasmid (Barac *et al.*, 2004; Weyens *et al.*, 2009a). Although CWs are systems that exploit the interactions between plants and their associated microorganisms in order to clean contaminated water, few studies have investigated the potential impact of endophytic bacteria inoculation in the performance of these systems (Shehzadi *et al.*, 2014; Ijaz *et al.*, 2015b).

Mixtures of organic and inorganic contaminants at two different concentrations were selected for our experiment. In the LC treatments, the plant itself with the indigenous microbial community managed to cope with the contaminants and the differences in performances among the different inoculations depended on the type of the

contaminant. For example, the capability of non-inoculated and inoculated plants to degrade BPA was similar while all inoculated plants demonstrated a better performance to remove antibiotics. As shown in a previous study (Syranidou *et al.*, 2016b) the indigenous community is highly enriched with BPA tolerant strains and potential degraders and this plant can efficiently clean BPA contaminated groundwater in short period of time and this phenomenon may have a positive impact on BPA removal from the water. Moreover, all the inoculated plants (especially B1- and B2-inoculated) enhanced zinc removal from water after 21 days compared to the non-inoculated even though *J. acutus* has been characterized as a potential zinc hyperaccumulator (Santos *et al.*, 2014). Concerning nickel removal, the non-inoculated plants, B1- and B2-inoculated plant decreased nickel concentrations in water at a same extent while the nickel concentration in the B3 and consortium-inoculated pots was higher.

Adding nickel resistant root endophytic inoculants negatively influenced the root biomass and nickel accumulation in the Ni-hyperaccumulator *Noccaea caerulescens*; however, co-inoculation of specific strains had positive effects on plant biomass and Ni translocation to internal tissues (Visioli *et al.*, 2015). Similarly, the dry weight of *Juncus acutus* inoculated with the consortium was not significantly different from that of the unexposed plants (Figure 6A). Moreover, the activity of the anti-oxidative enzymes in the roots of the consortium inoculated plants was lower (in some cases significantly) compared to the non-inoculated plants. This indicates a protective role of the consortium against the oxidative stress induced by the different contaminants. As shown by other studies (Qin *et al.*, 2014; Thijs *et al.*, 2014b; Belimov *et al.*, 2005), the IAA and ACC deaminase production by the B1 and B3 strains along with other plant growth promoting traits may participate in alleviating the stress and improve plant growth at the same time.

When the contaminant concentration increased, the beneficial role of the inoculated consortium to the plants is more pronounced in terms of removing specific contaminants from the surrounding water and stress alleviation. The decrease of the concentrations of the organic xenobiotics in the water followed the same general pattern as in the LC treatments. The performance of the plants in decreasing the BPA concentration in water was similar while the inoculated plants showed enhanced antibiotics removal. SMX could not be detected in water samples of consortium-

inoculated plants; it can be hypothesized that this greater SMX removal can be attributed to the endophytic consortium since the B3 strain has been characterized as a potential SMX degrader and B1 as a SMX tolerant strain. With respect to metals, the consortium-inoculated plants significantly decreased the zinc concentration in the water and slightly enhanced nickel removal in comparison to the non-inoculated plants. Moreover, cadmium was efficiently removed by all plant treatments. In several studies, species belonging to the genus *Juncus* have been found able to accumulate cadmium mainly in their belowground tissues (Ladislas *et al.*, 2013; da Silva *et al.*, 2015; Christofilopoulos *et al.*).

The weight of all plants was significantly affected by the high concentration of xenobiotics and metals while the root dry weight of all inoculated plants did not differ significantly from unexposed plants. Moreover, the activities of all measured anti-oxidative enzymes in the roots of consortium-inoculated plants was (from slightly to significantly) lower compared to the activity in the roots of non-inoculated plants. These results indicate that the inoculated consortium may assist the plant to cope with elevated environmental stress and that this effect is more prominent in roots that are in direct contact with the contaminants in comparison to leaves. These findings are in accordance with other studies (Li *et al.*, 2016; Khan *et al.*, 2015; Zhang *et al.*, 2015). Bioaugmentation with a selected consortium of two endophytic bacterial strains promoted the host plant growth and reduced the crude oil levels in soil (Fatima *et al.*, 2015). In another study, bulk soil, rhizosphere and endophytic strains of *A. pseudoplatanus* were selected according to their plant growth potential and TNT transformation capabilities and formed a consortium (Thijs *et al.*, 2014b). When this consortium was inoculated to *A. capillaris*, it protected the grass from oxidative stress and contributed to TNT transformation. In this study, we noticed that the level of contamination together with the type of contaminant affect the physiological status of either inoculated or non-inoculated plants in a different way.

The inoculations that we performed did not alter the root endophytic community; it changed only in response to the different levels of contamination. This is in agreement with the observation that not the inoculation with *B. phytofirmans* but the additions of different metal immobilizers influenced the composition of shoot and rhizosphere communities (Touceda-González *et al.*, 2015). Plant species and soil type seem to be

crucial factors in shaping the endophytic communities (Phillips *et al.*, 2008; Bulgarelli *et al.*, 2012). Plants may have the ability to regulate the *in planta* bacterial catabolic genotypes in relation to the level of environmental contamination (Oliveira *et al.*, 2014) while the entrance and transport of contaminants to various plant parts may also affect the endophytic microbiome (Su *et al.*, 2015a; Eevers *et al.*, 2016). Instead of waiting for the contaminants to enter the plant and induce the development of corresponding contaminant biodegraders, we propose to proceed with bioaugmentation of the indigenous microbes that are carriers of appropriate genes to assist their host plant to better cope with the stress induced by the contamination and to earlier remediate the contaminated area/water.

With respect to diversity index, no statistical differences were detected among the root communities exposed to the low contamination (Figure 12). At the highest level of contamination the microbial diversity of non-inoculated, B2- and B3- inoculated roots was negatively affected in comparison to the unexposed control roots. It seems that the roots inoculated with B1 strain and the consortium harbored a diverse microbiome that was less affected by the high levels of contamination.

## 4.5 Concluding Remarks

Inoculation with indigenous endophytic bacteria and especially the consortium was shown to have positive effects on the plant in terms of contaminant removal and stress alleviation. Also, the root communities were only altered in function of the contamination level. To our knowledge, this is the first study in which a positive impact on removal of EOCs together with metals was demonstrated after inoculation with endophytic bacteria. This study reveals that the exploitation of wetland helophytic plants and their associated bacteria shows promising potential towards implementing CW systems on a large scale with improved performance.

# Chapter 5

# Responses of *J. acutus* endophytic bacterial communities to contamination with metals and emerging organic contaminants and to inoculation with indigenous strains

*In preparation*

**SCOPE**

Plants and their associated bacteria play a crucial role in constructed wetlands. In this context, the impact of bioaugmentation with endophytic strains individually and as a consortium to *Juncus acutus* plants was investigated, in terms of elucidating the potential beneficial effects on the plant's metal phytoextraction capacity. The role of the inoculated consortium in accelerating metal uptake and -accumulation in the plant is highlighted. Moreover, the response of different endophytic communities to increased levels of contamination with metals and emerging contaminants was also evaluated. A significant change of the root endosphere community was observed due to presence of contaminants while the leaf community was not affected. It seems that metals influence negatively the bacterial diversity but the root community composition is affected at a different extent depending on the inoculant. This study highlights the effects of contamination and inoculation on the endosphere distribution patterns towards a better understanding of the driving mechanisms in phytoremediation applications.

## 5.1 Introduction

Emerging organic contaminants (EOCs) refers to pharmaceuticals, personal care products and endocrine disrupting chemicals which may cause important negative effects on the environment as well as on human health at very low concentrations, ranging from μg $L^{-1}$ to ng $L^{-1}$ (Jiang *et al.*, 2013). Spreading of antibiotic resistant strains in the environment and in wastewater treatment plants due to the extensive use of antibiotics (more than 200000 t per year) along with the potential risk of human exposure to them raises big concern to public health (Benotti and Brownawell, 2009). Sulfonamides are among the most frequently used antibiotics worldwide and sulfamethoxazole is one of the most applied sulfonamide antibiotics (Larcher and Yargeau, 2012b). It is a bacteriostatic agent that inhibits the folic acid synthesis in bacteria (Le-Minh *et al.*, 2010) and has been detected in effluents of wastewater treatment plants and in freshwater rivers in North America, Europe, Asia and Australia (Gavrilescu *et al.*, 2014). Another category of antibiotics that has been extensively used are the fluoroquinolones that inhibit enzymes involved in DNA replication (Le-Minh *et al.*, 2010). Among them, ciprofloxacin is one of the most prescribed FQs and has been found in wastewater, surface water and sediment samples (Marti *et al.*, 2014).

