UNIVERSITY OF MOLISE

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DOCTORAL THESIS

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Analysis and characterization of microbial biofilm associated with the roots of *Phragmites australis* and *Typha latifolia*

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“Analysis and characterization of microbial biofilm associated with the roots of *Phragmites australis* and *Typha latifolia*”

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Abstract

Phragmites australis and *Typha latifolia* are two macrophytes commonly present in natural and artificial wetlands. Roots of these plants engage in interactions with a broad range of microorganisms, collectively referred to as the microbiota. These interactions contribute to the natural process of phytodepuration, whereby pollutants are removed from contaminated water bodies through plants. The outermost layer of the root corpus, the rhizoplane, is a hot-spot for these interactions where microorganisms establish specialized aggregates designated biofilm. Earlier studies suggest that biofilm-forming members of the microbiota play a crucial role in the process of phytodepuration. However, the composition and recruitment cues of the *Phragmites* and *Typha* microbiota remain poorly understood. We therefore decided to investigate the composition and functional capacities of the bacterial microbiota thriving at the *P. australis* and *T. latifolia* root-soil interface. By using 16S rRNA gene Illumina MiSeq sequencing approach we demonstrated that, despite a different composition of the initial basin inoculum, the microbiota associated with the rhizosphere and rhizoplane of *P. australis* and *T. latifolia* tend to converge towards a common taxonomic composition dominated by members of the phyla Acidobacteria, Actinobacteria, Firmicutes, Proteobacteria and Planctomycetes. These differences were mirrored by a structural diversification of the microbiota at lower taxonomic ranks. This indicates the existence of a selecting process acting at the root-soil interface of these aquatic plants reminiscent of the one observed for land plants. The magnitude of this selection process is maximum at the level of the rhizoplane, where we identified different bacterial taxa enriched in and discriminating between rhizoplane and rhizosphere fractions in a species-dependent and –independent ways. This led us to hypothesize that the structural diversification of the rhizoplane community underpins specific metabolic capabilities of the microbiota. We tested this hypothesis by complementing the sequencing survey with a two-pronged approach. First, we inferred the functional potential of these communities through a predictive metagenomics approach using the software PICRUSt and we found that transporters and transcription

factors-encoding genes are a distinctive feature of the rhizoplane-enriched communities. In parallel, we used Scanning Electronic Microscopy, bacterial isolation and a biochemical assay to demonstrate that rhizoplane-enriched bacteria have a bias for biofilm-forming members. Together, our data will set the stage towards the rational exploitation of plant-microbiota interactions for phytodepuration.

CONTENTS

List of publications

This thesis is based on the work presented in the following paper:

Unravelling the composition of the root-associated bacterial microbiota of *Phragmites australis* and *Typha latifolia*

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Chapter 1

1. INTRODUCTION

1.1 Water, a resource to be safeguarded from pollution

Water is an essential resource for our life, yet water pollution is one of the most serious ecological problems of the planet. Therefore, preserving water quality is major challenge that humanity is facing in the twenty-first century all over the world (Schwarzenbach et al., 2010). Water pollution is definable generally as the degrading of water quality due to toxic substances which accidentally or intentionally enter in water bodies such as rivers, lakes, seas and oceans, dissolve in them, remain floating on their surface or deposit themselves on the bed of water systems (Goel, 2006; Schwarzenbach et al., 2010).

The increasing of urbanization, industrialization and over population can be identified as main causes of water pollution, since industrial and residential wastes, agricultural and surface runoff exert the major impact on the quality of water bodies (Dhote and Dixit, 2009). To avoid the contamination of receiving water bodies various conventional methods are applied to remove pollutants from wastewaters, yet these conventional methods result costly and appear not sustainable in the long term (Dhote and Dixit, 2009). To overcome these problems, from many decades the most researched field in biological and environmental sciences all over the world has been the development of bioremediation techniques, i.e. biological processes performed naturally by eukaryotic and prokaryotic organisms or derived from their interaction, which are able to mitigate the environmental pollution using a lower amount of energy and thus resulting less expensive and more eco-friendly approaches (Srivastava et al., 2017).

Interestingly, many studies demonstrated that aquatic plants in the natural wetlands ecosystems play a relevant role in the removal of pollutants (Williams, 2002).

1.2 Wetland ecosystems

Wetlands are land areas wet during a part or all the year definable as the interface zones between freshwater and soil (Srivastava et al., 2017). The complex wetland ecosystem is based on the interaction between vegetation, microorganisms, animals, soil and water. In recent years the characterization of natural wetlands gained center stage in biological science owing to their contribution to the process of phytodepuration, whereby polluted sites are reclaimed to their natural

status through the use of plants (Stout and Nüsslein, 2010; Faußer et al., 2012; Gupta et al., 2012; Sharma et al., 2013).

Interestingly, artificial wetlands, mimicking the processes occurring in natural environments, have been designed and engineered to be used as a low-cost useful technology for wastewater treatment (Mthembu et al., 2013; Chong-Bang et al., 2010; Yongjun et al., 2010). More precisely, these artificial systems exploiting the natural processes of phytodepuration have been effectively used to remove pollutants from municipal, industrial, livestock farming wastewaters and from mine drainage (Basker et al., 2014; Stefanakis et al., 2011).

However, although the effectiveness of phytodepuration in such natural or recreated wetlands has been widely demonstrated, the process is not fully understood. Consequently, this knowledge gap is currently hampering rational biotechnological manipulations of phytodepuration processes to improve the water depuration efficiency.

1.3 The phytodepuration process

Phytodepuration is the process based on the combined action of aquatic plants and microorganisms which results in the removing of contaminants from water and sediments and finally in the improvement of water quality in natural and artificial wetlands (Dominguez-Patino et al., 2012).

1.3.1 The role of plants

A pivotal role for plants in phytodepuration processes has been reported in many informative overviews addressed both land and wetland plant species (Zhang et al., 2010; McCutcheon and Jørgensen, 2008; Williams, 2002; Dietz and Schnoor, 2001; Macek et al., 2000; Susarla et al., 2002). Plants have shown the capacity to withstand relatively high concentrations of contaminants without toxic effects (Zhang et al., 2010). They can uptake some chemicals as nutrients (i.e., N, P) and in some cases quickly convert toxic compounds to less toxic metabolites (i.e., phytotransformation) (Shelef et al., 2013; Zhang et al., 2010). Moreover, they release root exudates and enzymes which stimulate the degradation of the organic chemicals in the rhizosphere (i.e., rhizosphere

bioremediation) and represent a source of organic carbon for the microbial metabolism (Zhang et al., 2010). Also, an important role of plants was recognized in the uptake and recovery of metal contaminants into above-ground biomass (i.e., phytoextraction). Likewise, plants can act as a 'biological filter', sequestering at the root-soil interface water pollutants (i.e., rhizofiltration) (Zhang et al., 2010). Finally, it has been demonstrated that plants can 'stabilize' contaminated sites by reducing the risk of soil erosion and increasing the water evapotranspiration flux, both useful strategies to reduce the risk of contaminant dispersal to other sites (i.e., phytostabilization) (Zhang et al., 2010).

Regardless of aforementioned specific processes, phytodepuration in wetlands systems can be summarized as the net outcome of both direct and indirect interactions between plant roots and microorganisms. Indeed, the capability of the root system to oxygenate the sediment was demonstrated as a key in sustaining the metabolic activities of aerobic microorganisms such as the rhizobacteria (Faußer et al., 2012).

Interestingly, aquatic plants, mainly of the order Poales, Cyperaceae, Juncaceae, Typhaceae as well as other monocots, have evolved dedicated aeration systems which run through all plant organs (Brix et al., 1992). These include belowground rhizomes interconnecting individual plants (Klimešová and Čížková-Končalová, 1996). Anatomically, a specialized plant tissue, designated aerenchyma, empowers wetland plants to channel oxygen to submerged tissues and, at the same time, to partly oxygenate the rhizosphere surrounding belowground organs (Armstrong et al., 2000; Colmer, 2003). At the molecular level, this task is accomplished through the mechanism of radial oxygen loss (ROL) (Colmer et al., 2006; Matsui and Tsuchiya, 2006, 2008). The oxygen released via ROL in the rhizosphere underpins, at least in part, the biochemical reactions, catalyzed by both plants and microorganisms, degrading and recycling (into plant nutrients) phytotoxic compounds. Furthermore, the root system offers a wide surface to host microorganisms and, through the diffusion of exudates and other organic compounds, stimulates the degradation of pollutant by resident microorganisms (Trapp and Karlson 2001; Trapp et al., 2007).

The plant species more represented in the natural wetlands, and therefore more utilized in phytodepuration applications, are *Phragmites* spp. and *Typha* ssp. These plants can adapt to different abiotic conditions and, therefore, have a worldwide diffusion (Bellavance and Brisson, 2010; Li et al., 2013). In addition, these are perennial plants capable of performing the water cleaning process in the site of their rooting all year round (Tsyusko et al., 2005; Srivastava et al., 2014; Bonanno and Cirelli, 2017; Eller et al., 2017; Mthembu et al., 2013) and finally, thanks to a rhizomatous propagation, can promptly colonize wetlands areas (Dhir, 2013; Juneau and Tarasoff, 2013).

1.3.2 The microbial involvement

In the wetland ecosystems a wide range of microorganisms is commonly observed as detrital microbial mat, biofilm, and planktonic-microalgal-bacterial assemblages (Battin et al., 2003; Srivastava et al., 2017). These microorganisms contribute substantially to the nutrient cycling (e.g., nitrification, denitrification, sulfate reduction, methanogenesis, metal ion reduction or oxidation) and energy flow (Srivastava et al., 2017). In particular, the presence of biofilm microbial assemblages has commonly been detected on different plant surfaces, such as the leaves of submerged plants, in the rhizosphere on sediment and, more often, on the root surface, i.e. the area identifiable as rhizoplane (Srivastava et al., 2017; Giaramida et al., 2013; Calheiros et al., 2009). The tight and preferential association of microorganisms forming biofilm on the root surface suggests a functional interaction of microbial cells with the plant roots and with roots products diffused in the surrounding. For example, it has been proposed that oxygen and root exudates (carbon compounds) can be "traded" by the plants to fuel the microbial metabolism needed to degrade phytotoxic compounds (Srivastava et al., 2017). Consistently, this has recently been demonstrated by Srivastava et al. (2017) who reported the capability of the aquatic plant associated biofilm to degrade the algal-derived organics, i.e. chiefly amines, aliphatic aldehydes and phenolics (Simpson, 2008) and to use such algal derived carbon to grow and multiply efficiently (Gasol and Duarte, 2000). Moreover, the microorganisms constituting the plant associated biofilm have been demonstrated able to degrade the dissolved organic matter (DOM) (Tranvik, 1998) such as PCBs (poly-chlorinated biphenyls) (Ghosh et al., 1999) and atrazine (Guasch et al., 2007). The rhizoplane of aquatic plants resulted also being enriched for ubiquitous methanotrophs (α and γ proteobacteria) which use methane as carbon source for their metabolism removing it from the aquatic ecosystem (Semrau et al., 2010). Moreover, thanks to particulate methane monooxygenase (pMMO), some bacterial species (e.g., *Methylosinus trichosporium*, *Methylococcus capsulatus*) resulted able to degrade a wide range of others toxic organic compounds and among them especially chlorinated ethenes (Pandey et al., 2014), via a cascade of enzymatic reactions which end with CO_2 as terminal product.

As reported from Hansel et al. (2001) and Carranza-Álvarez et al. (2008) microorganisms arranged as biofilms on the root surface perform also an important role in the removing of metal pollutants from water bodies. Sub-toxic levels of metals usually identified in wetlands can be the result of natural leaching of soil and sediments (Srivastava et al., 2017). However, these metals can reach toxic level when they are introduced into water bodies from industrial, agricultural and

municipal wastes (Zhou et al., 2008; Hansel et al., 2001; Carranza-Álvarez et al., 2008; Srivastava et al., 2017).

Interestingly, King and Garey (1999) and Hansel et al. (2001) reported that a consistent proportion of metal cations in water adheres to the negatively charged EPS of microbial biofilm matrix forming metal plaques around the roots of aquatic macrophytes and around all submerged plant parts. Through this mechanism, metals are sequestered from the water body and, not less important, the presence of plaques prevent other metals to enter and accumulate up to toxic level into plant tissues (Srivastava et al., 2017). Iron plaques are commonly detected around the roots of aquatic macrophytes and although their formation is principally due to iron oxidation process mediated by molecular O_2, their presence is more consistent when iron oxidizing bacteria such as *Ferroplasma* sp. and *Leptospirillum ferroxidans* are detected on the root surface (King and Garey 1999).