Bisphenol A (4,4-isopropylidenediphenol; 2,2-bis(4- hydroxyphenyl)-propane) is a chemical compound that is extensively used in everyday life and in food contact materials; its production exceeds 6 million tons annually (Michałowicz, 2014). Food is considered to be the main source of human exposure to BPA, but air, soil and the aquatic environment are also possible sources. It belongs to the category of endocrine disruptors that can cause adverse effects at low concentrations through a number of mechanisms such as mimicking or inhibiting the activity of endogenous estrogens and influencing the thyroid hormone function (Wetherill *et al.*, 2007a).

Constructed wetlands (CWs) are a promising alternative for treating these chemical compounds and preventing their leach to the environment (Verlicchi and Zambello, 2014). They are engineered, state of the art green systems used to treat effluents rich in pharmaceutical and personal care products by exploiting the plant-bacteria interactions together with the physicochemical processes (Zhang *et al.*, 2014). In such systems, selection of the appropriate plant species significantly influences the performance of

the CW since plants can take up the xenobiotics and/or enhance the role of the functional associated microbiota (Li *et al.*, 2014b). Recently, many studies have focused on investigating the efficiency of wetland plants to remove EOCs in hydroponic systems and the potential effects of these compound on their physiological status (Dordio *et al.*, 2011; Liu *et al.*, 2013; Christofilopoulos *et al.*, 2016).

The bacterial community is also a significant factor in regulating the removal rates in CWs (Meng *et al.*, 2014), which is strongly affected in terms of functionality and diversity by the presence of helophytes (Button *et al.*, 2016; Fernandes *et al.*, 2015). These plants provide oxygen (through their aerenchyma) and root exudates to the rhizosphere microoorganisms, thus creating favorable niches for microbial colonization in the waterlogged environment (Stottmeister *et al.*, 2003b; Blossfeld *et al.*, 2011). Besides rhizospheric microbiota, the endophytic community plays an important role in transforming the organic compounds *in planta*, thus reducing their toxicity and evapotranspiration (Afzal *et al.*, 2014). The contribution of plant-associated microorganisms in metal phytoremediation has also been highlighted through promoting plant growth in metal polluted areas, influencing the metal uptake and translocation by plants and increasing the metal bioavailability by excretion of metals (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 highly contaminated environments (Su *et al.*, 2015b; Zhang *et al.*, 2016b; Zhao *et al.*, 2015). Moreover, information relevant to the impact of contaminants on the endophytic bacteria of wetland plants is scarce. In such complicated environments, it is unclear whether the type and level of contamination, the plant species and phylogeny or a multifactor combination influence the endophytic assemblages. It is important to address these questions towards improving the performance of CWs. In this study, in which, as frequently happens in CWs, soil harboring a diverse community is absent, the response of the *J. acutus* endosphere composition to different levels of contamination is explored. The effect of bioaugmentation with indigenous endophytic strains on the community structure along a contamination gradient was evaluated. Also, the effects of the inoculated strains on the plant's ability to take up metals were investigated together with the colonization efficiency of the strains.

## 5.2 Materials and Methods

### 5.2.1 Experimental design

The large extent of the experimental design has already been described elsewhere (in the previous chapter) but a few modifications were made in this experiment. In detail, the three endophytic strains (B1- *Sphingomonas* sp. U33, B2- *Bacillus* sp. R12, B3- *Ochrobactrum* sp. R24) 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, no contaminants were added in four treatments in order to investigate the potential effects of inoculation to the endophytic community in the absence of contaminants. A two-factorial study design was followed with factor 1 contaminant concentration (3 levels, zero, low, high), and factor 2 bioaugmentation treatments (5 levels, no inoculation, strain 1, strain 2, strain 3, consortium). In total, there were five different treatments (one non-inoculated control and 4 bioaugmented treatments) concerning the inoculation effect and three different concentrations of the mixture of contaminants (one without contaminants-NO, one low concentration-LC and one high concentration-HC) concerning the contamination effect. The experiment lasted for 21 days and was irrigated with 50 mL tap water every week.

### 5.2.2 Sample collection

Fresh root (0.3 g) and leaf (1 g) tissue samples 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 solution for 30 sec 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 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$^{o}$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).

### 5.2.3 16S rRNA gene amplicon libraries preparation

The forward 799F primer (AACMGGATTAGATACCCKG) and the reverse primer 1193R (ACGTCATCCCCACCTTCC) were used for the amplification of the V5-V7 hypervariable region of the bacterial 16S rRNA gene, producing a ~400 bp fragment (Schlaeppi *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 samples were run on a 1.5 % agarose gel and the bacterial DNA was selected over the not always present mitochondrial DNA (approximately 800 bp). The bacterial amplicons were collected from the agarose gels using the x-tracta tips (Sigma-Aldrich, Diegem, Belgium) and purified from agarose with the QIAquick gel extraction kit (Qiagen, Venlo, Netherlands). For multiplexed pyrosequencing, a sample-specific 10 bp barcode (MID) was fused to the forward primer, followed by the key and a Lib-L Adaptor A sequence. Every PCR reaction contained 1 × FastStart High Fidelity Reaction buffer (Roche) with 1.8 mM $MgCl_2$ (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 ($1^{st}$ PCR) or 20 ($2^{nd}$ 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 from PCR ingredients using the QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands). The concentration of purified DNA was determined with the Quant-iT Picogreen dsDNA assay kit (Life Technologies Europe, Ghent, Belgium) according to the manufacture's protocol and equimolar mixtures of different samples were prepared and diluted in TE buffer. 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.

### 5.2.4 16s rRNA gene sequences analysis

Sequence reads were processed and analyzed in QIIME (version 1.9.0) (Caporaso *et al.*, 2010). Primers and barcodes were trimmed off, chimeric sequences and sequences shorter than 200 bases were discarded, reads with erroneous barcodes or forward primer sequences were removed and the sequences were assigned to samples according the barcoded primers (Ampliconnoise). Truncation of the reverse primer and any subsequent sequence at the 3' end was followed. Sequences were clustered at 97% sequence similarity, one representative sequence per OUT was selected and all the representative sequences were classified with RDP (RibosomalDatabase Project) classifier using the Greengenes reference set. Plant-associated sequences such as mitochondria and chloroplast were discarded and a phylogenetic tree was constructed using PyNAST with a minimum alignment length of 150 bp and a minimum identity 75%.

Rarefied OTU tables were generated and all samples were subsampled to 2870 sequences per sample. For alpha diversity analyses, the indices (inverse Simpson diversity index, Chao richness estimator, Shannon, Simpson, Faith's PD and number of observed species) were calculated based on 1000 iterations an plotted in R 3.1.1 (package: phyloseq) (R Development Core Team, 2009).

Principal Coordinate analysis (PCoA) was performed with the phyloseq package in R 3.1.1 using the unweighted Unifrac from QIIME produced from the rarefied OTU-tables. Hierarchically clustering of the normalized samples was performed using the unweighted Unifrac using UPGMA with jackknife tree node support. To evaluate the similarity of community assemblages among the samples, an analysis of similarities (permutation based statistical test ANOSIM) was performed using the unweighted Unifrac distance matrix.

Mantel test analysis was performed in order to test any significant correlation between the level of mix contamination concerning the metal addition and endophytic bacteria composition using the unweighted Unifrac matrix. This test determines whether the distance between measured samples is significantly correlated for paired matrices. In other words, the bacterial community distances for each possible pairwise combination of samples under two levels of metal concentration was measured for each inoculation treatment.

LEfSe [Linear Discriminant Analysis (LDA) Effect Size] was used to detect the bacterial taxonomic biomarkers across the different treatments. As an input, the relative abundances (RA ‰) of the OTU tables were used. To express this, the OTU count in each sample was divided with the sum of all OUT counts in that sample and multiplied by 1000. Heatmap was constructed based on RA values of the most significant OTUs. 88Venn-diagrams

### 5.2.5 Metal analysis in plant parts

Preweighted (0.2 g) dried plant samples were digested with 9 mL $HNO_3$ (>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).