Sulfate reducing bacteria constituting biofilm on aquatic macrophytes roots also contribute to the metals removing process. Reducing sulfate in sulfides, they determine the lowering of water pH to values required from microbial cells to bioabsorb the metal ions from water (Han and Gu, 2010). Moreover, the hydrogen sulfide produced from these microorganisms reacts with metal ions and forms metal sulfide, which under acidogenic conditions precipitates sequenstring metal ions from water body (Webb et al., 1998; Machemer and Wildeman, 1992).

Together, these experiments clearly point to an active involvement of microorganisms in the phytodepuration process and an evident effective interaction of microbes with plants, particularly evident for microorganisms constituting biofilm on the rhizoplane. However, the knowledge about the entire microbial community underpinning the phytodepuration process and the factors which influence its composition are still incomplete.

These are becoming crucial questions in this research field, as advances in sequencing technologies and computational analysis have confirmed that plants are not autonomous entities but rather are sites of colonization for a myriad of microorganisms, collectively referred to as the plant microbiota, whose interactions at given plant sites define distinct biomes, the plant microbiome (Schlaeppi and Bulgarelli, 2015).

1.4 The plant microbiota

Microorganisms colonize almost all ecological niches. Plants represent, as the rest of most multicellular eukaryotic organisms, effectual provider of nutrients for microorganisms and thus result good hosts for them.

Along the plant structure diverse abiotic factors such as temperature, moisture, oxygen availability, wind exposure, etc. interact with biotic factors, such as the wide range of compounds produced by plant cells, creating outside and inside plant tissues different microhabitats for microbial colonization, epiphytes and endophytes respectively (Schlaeppi and Bulgarelli, 2015). One of the most characterized plant microhabitats is the rhizosphere, where microorganisms are associated to the thin soil layer around roots under the influence of numerous plant exudates (Walker et al., 2003). Instead, the rhizoplane microhabitat hosts microorganisms which live in a more tight interaction with roots, adhering on their surface. Then, endosphere is identifiable as the microhabitat which permits the microbial survival inside the plant tissue and finally the phyllosphere, the microhabitat colonized by microorganisms which proliferate on the stem and leaf surface (Hardoim et al., 2008; Lindow and Brandl, 2003).

Microorganisms interact with the host as their pathogens, mutualists or commensals. Although for economical reason the pathogens were the most studied ones, in the last decades great attention has been paid to other plant-associated microorganisms, especially after the demonstration of their important role in maintenance of plants health and for the improvement of their growth. Well know is the beneficial action of mutualists such as rhizobia for leguminous plants or of nitrogen fixing bacteria for their hosts (Masson-Boivin et al., 2009). Yet, in recent years also commensals, which by definition do not advantage the host plant, have been suggested as indirectly implicated in the plant protection and development under specific conditions.

Since it seems that plants can count for their survival not only on their own genes, but also on the accessorial pool of microbial genes as an “extended" trait to reach the adaptation to the environment, the plant and its microbiota are considered as an unique entity, an holobiont, whose genome, the holobiome, is composed both by plant genome and microbiome subjected to a mutual evolution process (Theis et al., 2016).

Effectively, the coevolution of plant host with its associated microbial community defines for each plant a consistent microbiota which differentiate it at specific and subspecific levels (Hartmann et al., 2009). The advantages obtained from the coevolution of plant genome and the associated microbiome appear clear since many plant hosts have shown their tight interaction with individual

members of microbiota. This is the case of Plant Growth Promoting Rhizobacteria (PGPRs) which are able to confer protection to plants against pathogens and to enhance plant's capabilities to uptake nutrients from soil (Lugtenberg and Kamilova, 2009). Specifically, it has been demonstrated that rhizosphere bacteria can contribute to the plant uptake of scarcely mobile minerals, such as iron and phosphorus, the biochemical fixation of atmospheric di-nitrogen into ammonia, the production of phytohormons, such as Indole Acetic Acid, and other signaling compounds such as volatile compounds (Berendsen et al., 2012, Turner et al., 2013, Lugtenberg and Kamilova, 2009; Yang et al., 2009; Berg et al., 2014). Likewise, non-pathogenic rhizobacteria can trigger plant immune responses in distal organs, a process known as Induced Systemic Resistance, and compete, either directly or indirectly, with pathogenic bacteria for the colonization of the root-soil interface (Reviewed by Bulgarelli et al., 2013, Figure 1).

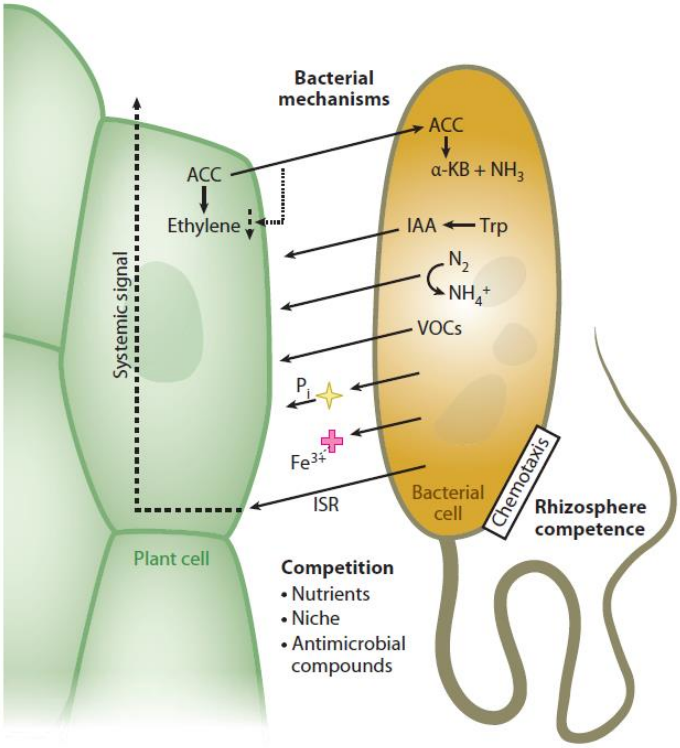

FIGURE 1. Biochemical mechanisms by which rhizobacteria influence plant growth and health (from Bulgarelli et al., 2013).

Therefore, this positive influence of microbiota on plant could be considered an important manipulation target for crop improvement and management.

Yet, the knowledge about the composition of microbiota of major part of plants remains limited as well as the understanding of the molecular interaction between constituting

microorganisms with plant host. Moreover, about the picture of other abiotic and biotic determinants of the structure and function of the plant microbiota is far to be completed (Schlaeppi and Bulgarelli, 2015; Waldor et al., 2015).

In addition, the biological complexity of existing ecosystems makes it at the same time difficult and fascinating to infer general principles of plant-microbiota interactions (Alegria Terrazas et al., 2016). On these assumptions, the characterization of the microbiota of land plants has been gaining momentum both in basic and translational science (Hacquard et al., 2015). Yet, elucidating the functional significance of aquatic plants microbiome can be considered a research field in its infancy (Bowen et al., 2017; Cerri et al., 2017).

Consequently, one of the principal research targets in environmental science is the possible manipulation of the microbiota as eco-friendly strategy to increase the phytodepuration efficiency, an approach expressly similar to the one proposed to sustainably increase crop production and reduce the input of chemicals and the emission of greenhouses gases in natural ecosystems (Bakker et al., 2013; Adesemoye et al., 2009; Singh et al., 2010; Berg et al., 2014).

Thanks to its important contribution to the plant survival and development, the microbiome, as previous specified, may be considered a second plant genome whose composition is actually strongly influenced by the plant genome (Turner et al., 2013; Berg et al., 2014). Yet, only for a limited number of species the first principles underpinning the important interaction between plants and their microbiotas have been defined, whereas for the majority of other plants, the intertwined relationship host-microbiome is still poorly characterized (Turner et al., 2013). Moreover, also the abiotic conditions of different microenvironments such as temperature, soil properties, moisture, pH, and nutrients amount influence broadly, directly and indirectly, the composition of plant microbiota. According to that, also microbiomes associated with different compartments of the same plant such as above-ground, below-ground and internal tissues compartments are distinct from each other (Berg et al., 2014; Turner et al., 2013; Berg and Smalla, 2009). However, although microorganisms colonize most plant compartments, since the well demonstrated influence of soil habitat on plants productivity and the necessity to adapt the plant cultures to different soil conditions, the larger part of researches targets to the characterization of rhizosphere microbiome (Berg et al., 2014; Alegria Terrazas et al., 2016). Only few other plant compartments have been studied in this respect (Vorholt, 2012) and, with few notable exceptions (Edwards et al., 2015), the rhizoplane microbiota received limited attention.

1.5 How to study the plant microbiota

The study of plant microbiota ultimately aims at identifying the structural and functional composition of the entire microbial community associated to different plant compartments. Since each microbial community is constituted by a wide range of microorganisms which interact with each other and with the host, both culture- dependent and -independent approaches are required to capture as much as possible of the microbiota diversity (Turner et al., 2013).

1.5.1 Culture-dependent approaches

The culture-dependent approach for studying the microbial community associated to plant compartments involves the isolation of microorganisms from specific microenvironments on artificial media. Fundamental and critical in this type of studying approach is the choice of adequate media to use as substrates for the microorganisms growth. The useful and commonly applied strategy is the use of media which mimic as much as possible the environmental conditions of the ecological niche which microbes live in. However, no medium can perfectly reproduce all the abiotic and biotic factors and their interactions influencing the microbial life and survival. Historically, this technique has been considered limited by the fact that many soil bacteria, from which the plant microbiota is largely assembled, were considered recalcitrant to in-vitro cultivation: it was estimated that only a limited portion of microorganisms (i.e., less than 1%) can be obtained in pure culture through classic microbiology culture methods (Vieira and Nahas, 2005). However, recent breakthrough discoveries are challenging this vision and culture dependent approaches are regaining center-stage in the characterization of the plant microbiome (see paragraph 1.5.3).

1.5.2 Culture-independent approaches: DNA sequencing-based methodologies

Since the late 1970s the development and continuous implementation of culture-independent techniques targeted to the study of microbial phylogenetic markers, combined with the more recent advancement in computational analysis has permitted a pronounced outburst in characterization of

microbial communities associated to a wide range of different environments (Schlaeppi and Bulgarelli, 2015). In particular the introduction of high throughput sequencing (HTS) technologies which perform the sequencing of multiple DNA molecules in parallel, it is now possible the obtainment of thousands to millions of sequences in more than one sample at a time, revealing the abundances of even rare microbial species (Bentley et al., 2008; Margulies et al., 2005; Turner et al., 2013). Combined to this, the availability of many open source tools for data analysis has favored the rational organization and classification of complex sequencing data in consistent sequencing datasets (Caporaso et al., 2010; Meyer et al., 2008). Not less important, the development and availability of public databases where sequencing data are constantly annotated and implemented has facilitated much more the expansion of knowledge about a wide range of microbiotas (Cole et al., 2014; DeSantis et al., 2006; Fish et al., 2013), also permitting the comparison between microbial communities associated to different environments and the reduction of analysis costs.

These sequencing-based methodologies can be actually classified in the methods indicated as targeted amplicon sequencing and the metagenome approach. The target amplicon sequencing is applied to a specific set of genes of the studied microbial community, whereas the metagenome approach is aimed at providing a general overview about the functional role of microorganisms in a microbiota through the study of genes considered as genetic markers for specific functions and metabolic capabilities (Alegria Terrazas et al., 2016; Turner 2013). Since both approaches provide fundamental information to deeply understand the composition and organization of microorganisms in the whole community, the preferential strategy to study the microbiota should be the combination of both approaches. However, the application of such methods is often made difficult by the diversity and complexity of studied environments. In addition to the fact that the existing environments are all constituted by numerous different niches for microbial colonization and thus are associated to a multitude of microbiotas, each environment is also characterized by an intrinsic complexity due to the fact it is populated by organisms belonging to all domains of life (Turner et al., 2013, Alegria Terrazas et al., 2016). This means that sequencing-based methodologies must be set up and adapted for the study of microbes as diverse as multicellular eukaryotes (e.g., fungi), prokaryotes and viruses. Therefore, usually the characterization of the entire microbiota derives from the characterization of all portions of microorganisms represented in that. However, the prokaryotic and eukaryotic portions of microbiota represented by categories of bacteria, archaea and fungi are the most investigated ones for their supposed and in some cases demonstrated contribution to the maintenance of plant health and development (Turner et al., 2013, Alegria Terrazas et al., 2016).