### 5.2.6 Colonization and distribution of endophytes within host plants

Strains were tagged with fluorescent proteins in order to monitor their colonization to the host plant under similar experimental conditions. The labeled strains were inoculated ($10^9$ cells $ml^{-1}$) to plants without and with addition of contaminants. Their colonization efficiency was investigated with confocal microscopy (CLSM).

### 5.2.7 PCR and qPCR of antibiotic resistance genes

The presence of various ARGs such as the sulfamethoxazole (sul) resistance and ciprofloxacin (qnrS and aac(6′)-Ib) were determined by pcr (Xu *et al.*, 2015; Xiong *et al.*, 2015).

### 5.2.8 Statistical analysis

Statistical analysis was performed with R. Differences in the metal concentration and alpha diversity indices in different plant parts among the treatments was estimated with an analysis of variance (2 way ANOVA). After detecting significant differences, a multiple Tukey comparison test was performed. Correlation analysis between the diversity indices and the different metal concentrations in was performed in R (package: Hmisc and corrplot), based on Pearson's product moment correlation coefficient.

## 5.3 Results and Discussion

Phytotechnologies applied to sites with mixed contaminations is a complex issue, since possible interactions between organic xenobiotics and metals may take place as well as with the soil and rhizosphere microbiota. Moreover, the presence of different contaminants causes toxicity thus affecting plant growth and performance (Chirakkara *et al.*, 2016). Taking into account that this is the case in almost every CW, new remediation practices should be investigated towards this direction.

In this context, two different concentrations of a mixture of contaminants were added to microcosms planted with the helophyte *J. acutus* and inoculated with different endophytic bacteria. The wetland plant showed efficient to clean the surrounding water from organic xenobiotics and metals after 21 days (Syranidou *et al.*, 2016a), without showing symptoms of toxicity. Indeed, the inoculation of a tailored endophytic consortium enhanced the plant's performance and alleviated the stress induced by the contaminants, especially at the high concentration treatments. In this experiment, the effects of inoculation and of the level of contamination on the root and leaf endophytic communities were explored. In addition, the extent of enhancing the metal accumulation in the belowground and aboveground tissues of the helophyte was investigated.

### 5.3.1 Metal uptake by plants

The metal concentration in *J. acutus* roots and leaves tended to increase in the treatments with high concentrations of mixed contamination (Figure 20, 21 & 22). In every treatment, the roots accumulated higher amounts of metals compared to the leaves. Similar metal concentrations have been determined in *J. acutus* plant parts after growing in water contaminated with organic xenobiotics and metals at various concentrations (Christofilopoulos *et al.*, 2016). Besides, the helophyte accumulated more Zn in its tissues compared to Ni and Cd. This may be attributed to the higher Zn concentrations used in the two different treatments and to the fact that *J. acutus* has been proposed to be hypertolerant to zinc (Mateos-Naranjo *et al.*, 2014) or even a possible hyperaccumulator (Santos *et al.*, 2014).

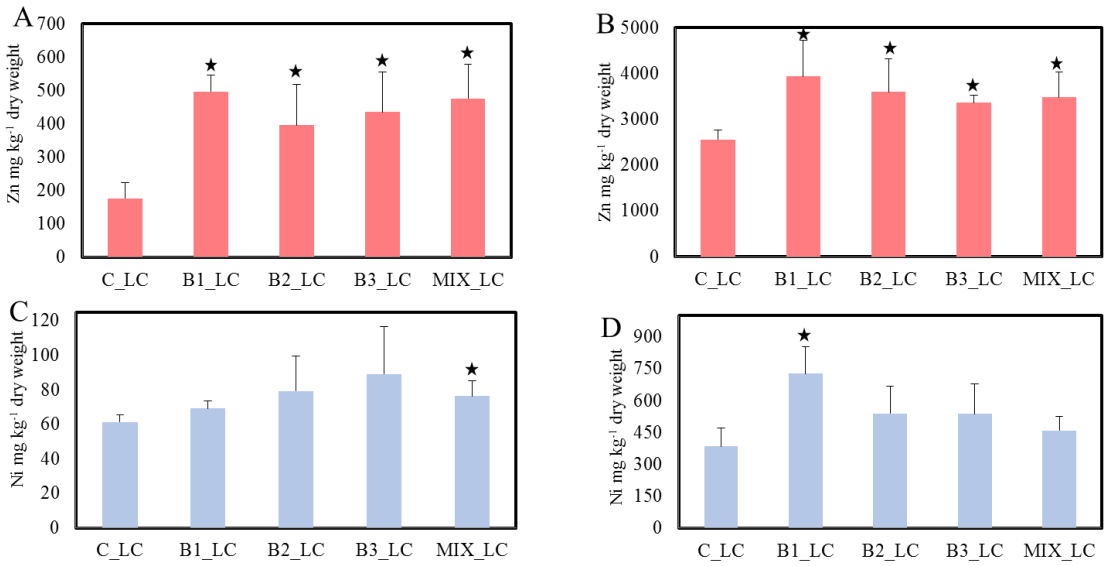



Figure 20. The Zn (A&B) and Ni (C&D) concentrations in the leaves (A&C) and roots (B&D) of *J. acutus* when the low concentration of contaminants was added (data with asterisk are significantly different (p<0,05) compared to the non-inoculated plants).

Inoculation with the indigenous endophytic bacteria enhanced the metal accumulation in roots and leaves (Figure 1). Regarding Zn, two way ANOVA revealed a significant effect of the level of contamination (Roots: F: 164, p= $9*10^{-15}$, Leaves: F: 126, p= $1.2*10^{-12}$), as well as of inoculation (Roots: F: 6.30, p= 0.0003, Leaves: F: 13, p= $2*10^{-6}$) on the metal accumulation capacity of plants. An interaction effect was also observed (Roots: F: 3.1, p= 0.027, Leaves: F: 6.68, p= 0.0005) while the effect of different initial concentrations of contaminants was by far the most prominent. Due to the observed interactions between the factors the effect of inoculation on the plants' ability to accumulate Zn was investigated separately. When 200 mg $L^{-1}$ Zn were added, all the inoculants significantly enhanced the phytoextraction capacity of the plant (significantly higher Zn concentrations in roots and leaves) compared to the non-inoculated (Figure 20A, 20B). At elevated Zn concentrations, the B1, B3 and consortium inoculated plants 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 (Figure 21A, 21B). Correspondingly, bioaugmentation with endophytic bacteria was shown to stimulate the host to take up zinc in the plant interior (Ma *et al.*, 2016b, 2015c; He *et al.*, 2013). For example, the endophytic strain *Sphingomonas* SaMR12 isolated from *Sedum alfredii*, promoted Zn absorption and

translocation to shoots in comparison to the non-inoculated plant, across a Zn gradient (Chen *et al.*, 2014).

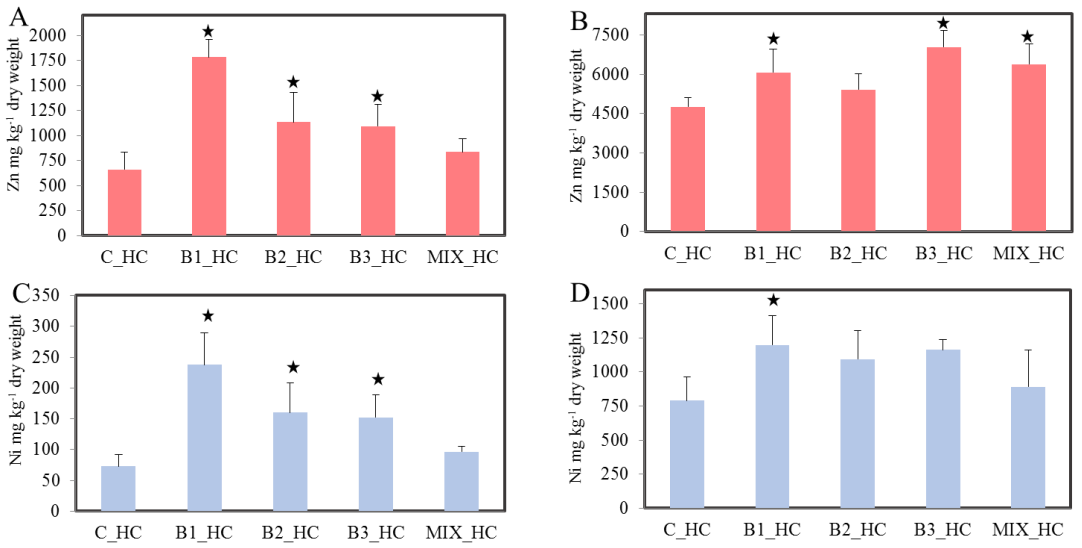



Figure 21. The Zn (A&B) and Ni (C&D) concentrations in the leaves (A&C) and roots (B&D) of *J. acutus* when the high concentration of contaminants was added (data with asterisk are significantly different ($p<0,05$) compared to the non-inoculated plants).