1.5.2.1 Targeted amplicon sequencing for the characterization of the microbiota

The targeted amplicon sequencing approach is the most applied strategy to study the microbiota. This technique allows scientists to identify the members of microbial communities or to compare the microbiota composition in different samples through the investigation of known phylogenetic markers. These phylogenetic markers permit the taxonomical classification of microorganisms grouping them on the basis of their phylogenetic similarity (Knief, 2014; Alegria Terrazas et al., 2016; Turner et al., 2013). The prokaryotic and eukaryotic genes encoding for the small subunit of ribosomes are the most commonly used phylogenetic markers in targeted amplicon sequencing surveys. These ribosomal genes are characterized by large sequence regions with a slow attitude to incur in sequence modifications thus only slowly subjected to evolutionary changing. Yet, these genes contain also regions with high attitude of being subjected to sequence modifications and to DNA evolving, indicated for this reason as hyper-variable regions. These aspects made the genes encoding for small ribosomal subunits the optimal candidates for the microbial phylogenetic study. Firstly, the presence of conserved sequences within microorganisms permits the usage of a wide range of PCR primers which can perform efficiently the amplification of the phylogenetic markers of all the different members of microbiota. Next, the sequence modifications which occur in the hyper-variable regions differentiate the microbes from each other generating 'molecular fingerprints' useful to their discrimination at a far more detailed taxonomic level. For these reasons, the 16S rRNA gene sequencing approach has been the method preferred to study the composition of bacterial and archeal communities since many decades, resulting in the availability of a large *in silico* dataset about 16S rRNA sequences (Schlaeppi and Bulgarelli, 2015). On the same principle, the 18S rRNA gene, which encodes for the small ribosomal subunit in eukaryotes, is used as phylogenetic marker to discriminate specific members of the microbiota, such as oomycetes, protists and nematodes. However, when the fungal community is the investigation target, the profiling of Internal Transcibed Spacer (ITS) is often applied. This exception for fungi is due to the fact, that the gene encoding for the fungal small ribosomal subunit is characterized by a short sequence subjected to the rapid mutation of DNA and this makes difficult its usage as phylogenetic marker. However, the region comprised between genes encoding for ribosomal subunits, this named ITS, presents the same characteristics of the other phylogenetic markers, i.e. the same proportion between conserved regions and hypervariable sequence (Alegria Terrazas et al., 2016).

Regardless to the target of PCR amplification, the principal limitation of this approach is the choice of primers which mainly influences the outcome of analysis. For this reason, each targeted amplicon sequencing procedure should pass through a preliminary set up of amplification protocol testing the amplification efficiency of diverse primer pairs before being applied to a full-scale analysis (Walters et al., 2015). Moreover, in the specific case of the study of microbiota associated with plants the majority of primer pairs generates also the undesired amplification of not targeted plant sequences together with the desiderated microbial ones. More precisely, since of their sequence similarity with sequence regions of 16S rRNA gene, host-derived plastidial and mitochondrial sequences are normally obtained as "contaminants" among the microbial amplicons pool, thus they need to be filtered from the final dataset to provide a realistic characterization of plant microbiota. The proportion of host derived "contaminant" sequences could be also reduced through suitable modification of PCR protocol regarding for example PCR steps temperature setting, number of amplification cycles and obviously the choice of primers pair which produce the lowest interfering amplification of host sequences (Lundberg et al., 2013). Intuitively, this fine tuning of PCR protocol is especially required for the study of endophytic microbiota since in this case the starting samples for the microbiota analysis are inevitably composed of a large portion of plant material and tissues.

The filtering of contaminant sequences and the analysis of entire sequencing dataset is normally conducted through *in silico* analysis. This process is based on the prior analysis of sequence quality and length, and the removal of all possible PCR artifacts. Subsequently, the highquality sequences are assigned to their source samples and clustered into Operational Taxonomical Units (OTUs) which identify closely related microorganisms, whose 16S rRNA gene sequences present a 97% identity threshold. Basically, OTUs can be considered the individual community members in amplicon sequencing surveys. Moreover, since each OTU is associated to a specific taxonomic classification, the pool of obtained OTUs permits to reconstruct the taxonomical classification of the entire microbial community associated to the investigated plant microbiota. Also, the association of sequences to specific OTUs simplifies the filtering process of those sequences belonging to host plastidial and mitochondrial DNA. The handling of obtained huge amount of sequencing data and the matching of enormous number of sequences with the available database of OTUs are made possible by the use of continually implemented software for the investigation of microbial ecology, such as QIIME (Quantitative Insights Into Microbial Ecology), the most employed one (Caporaso et al., 2010, 2012). Subsequently, after definition of OTUs constituting the studied microbiota, bioinformatics tools are often used to perform the statistical analysis of the OTUs properties such as their presence/absence, their relative abundance in the microbiota and whether these statistical parameters

are significantly associated to any given biotic or abiotic factor putatively influencing microbiome composition.

1.5.2.2 Metagenomic approaches for microbiome investigations

The metagenome approaches for studying the microbiome aim at identifying the functional genes detectable in the microbial community. Since the genes investigated are related to specific metabolic functions, the metagenome is the way to determine the putative role of microbes within the microbial community. This type of investigation can be conducted through the approach named gene-targeted metagenomic, a reduced complexity approach focused on the amplicon sequencing of genes encoding only for specific functions used as functional markers. This method is performed similarly to the one dedicated to the phylogenetic markers, but it results in the obtainment of specific database regarding the functional markers of interest in the considered microbiota (Fish et al., 2013, Alegria Terrazas et al., 2016).

Conversely, a methodology designated shotgun metagenomic approach aims a targeting of the totality of DNA collected from the studied environment. This approach is combined with dedicated *in silico* analyses aimed at annotating (both taxonomically and metabolically) and characterizing the putative function encoded by the sequenced material. Similar to the amplicon sequencing approach, the existence of open-access, dedicated analytical servers, such as the widely used MG-RAST (Meyer et al., 2008) are streamlining the associated analytical strategies.