With respect to nickel, the beneficial effects of inoculation to *J. acutus* phytoextraction capacity were less pronounced. Nevertheless, significant effects on plant Ni concentration were observed due to contamination (Roots: F: 52, p= $2*10^{-8}$, Leaves: F: 18.67, p= 0.0001) and due to inoculation (Roots: F: 3.8, p= 0.01, Leaves: F: 8.5, p= $9*10^{-5}$). An interaction effect between the two factors was detected for the leaves (F: 5.58, p= 0.002). At low Ni concentration, only the B1 inoculated plants accumulated significantly more Ni in the roots and only in the leaves of the consortium inoculated plants a significantly higher Ni concentration was detected in comparison to the non-inoculated plants (Figure 20C, 20D). When 40 mg $L^{-1}$ Ni was added, 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 accumulated significant Ni amounts in their leaves in comparison to the non-inoculated plants (Figure 21C, 21D). Along a Ni gradient, it was demonstrated that inoculation of an endophytic strain stimulated Ni uptake in roots and shoots of *Alyssum serpyllifolium* (host plant) and *Brassica juncea* (non-host plant) (Ma *et al.*, 2011b). This effect can be attributed to the plant growth promoting traits of the inoculant (ACC deaminase,

siderophores, IAA and P solubilization) and the plant polymer hydrolyzing enzymes (cellulase and pectinase). Likewise, co-inoculation with specific endophytic strains enhanced Ni concentration in the shoots and roots of *Noccaea caerulescens* (Visioli *et al.*, 2015). Supplementation with endophytic strains may also have a protective role to the host by reducing the metal accumulation in the plant parts and by improving the physiological status of the plant (Mesa *et al.*, 2015b).

Cadmium was not detected in leaves of all plants regardless of the initial concentration and inoculation effort. A significant impact of the initial amount of Cd contamination to roots was revealed (F: 12.8, p= 0.001). However, no significant differences were observed among the Cd accumulation capacity of inoculated and non-inoculated plants at low and high Cd exposure (Figure 22A, 22B). When one rhizospheric and one endophytic transconjugant were inoculated separately and as a mixture to willow cuttings contaminated with Cd and toluene, a decrease in the amount of Cd in leaves and roots was demonstrated for the willows inoculated with the individual strains (Weyens *et al.*, 2013). Inoculation with both strains did not affect Cd uptake by the roots and leaves. In other studies, addition of endophytic strains enhanced the Cd accumulation in various plant parts in comparison to the non-inoculated ones (Luo *et al.*, 2011; Babu *et al.*, 2013).

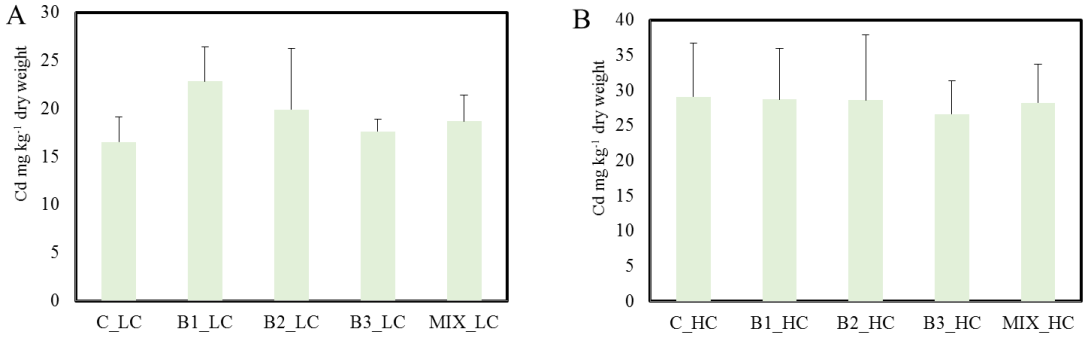



Figure 22. The Cd concentrations in the roots of *J. acutus* when the low (A) and high (B) concentration of contaminants were added (data with asterisk are significantly different (p<0,05) compared to the non-inoculated plants).

Generally, endophytic bacteria can alter the phytoextraction capacity of their host plant through several mechanisms such as secreting a variety of metabolites (Ma *et al.*, 2016a). Most of the times, the inoculation of bacterial strains with plant growth

promoting traits along with tolerance to the target compound has a positive phytoremediation outcome (Rajkumar *et al.*, 2009; Afzal *et al.*, 2014). The results of our experiment corroborate the before-mentioned observations. After considering the total amount of metals accumulated in the whole plant biomass, it was demonstrated that significant (in almost all cases) amounts of Ni and Zn were accumulated inside inoculated plants in comparison to non-inoculated ones (data not shown). It is worth to notice that the consortium had the most pronounced effect on the accumulation of all three metals in *J. acutus*: the consortium inoculated plants accumulated approximately three times more Ni, two times more Zn and 1.5 times more Cd in comparison to the non-inoculated ones.

### 5.3.2 Response of bacterial communities

In order to investigate the interactions and changes that take place in a phytoremediation setup of a multi-contaminated matrix after bioaugmentation with indigenous endophytic strains, the total DNA extract of samples of leaves and roots was analyzed using next generation sequencing. A total of 358035 sequences were obtained from 66 samples, with on average 5680 sequences per sample; the average length of the sequences was 353 bp. After the sequence quality filtering, removing of all sequences belonging to mitochondria, chloroplasts, archaea and eukaryotes and deleting of samples with high numbers of false sequences or low numbers of sequences, 63 samples remained with 214554 assembled high-quality sequences in total and with 3405.6 sequences per sample. The OTU tables were rarefied to 2870 sequences per sample and the taxonomic composition was performed using the Greengenes database.

Bacterial diversity of the samples was estimated with the rarefaction analysis, indicating approximate saturation. Moreover, increased levels of mixed contamination showed a negative effect on the alpha diversity of root communities using phylogeny-based methods (Figure 23A). The number of observed species (97% similarity) of root communities ($F:5.24$, $p= 0.011$) was significantly affected by the contamination according to two-way ANOVA tests, while no effect of inoculation ($F:0.86$, $p= 0.50$) or interaction effect ($F:0.44$, $p= 0.88$) were noticed. A significant effect of contamination can explain the differences in the Shannon diversity ($F: 33.4$, $p= 0.0001$), while the effect of different inoculants was minimal ($F: 0.53$, $p= 0.72$). The pattern was similar for the phylogenetic diversity index and Simpson diversity. Interestingly,

statistically significant differences in the number of observed species were detected between the high contaminated communities with the low and non-contaminated communities (p= 0.013 and p= 0.002 respectively) while no significant differences were noticed between the low and non-contaminated treatments (p= 0.472). A similar pattern was observed for the rest of diversity indices.

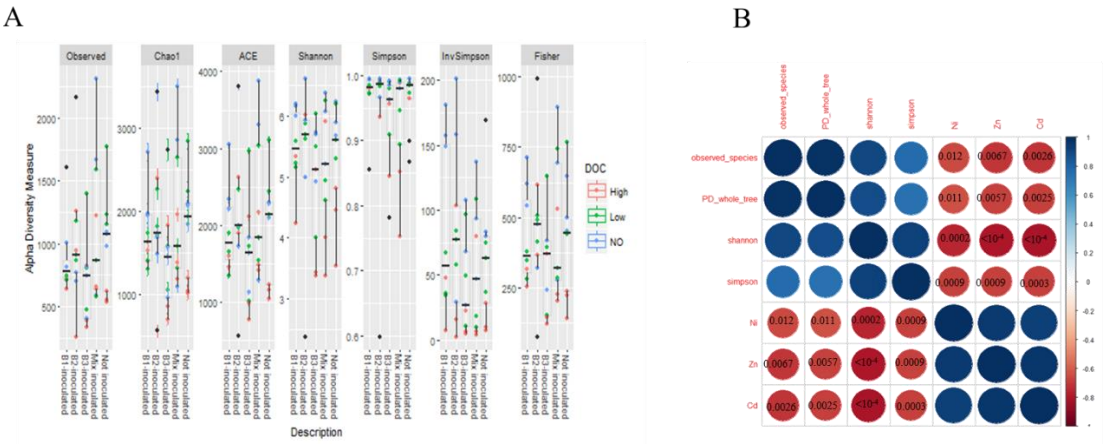



Figure 23. Alpha diversity indices of root endophytic communities among the different bioaugmented treatments (A) and the correlation of them with the metal concentration in the roots (the significant correlation coefficients are indicated) (B). The scale colors denote whether the correlation is positive (closer to 1, blue circles) or negative (closer to −1, red circles) between the diversity indices and metal concentrations.