However, this shotgun metagenomic suffers from some intrinsic limitations. In particular, the difficulty in defining the suitable sequencing depth (i.e., number of reads) per individual samples. Likewise, the high number of replicates required to obtain representative results contribute to inflate the costs of the analysis including those for the larger downstream computational effort (Knight et al., 2012).

Another approach to study the microbiome is the predictive metagenomic. This method aims at inferring the composition of a microbial community through available phylogenetic marker information (e.g. 16S rRNA gene profiles). This is achieved by deriving microbial metabolic capacities from database containing the entire genome of microorganisms associated to the same phylogenetic markers. The predictive analysis of microbiomes from samples associated to different niches is often performed through dedicated bioinformatics tools, such as the widely used PICRUSt

(Langille et al. 2013). This software predicts the functional composition of a microbial community from its 16S rRNA profile. The PICRUSt analysis process is organized onto two steps, the step of "gene content inference" and the step of "microbial inference" (see material and methods for details). The clear advantage of this method is that it is conducted fully *in silico* starting from the phylogenetic marker profiles which, nowadays, are routinely obtained in many laboratories. Conversely, the intrinsic limitation is that is based on an algorithm and probabilistic calculations, therefore it could fail to precisely characterize the functional genes in the microbiota (Bulgarelli et al., 2013). Nevertheless, it remains a useful preliminary analysis to evaluate the rate of diverse functions in a microbiota, to compare the functions detected in different microbiotas, understanding how much they differ from each other and also to establish the next steps of analysis (Alegria Terrazas et al., 2016). Figure 2 summarizes the main steps in cultivation-independent analysis of the plant microbiota and microbiome.

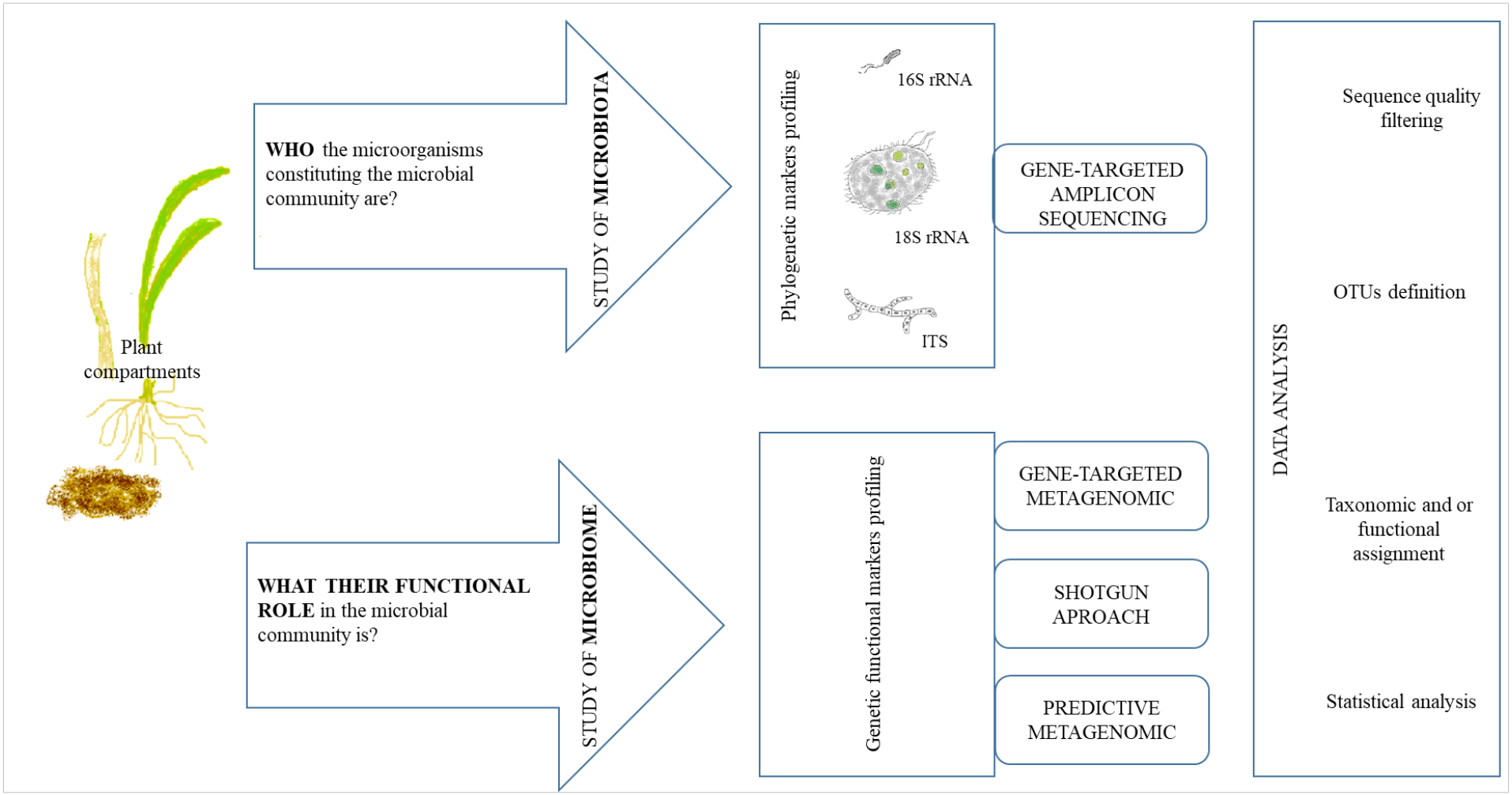

FIGURE 2. Cultivation-independent methods to study the plant microbiota and microbiome (modified from Alegria Terrazas et al., 2016)

1.5.3 Establishing causality: the emergence of Synthetic Communities (SynComs) of the plant microbiota

One of the perceived barriers severely impairing the advancement of this research field was the fact that the plant microbiota is represented by soil-dwelling bacteria (Bulgarelli et al., 2013) that, historically, have been considered recalcitrant to *in vitro* cultivation: it has been estimated that a very minor proportion, often less than 1%, of soil bacteria can be readily isolated on microbiological media (Vieira and Nahas, 2005). However, the realization that a) the plant microbiota is akin to a gated community, whereby a limited number of microorganisms can successfully thrive and b) these microorganisms are largely represented by members of taxa routinely isolated in laboratory (e.g., Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Hacquard et al., 2015)) challenged the paradigm of (un-)culturability of plant-associated microbial communities. For instance, a breakthrough study recently revealed that the majority (~58%) of the *Arabidopsis thaliana* root microbiota, identified in sequencing surveys, can be recapitulated in a bacterial collection (Bai et al., 2015). The establishment of such indexed bacterial collections is a fundamental pre-requisite allowing scientists to combine isolated members of the microbiota into 'microbial consortia' of known composition, designated Synthetic Communities (SynComs). These can be transplanted into germ-free plants and their impact on given plant phenotypes properly discerned. Combined with whole genome information, this is a powerful tool to formulate testable hypotheses and gain novel insights into plant-microbiota interactions. Perhaps not surprisingly, SynComs are taking center stage in microbiota science. In *A. thaliana*, SynComs have successfully been adopted to identify the host genetic determinants of the leaf-associated communities (Bodenhausen et al., 2014), demonstrate that components of the plant immune system shape the root microbiota (Lebeis et al., 2015) and infer the contribution of the microbiota to phosphorus starvation (Castrillo et al., 2017).

Operationally, the application of the SynComs approach is strictly dependent from a) the definition of the microbiota composition through cultivation-independent approaches, such as the 16S rRNA gene sequencing for bacteria and archaea, and b) the direct isolation of strains from the plant specimens. Although it results often difficult to translate OTUs to strains due to the variability within OTU (97% sequence identity for definition), selecting multiple strains per OTU can overcome this limitation (Callahan et al., 2016; Tikhonov et al., 2015). Therefore, both cultivation-independent and dependent approaches are necessary to ultimately determine whether structural and functional configurations of the microbiota are causally related to the given plant phenotypes.

Despite experiments conducted with the model *A. thaliana* have greatly enhanced our understanding of the interactions between plants and their associated microbial communities, the existence of species-dependent recruitment cues for these microbes (Bulgarelli et al. 2013, Hacquard et al., 2015) as well as the distinctive environmental variables impacting on these interactions are now calling for dedicated investigations of the plant microbiota in diverse natural and managed ecosystems, such as wetlands.

2. OBJECTIVES

Many studies demonstrated the potential and importance of microbiota for land plant nutrition, maintenance of their health state and development. Moreover, whereas the microbiota sustains indirectly plant growth, is also directly active in metabolizing and mobilization of chemicals and pollutants from the surrounding, usually performing a synergic action in association with plants which are the final up-taker of metabolized products in/on their tissues. Also in wetlands the important involvement of microorganisms associated with plants in removing pollutants from water and sediments has been demonstrated, in the process usually indicated as phytodepuration. In particular, microorganisms which colonize the rhizoplane of aquatic plants and form biofilm assemblages on their root surface have revealed an interesting ability in removal of pollutants from wetlands. Yet, the wetland plants microbiota is a research field in its infancy and in particular the microbiota of rhizoplane compartment received less attention compared to the communities inhabiting the rhizosphere. Therefore, the composition of the microbial assemblages on the root surface and their real potential contribution to phytodepuration remain largely unknown. Earlier studies suggest that Proteobacteria dominate the root-soil interface of *Phragmites australis* and *Typha latifolia*. However, these studies were conducted with low-resolution techniques and this makes it difficult to infer general principles.

To gain novel insights into the functional significance of the wetland plants microbiota, this doctoral thesis presents:

- The characterization through Illumina MiSeq technology of the bacterial communities associated with the roots of *Phragmites australis* and *Typha latifolia* and comparison with the ones inhabiting the surrounding compartments, to understand the effect of microhabitat conditions in shaping the microbiome;
- A comparison between the two plants microbiotas to point out the role of plant species factor in recruitment of microorganisms at root surface;
- A predictive metagenomic investigation aimed at elucidating the functional potential of these communities;
- A Scanning Electron Microscopy (SEM) analysis to go insight the spatial organization of the rhizoplane microbiota on the root surface;
- The isolation of culturable portion of microorganisms from the rhizoplane of both plant species as a first step towards the establishment of SynComs for *P. australis* and *T. latifolia* and to test their ability to form biofilm *in vitro*.

Chapter 3

3. MATERIALS AND METHODS

3.1 Samples collection and preparation

From the wetland located in the naturalistic area of "Le Mortine Oasis" (Campania, southern Italy) five root systems of five *Phragmites australis* and *Typha latifolia* plants respectively were sampled in sterile bags (Figure 3). Also four surface water samples (1L) were collected in sterile bottles from sampling points distributed along the *Phragmites* and *Typha* rooting sites. The samples were immediately transported to the laboratory in a portable cooler at 4 °C. 1 g of the soil surrounding the *Phragmites australis* and *Typha latifolia* roots was collected in sterile Petri dishes and used to investigate the rhizosphere microbial communities. Four root segments (2 cm in length and 0.3-0.5 cm in thickness) were obtained from each plant system. Subsequently, the root segments were washed by shacking three times in 10 ml of sterile tap water, twice in 10 ml of sterile distilled water and once in 20 ml of sterile 0.85% NaCl (Kirzhner et al., 2009; Li et al., 2013).

FIGURE 3. Sampling. The sampling site in the natural area of "Le Mortine Oasis" (41°28'11.4"N 14°05'26.6"E) **(A)** and examples of *P. australis* **(B, left)** and *T. latifolia* **(B, right)** root systems sampled from the wetland.

3.2 Biomolecular analyses

Water samples (1L) were filtered through sterile mixed esters of cellulose membranes (S-Pak™ Membrane Filters, 47 mm diameter, 0.22 μm pore size, Millipore Corporation, Billerica, United States) and the DNA was extracted from the filters using the PowerWater® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, United States) following manufacturer's recommendations. The DNA samples generated from the wetland water (W1, W2, W3, W4) were stored at -20°C until further use.