Bacterial diversity in leaf communities did not seem to be affected by the presence of contamination or by the inoculation (Figure 24A). For example, the number of observed species remained stable independently of level of contamination (F: 0.46, p= 0.69) and different inoculants (F: 0.96, p= 0.57). Similarly, the exposure of the plant to metals and organics did not affect the phylogenetic diversity in leaf microbiota (F:3.9, p= 0.2). Also, only a limited effect was detected due to inoculation of endophytic strains (F: 0.4, p= 0.8). Shannon and Simpson diversity followed the same pattern (F: 1.6, p= 0.38 and F: 0.44, p= 0.7 respectively) for the contamination and the inoculation (F: 0.56, p= 0.7 and F: 2, p= 0.36 respectively) effects.

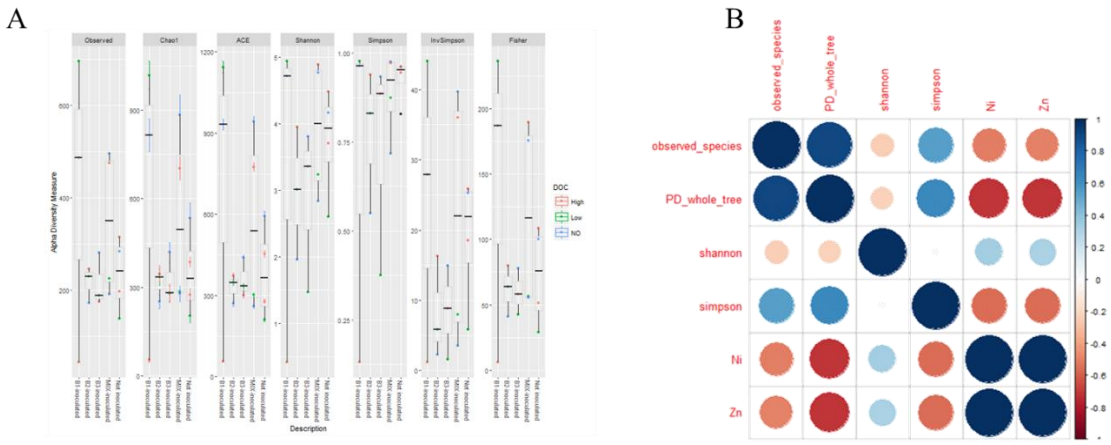



Figure 24. Alpha diversity indices of leaf endophytic communities among the different bioaugmented treatments (A) and the correlation of them with the metal concentration in the leaves (the significant correlation coefficient are indicated) (B). The scale colors denote whether the correlation is positive (closer to 1, blue circles) or negative (closer to −1, red circles) between the diversity indices and metal concentrations.

Correlation analysis revealed that the number of observed species, phylogenetic diversity, and Shannon and Simpson diversity of the root communities negatively correlated with increased concentrations of metals in roots (Figure 23B). Moreover, this negative correlation between diversity indices and each metal was significant. The number of observed species was negatively affected by the nickel, zinc and cadmium concentrations in the root compartment (Ni: p= 0.012, r= -0.46, Zn: p= 0.0067, r= -0.49, Cd: p= 0.0026, r= -0.54), as well as the phylogenetic diversity (Ni: p= 0.011, r= -0.46, Zn: p= 0.0057, r= -0.50, Cd: p= 0.0025, r= -0.54). The increased metal concentrations affected significantly the Shannon diversity (Ni: p= 0.0002, r= -0.63, Zn: $p<10^{-4}$, r= -0.67, Cd: $p<10^{-4}$, r= -0.71) and Simpson diversity (Ni: p= 0.0009, r= -0.58, Zn: p= 0.0009, r= -0.58, Cd: p= 0.0003, r= -0.62). No significant correlation was observed between the leaf communities and the metal concentrations in this plant compartment (Figure 24B).

A negative trend in endophytic bacterial diversity in response to increased levels of contaminants has also been mentioned by other studies (Peng *et al.*, 2013; Su *et al.*, 2015b). For example, the presence of PAHs affected the endophytic community of *Spartina alterniflora* (Su *et al.*, 2015b). In this study, it was demonstrated that phenanthrene had a stronger impact on the diversity of the root bacterial endophytic community than pyrene, which may be attributed to their different ability to enter in the

plant. Furthmore, increased metal concentration were also reported to have negative or adverse effects on the microbial community (Mucha *et al.*, 2013; Khan *et al.*, 2010). In the more sensitive environment such as CW in comparison to soils, multi-metal contamination caused changes on the bacterial community (Zhang *et al.*, 2016a).

Sulfamethoxaxole and ciprofloxacin have been detected in the parts of several plant species (Goldstein *et al.*, 2014; Prosser and Sibley, 2015; Liu *et al.*, 2013). The presence of antibiotics (ciprofloxacin and sulfamethoxazole) in the plant parts may negatively affect the diversity and structure of the endophytic bacterial communities. For example, SMX addition to soil lead to a strong inhibitory effect in soil microbial communities (Wang *et al.*, 2016) while ciprofloxacin alters the soil microbiota composition at lower taxonomic levels (Lin *et al.*, 2016). The combination of tetracycline, sulfamonomethoxine and ciprofloxacin stimulated the growth of resistant cultivable soil bacteria compared to the population in control and soil contaminated with single applications (Ma *et al.*, 2014). Long-term mixed contaminations and especially the presence of polycyclic aromatic hydrocarbons, zinc, and polychlorobiphenyls concentrations are strong factors that shape the soil microbial community structure (Kaci *et al.*, 2016). The PhyloChip analysis revealed a significant relationship between the community and the contaminant concentration.

The Principal Coordinate analysis revealed that the *J. acutus* root endophytic communities seemed to alter in function of the level of contamination. As seen in Figure 25A, separate groups are formed, moreover this separation is statistically significant (ANOSIM R: 0.33, $p<0.001$). Each group involves samples of treatments contaminated with the same concentration of the mixture of metals and emerging organic contaminants (High, Low, No), indicating an induced shift in the root community composition. Root endophytic communities from the non-contaminated treatments were clearly different from the others, whereas a slight overlap was noticed between communities of low and high-contaminated treatments. This phenomenon has been also observed in the dominant root endophytic species of *Cucurbita pepo* as a response to DDE addition (Eevers *et al.*, 2016). Significant differences were detected in the culturable endophytic communities isolated from *Halimione portulacoides* across a gradient of metal(loid) contamination (Fidalgo *et al.*, 2016).

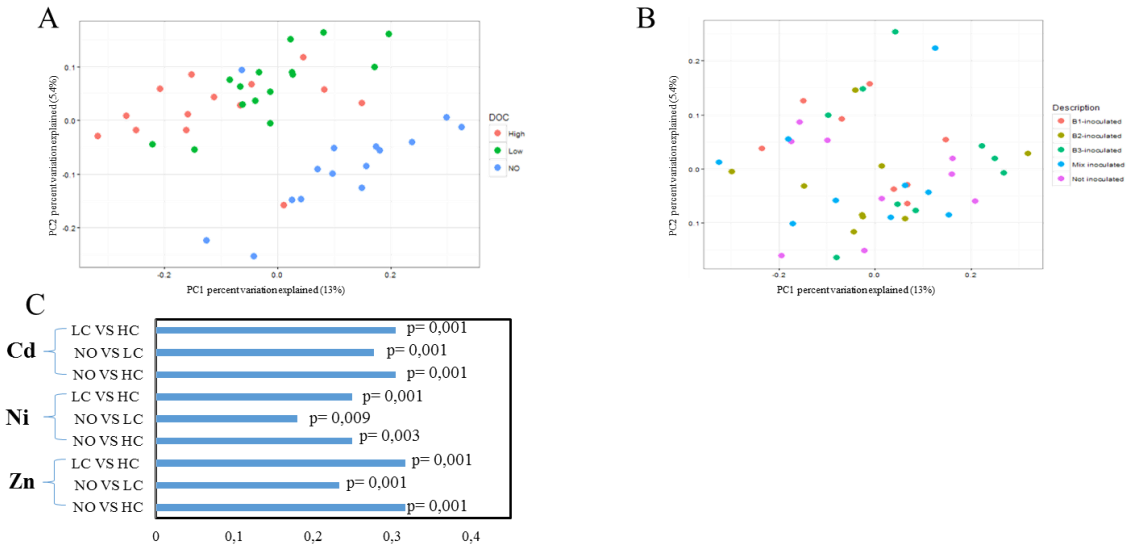



Figure 25. Similarity of the root bacterial communities across mixed contamination gradient (A) and inoculation (B). Distance between the samples are based on similarity in OTU composition, calculated using the unweighted UniFrac distances and visualized in Principal Coordinates Analysis (PCoA). Phylogenetic correlation of root bacterial communities across metal gradient in roots using the Mantel test (999 permutations) (C).

With respect to the composition of the leaf endophytic community, no clear pattern was detected (Figure 26A). It seems that almost all samples are grouped together regardless of the level of contamination. This convergence among the leaf composition may be attributed to the lower metal concentrations in this plant compartment and implies that this compartment is affected at a lesser extent by the mixed contamination. On the contrary, the cultivable leaf endophytic communities of *Spartina alterniflora* varied among plants harvested from oil contaminated sites (Kandalepas *et al.*, 2015). The limited dispersal possibility of seeds along with the oil presence and stoxastic phenomena contribute to this dissimilarity. Host plant, sampling site and time have been identified as significant factors that influence the leaf community at non-contaminated areas (Ding *et al.*, 2013).

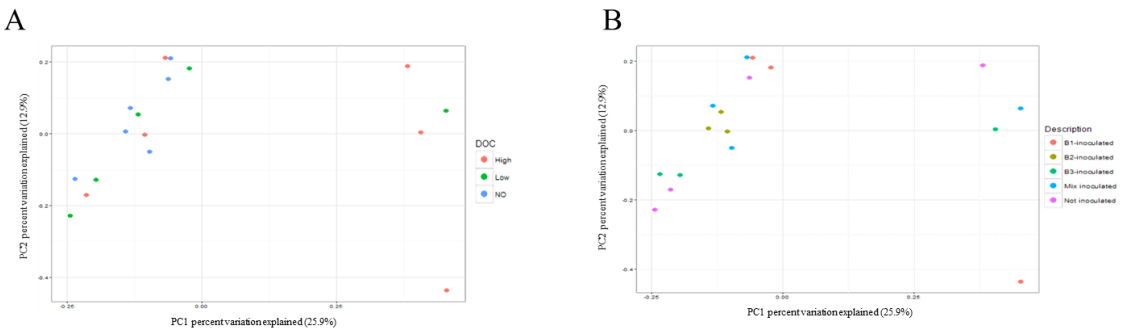



Figure 26. Similarity of the root bacterial communities across mixed contamination gradient (A) and inoculation (B). Distance between the samples are based on similarity in OTU composition, calculated using the unweighted UniFrac distances and visualized in Principal Coordinates Analysis (PCoA).

The plant itself in combination with the environmental parameters such as the type and concentration of contaminants can selectively enrich specific endophytic bacterial genotypes (Siciliano *et al.*, 2001). For example, the concentration of genes encoding for PAH-ring hydroxylating dioxygenses was stimulated in endophytic populations isolated from plants growing in more contaminated areas (Oliveira *et al.*, 2014). Moreover, the abundance of these genes was significantly higher in a root endosphere treated with phenanthrene in comparison to the control community while no significant differences were detected between the abundance of endophytic genes in pyrene contaminated pots and control (Hong *et al.*, 2015). However, it is difficult to attribute this *in plant* enhancement of functional traits either to plant or to contaminant selection. For sure, the host plant also plays an important role, influencing the endosphere composition and thus developing species specific relations in a contaminated environment (Lumactud *et al.*, 2016; Phillips *et al.*, 2008).

In uncontaminated environments, it has been demonstrated that the host genotype affects the endophytic community to a lesser extent than the soil type or the soil microbial community (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012). The 16S rRNA gene pyrosequencing of bulk soil, rhizosphere, and root samples of eight *Arabidopsis* ecotypes grown in two soil types revealed that among all the 778 OTUs, only 12 OTUs showed host genotype–dependent quantitative enrichment in the root endophyte compartment (Lundberg *et al.*, 2012). With respect to seed endophytic community, it is seems that there exists a plant-selection mechanism that determines the bacteria transmitted from one generation to the next (Truyens *et al.*, 2015a).

Mantel correlation coefficients indicated that significant relationships existed between root communities exposed to different levels of mixed contamination (Figure 25C). When the root communities from the different inoculation treatments were investigated separately, different correlations between communities and the concentration of contaminants were revealed. The root endosphere of non-inoculated and B3 inoculated plants was significantly correlated with the increasing metal concentrations in the plant part, while no association was observed between the other endophytic communities and each metal, indicating treatment-specific responses.

As seen in Figures 25B and 26B, inoculation of indigenous endophytic bacteria did not alter the endophytic community structure in plants, which is in accordance with other studies (Conn and Franco, 2004). Despite the fact that differences were detected in plant's phytoextraction capacity, adding of indigenous bacteria was not strongly influencing the community. In accordance with this observation, inoculation with *Burkholderia phytofirmans* PsJN to maize cultivars did not change the shoot and rhizosphere communities (Touceda-González *et al.*, 2015). However, a strong effect on the soil microbial community has been observed after inoculation with a soil strain while inoculation with a consortium had minor effects, thus this strategy was proposed for treating soil contaminated with phenanthrene (Festa *et al.*, 2016).

The pattern of the main endophytic taxonomic profiles remains more or less similar across the treatments, indicating a resilience of the communities (Figure 27 & 28). Since the endosphere is a relatively stable environment concerning environmental conditions and amounts and types of carbon sources, the communities are protected from detrimental effects of high concentration of contaminants (Mench *et al.*, 2009). In total, 20 phyla have been detected across the different treatments while the phylum Proteobacteria dominates the *J. acutus* endosphere in both plant compartments. The root community mainly consisted of Alphaproteobacteria followed by Betaproteobacteria, and members of Bacteroidetes and Actinobacteria. Addition of sulfonamides compounds (including sulfamethoxazole) has been found to significantly enrich Alphaproteobacteria while addition of fluoroquinolones (including ciprofloxacin) significantly selected Actinobacteria among other classes (Xiong *et al.*, 2015). The leaf microbial community contained bacteria belonging to Alphaproteobacteria, Gammaproteobactria, and members of Firmicutes and

Actinobacteria. It is important to notice that the leaf community of B2 inoculated plants was dominated by the family Bacillaceae in accordance with the inoculant. Moreover, the colonization efficiency of specific inoculants was investigated with confocal microscopy. As seen in figure 30, the B3 strain efficiently colonized *J. acutus* root surface in presence of mixed contamination.

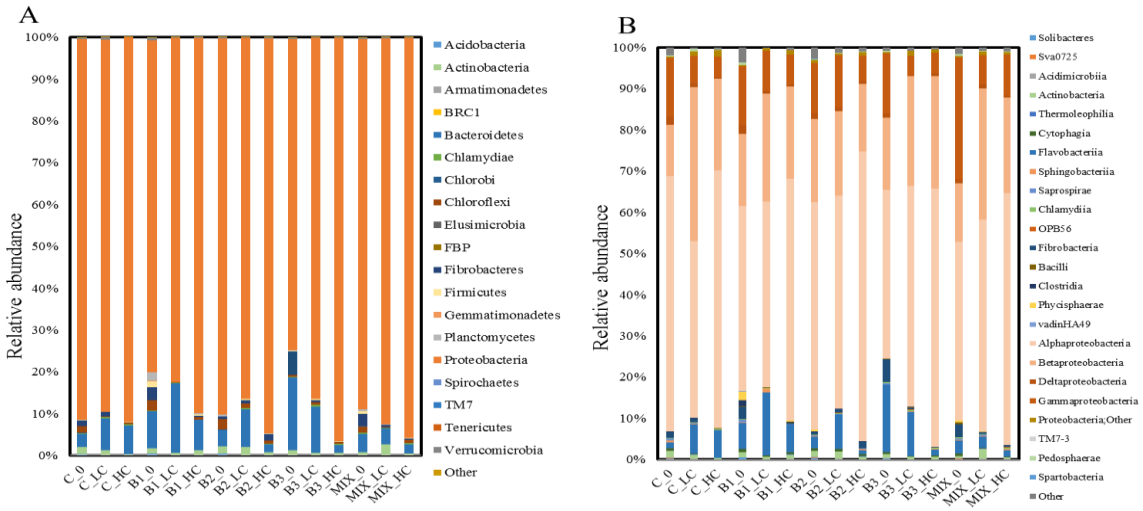



Figure 27. Community composition of major (A) bacterial phyla and classes (B) of the root communities among treatments.

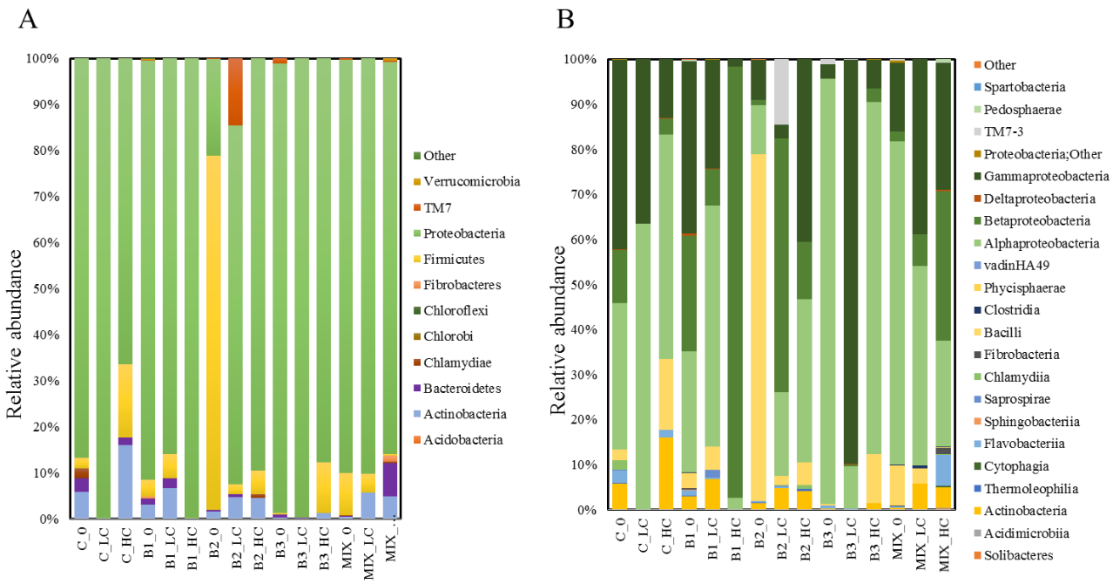



Figure 28. Community composition of major (A) bacterial phyla and classes (B) of the leaf communities among treatments.

Changes in the community members were observed in response to increasing levels of contamination (Figure 29). The root and the leaf communities exposed to different concentrations of mixed contamination were compared in order to identify the potential indicator species. Only 34 root OTUs and 10 leaf OTUs (LEfSE, $p < 0.05$, $\log_{10}$ LDA score >3) were enriched or depleted across the contamination gradient. With respect to root endosphere, members of Alphaproteobacteria mainly affiliated with Sphingomonadaceae were enriched at the low level of contamination while members belonged to Rhodospirillaceae were depleted at high contamination. Betaproteobacteria affiliated with Oxalobacteraceae were significantly enriched due to high contamination treatments whereas Comamonadaceae reduced. The Venn diagram revealed that the root endosphere exposed to low contamination shares many families with both the roots of unexposed plants and the roots of plants from the high contamination treatments, implying that these communities are at the 'transition' between the two extremes.

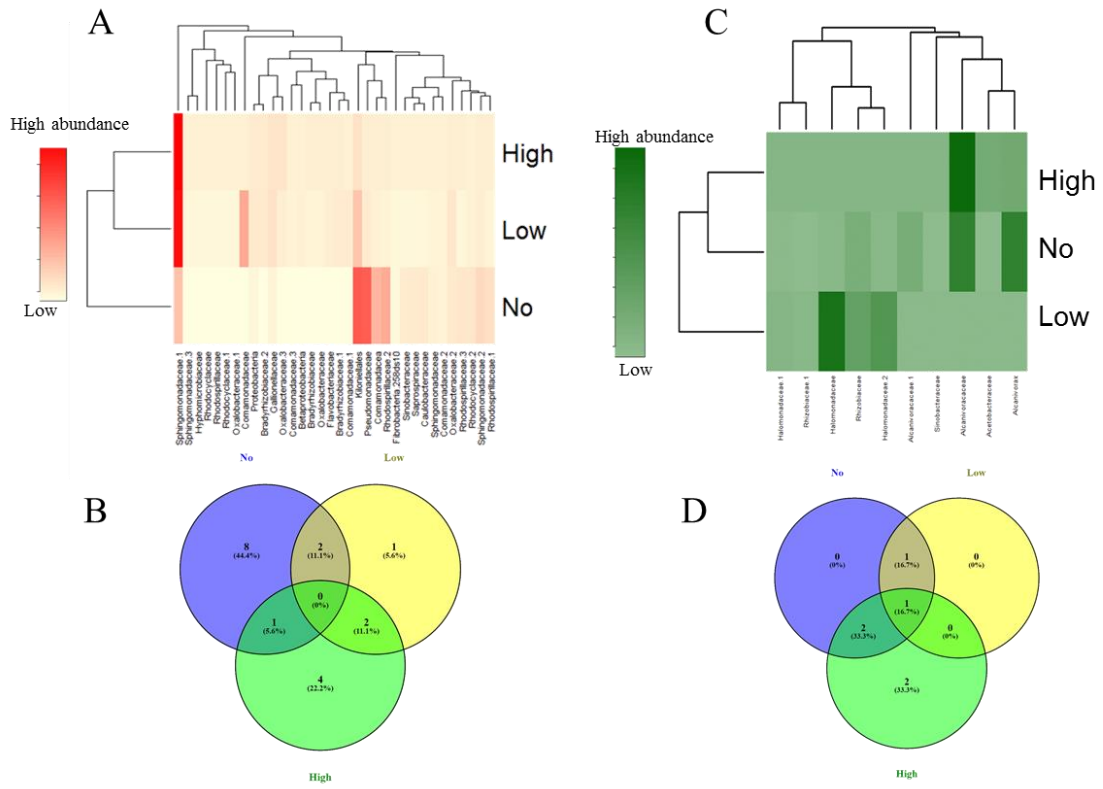



Figure 29. Heatmap showing differences in the normalized abundances of significant root (A) and leaf (C) OTUs and Venn diagram showing root (B) and leaf (D) families that significantly changed across mixed contamination gradient (No: treatments without contamination, Low: low contamination treatments, High: high contamination treatments).

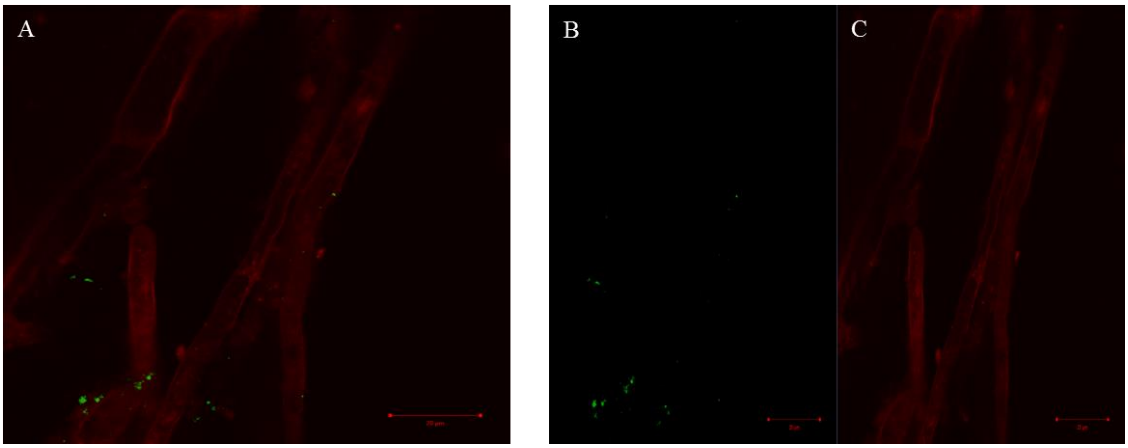



Figure 30. Confocal laser scanning micrographs of gfp-tagged B3 strain colonizing roots of *J. acutus* (A), solution with live gfp-labelled B3 bacteria (B) and root tissue autofluorescence (C).

Until now, only few studies have investigated the effects of host genotype–dependent variation on the root bacterial microbiota profiles and the responses of endophytic communities to contamination with high-resolution techniques. As a result, more studies are needed to shed light on the underlying mechanisms that drive the interactions between plants and their bacterial community in response to increased levels of stress.

### 5.3.3 qPCR

The beneficial role of plasmids in manipulating the microbial community has been already demonstrated (Bell *et al.*, 2014). Inoculation of endophytic bacterial-carriers of introduced plasmids with specific catabolic genes enhanced the phytoremediation efficiency of the host plants (Taghavi *et al.*, 2005; Barac *et al.*, 2004; Weyens *et al.*, 2012). In this experiment, the antibiotic resistance genes to sulfamethoxazole were identified in the root endosphere and in the B3-inoculated strain. Further experiments will reveal to what extent these genes were distributed within the root communities and enhanced the *in planta* sulfamethoxale degradation. SMX resistance genes have also been detected in roots and leaves of lettuce and endive growing in a manure amended soil (Wang *et al.*, 2015).

## 5.4 Concluding Remarks

Bioaugmention with indigenous endophytic bacterial strains was shown to improve the metal phytoextraction capacity of the wetland plant *J. acutus*. Moreover, the addition of the consortium enhanced the metal accumulation of Cd, Ni and Zn in the plant biomass since it combines multi-resistant strains with plant growth promoting characteristics. The increased concentration of the mixture of contaminants 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 unchanging across the contamination gradient the plants were exposed to. Specific OTUs were enriched in the root compartment in response to high levels of mixed contamination. However, it has not yet been examined whether plants growing in multi-contaminated soils/water alter the survival potential of specific resistant and/or beneficial microbes. Thus, it is critical to explore the diversity, distribution, and activity of endophytic microbial communities associated with various plants in phytoremediation studies and monitor the changes induced due to contamination. 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 contamination, 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.

# Chapter 6

# General Discussion and Conclusions

Phytoremediation is a green remediation alternative for soil, air or water restoration that offers additional benefits such as the increase of local biodiversity and the production of plants with added economic value. Constructed wetlands are a state of the art sustainable application of phytotechnologies that are used to treat various kinds of wastewater. It is considered as an appropriate method for treating wastewater effluents derived from low population communities. To overcome the limitations and encourage their use at larger scale, the exploitation of helophyte-endophytic bacteria partnerships is proposed (Sauvêtre and Schröder, 2015; Shehzadi *et al.*, 2014; Ho *et al.*, 2012). These systems are appropriate for investigating the plant-microorganisms interactions since they are simplified artificial systems mimicking natural ecosystems. They are mainly based on the plant-mediated and microorganisms-mediated strategies for removal of mixed contaminations and their evolved interactions.

Inclusion of symbiotic bacteria in plant-based remediation systems is proposed as highly promising but it needs further investigation. It is based on the synergistic relationship between plants and their associated microorganisms in the perspective of boosting the population or the metabolic activity of functional bacteria. Therefore, this mutualistic association represents a powerful emerging approach to sequester, degrade, transform, assimilate, metabolize, or detoxify contaminants from soil, sediment, or (ground)water. Until recently, the application of natural endophytic strains for improving the remediation capacity of host plants was limited due to lack of beneficial strains. During the last years, an increasing amount of beneficial associated bacteria have been discovered and many studies have highlighted their role in accelerating the performance of host plants to treat efficiently metals or organics (Shehzadi *et al.*, 2015; Dimitroula *et al.*, 2015; Ijaz *et al.*, 2015a).

In this thesis, *J. acutus* was selected as the model wetland plant and tested for its ability to treat BPA contaminated water. After assuring the efficiency of this plant, the potential role of root exudates in enhancing BPA removal was investigated. Since

biostimulation did not seem an appropriate strategy for increasing the plant's capacity to clean BPA contaminated groundwater, the endophytic community of this autochthonous wetland plant was explored in terms of revealing the genotypic and phenotypic characteristics. The results showed an associated community highly enriched with strains capable of expressing plant growth promoting characteristics and tolerating various metals. Furthmore, the isolates showed resistance to emerging organic contaminants (including endocrine disruptor and antibiotics) and the potential to degrade them.

Exploiting plant associated bacteria carrying specific characteristics offers potential in for more efficient remediation. The plant provides a stable environment rich in carbon sources and nutrients and in turn the bacteria reduce the stress experienced by the host plant, promote the growth of the plant and/or detoxify the contaminant. The concept of using endophytic bacteria in remediation strategies is still quite recent , thus there exists only a limited number of studies focusing on the specific host plant-bacteria associations in function of removing multi contaminants in soil or water (Ma *et al.*, 2016b; Mesa *et al.*, 2015a). In engineered systems such CWs this is a prerequisite since the co-occurrence of various metals and organic xenobiotics is the most often case.

In this context, we performed bioaugmentation experiments after identifying and characterizing the strains with high potential. A mixture of contaminants including metals and emerging organic contaminants was added to plants inoculated with endophytic individual strains and as a consortium.

During the last years, an increasing amount of data has been published demonstrating the wide dispersion of emerging contaminants in natural environments and highlighting their ability to enter and accumulate in various edible plant plants. Dissipation in soil is faster in rhizosphere in comparison to the bulk soil, whereas some compounds such as fluoroquinolones, may persist for several months to years. However, information about the microbial contribution to antibiotics dissipation in nature is scarce. Moreover, the induced shifts in soil microbial structure in wastewater treatments such as CWs have started to be investigated the last 2-3 years (Lin *et al.*, 2016; Wang *et al.*, 2016; Ma *et al.*, 2014; Liu *et al.*, 2014).

Inoculation of a tailored consortium seemed an appropriate strategy since it appeared to have a positive effect on plant's performance when dealing with mixed

contaminations. The consortium-inoculated plants removed efficiently metals and emerging organic contaminants from water in a shorter period of time in comparison to all the other treatments. Especially in case of high contamination, the beneficial effect of bioaugmentation with selected endophytic bacteria was more prominent. The consortium also contributes to alleviate the stress induced to the plant by the presence of mixed contamination and enhanced (in most cases significantly) the metal phytoextraction capacity of *J. acutus*. This indicates that application of plant growth promoting endophytes with multi-tolerance to metals and organics may result in the promotion of plant establishment, growth and development in stressful environments. Moreover, understanding and further exploitation of the developed interaction may point towards implementation of endophytic bacteria for accelerating large-scale application of phytoremediation with beneficial results.

It is of high importance to address the underlying mechanisms that drive plant bacteria interactions in order to direct them towards efficient phytoremediation applications. This is the first study that uses next generation sequencing techniques in order to get insight to the responses of the endophytic community of a wetland plant to increased levels of mixed contamination. It seems that metals influence negatively the bacterial diversity but the root composition is affected at a different extent depending on the inoculant. For example, the root endosphere community of consortium inoculated plants did not show any association with the increased metals concentration in roots. In general, it seems that the increased levels of contaminants mixture caused a shift in the root microbial communities but it did not affect the leaf communities.

In conclusion, bioaugmentation with the tailored consortium enhanced the phytoremediation efficiency of the wetland plant but did not significantly alter the endophytic community composition. This thesis suggests that the exploitation of synergistic relationships between plants and their endophytic bacteria in multi-contaminated CWs is promising.

## Future directions

The importance of plant–endophyte partnership for the removal of pollutants from soil and water is still underestimated. More studies should be conducted in multi-

contaminated environments focusing on the prospects of using tailored mixtures of indigenous endophytic bacteria. Presence of plants can modify the microbial communities and *vice versa* in natural or contaminated ecosystems. An integrated effort is needed to assess the host-microbiota interactions towards manipulation of this meta-organism. Unraveling the signaling routes that governs these interactions is necessary; this will reveal the processes of the selective enrichment of key functional taxa by hosts. Of particular interest will be the ability to make interventions in this meta-organism concept towards optimization of phytoremediation applications in a more resilient and robust direction.

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