0.5 g of each soil sample was subjected to the DNA extraction using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, United States) as the manufacture's producer recommend. DNA extracted from *Phragmites australis* (*P*r1, *P*r2, *P*r3, *P*r4) and *Typha latifolia* (*T*r1, *T*r2, *T*r3, *T*r4) rhizosphere was stored at -20°C until further use. Furthermore, three of the washed root segments were subjected to a double step ultrasound treatment using the Vibra-Cell™ ultrasonic processor VCX 130 (Sonics and Materials, Inc. Newtown, United States) set at the constant frequency of 20 kHz and at the amplitude of 30%, with a 6 mm probe. Firstly, the roots were sonicated in 10 ml of sterile sonication buffer (0.85% NaCl and 0.1% Tween 80) for 2 min and 30 sec in 15 ml Falcon tube, then the roots were transferred into 10 ml new sterile sonication buffer and subjected to a second ultrasound treatment in the same conditions, for 5 min. This procedure ensured through the first sonication step the removal of cells not firmly attached onto the root surface and the detachment and subsequent collection of the rhizoplane cells during the second step. After the ultrasound treatments, the roots were recovered and fixed using 3% glutaraldehyde in a 0.1 M phosphate buffer at pH 7.2 for 24 hours. Therefore, samples were washed three times using the same buffer and dehydrated through an ethyl alcohol series (30, 50, 70, 95, and 100%, for 5 min at each step). After dehydration, they were dried using an Emitech K850 Critical Point Dryer (Quorum Technologies Ltd, England, United Kingdom), mounted on aluminum stubs and coated with gold using an Emitech K550 sputter coater (Quorum Technologies Ltd, England, United Kingdom). Finally, the prepared samples were observed using a ZEISS DSM-940A Scanning Electron Microscope (Carl Zeiss, Jena, Germany) at 10 kV and 30x and 2000x magnification images were acquired. Instead, the suspension of rhizoplane cells was divided in two aliquots (5 ml) representing replicates of each sample (indicated as "a" and "b"). The 5 ml aliquots were brought to a final volume of 100 ml with sterile MilliQ water and filtered through sterile mixed esters of cellulose membranes (S-Pak™ Membrane Filters, 47 mm diameter, 0.22 μm pore size, Millipore Corporation, Billerica, United States). DNA was finally extracted from the filters using the PowerWater® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, United States) following the recommended protocol. More precisely, for each biofilm suspension two DNA samples were originated as replicates and they were indicated as "a" and "b" respectively. A total of 10 DNA samples were obtained from the rhizoplane of *Phragmites australis* (*P*1a and *P*1b; *P*2a and *P*2b; *P*3a and *P*3b; *P*4a and *P*4b; *P*5a and *P*5b) and *Typha latifolia* (*T*1a and *T*1b; *T*2a and *T*2b; *T*3a and *T*3b; *T*4a and *T*4b; *T*5a and *T*5b). The DNA samples were stored at -20°C until further use. DNA samples were quantitated using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, United States) and they were subjected to the amplification of the hypervariable V4 region of the 16S rRNA gene through a nested-PCR approach to generate amplicon libraries. The

PCR reactions were performed using Kapa HiFi HotStart PCR kit (Kapa Biosystems, Wilmington, United States) in a G-Storm GS1 Thermal Cycler (Gene Technologies, Somerton, United Kingdom). For the first amplification step the PCR mix contained 50 ng of DNA, 4 µl of 5X Kapa HiFi Buffer, 10 ng Bovine Serum Albumin (Roche, Mannheim, Germany), 0.6 µl of a 10 mM Kapa dNTPs solution, 0.6 µl of 10 µM solutions of the 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1392R (5'- ACGGGCGGTGTGTRC-3') PCR primers, 0.25 µl of Kapa HiFi polymerase and sterile MilliQ water up to the final volume of 20 µl. The reaction was performed with an initial denaturation at 94°C for 3 min, then 20 cycles of denaturation at 98°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 1 min and 30 sec and a final elongation step at 72°C for 10 min. The second amplification step was conducted using 2 µl of the first amplification product as template, 4 µl of 5X Kapa HiFi Buffer, 10 ng Bovine Serum Albumin (Roche, Mannheim, Germany), 0.6 µl of a 10 mM Kapa dNTPs solution, 0.6 µl of 10 µM solutions of the 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVG GGTWTCTAAT-3') PCR primers, 0.25 µl of Kapa HiFi polymerase and sterile MilliQ water up to the final volume of 20 µl. To generate the amplicon libraries both primers used in this PCR step presented flow cell adapter sequences at their 5' termini and the primers 806R also 12-mer unique 'barcode' sequences to provide the simultaneously sequencing of several samples (Caporaso et al., 2012). This PCR reaction was performed using the following conditions: initial denaturation at 94°C for 3 min, 25 cycles of denaturation at 98°C for 30 sec, annealing at 50°C for 30 sec, elongation at 72°C for 1 min and a final elongation step at 72°C for 10 min. Reaction negative controls (rNTCs) were generated in all the individual PCR reactions and for all the barcodes used in the second amplification. Furthermore, four no-template samples were amplified through both nested-PCR steps and thus they were tagged by their own barcodes in the second amplification step to be used as sequencing negative controls (sNTCs). 5 µl of amplified samples and controls were checked on 1.5% agarose gel. The samples which showed the expected size amplicon and whose rNTCs presented no detectable amplicon were used for the amplicons library construction. The four sNTCs were also used to generate the amplicons library. The amplicons and the sequencing negative controls (sNTCs) were purified using Agencourt AMPure XP kit (Beckman Coulter, Brea, United States) with a ratio of 0.7 µl AMPure XP beads per 1 µl of sample and then 3 µl of each sample were quantified using Picogreen (Thermo Fisher, United Kingdom) according to the manufacturer's recommendations. After that, individual barcode samples were pooled at equimolar ratios to generate the amplicon libraries. All library QC and processing was carried out by the Genome Technology group at James Hutton Institute (Invergowrie, United Kingdom) and high-quality libraries were run

at 10 pM final concentration on an Illumina MiSeq system with paired-end 2 × 150 bp reads for FASTQ file generation (Caporaso et al., 2012).

3.2.1 16S rRNA gene sequence data analysis

The FASTQ files obtained from the MiSeq machine were processed using the QIIME software version 1.9.0 (Caporaso et al., 2010). Firstly, forward and reverse files from libraries were decompressed and merged through the command join_paired_ends.py setting the minimum overlap of 5 bp between reads. Then the overlapping paired end (PE) reads were subjected to demultiplexing and quality filtering running the command split_libraries_fastq.py with a minimum PHRED score of 20. Subsequently, the high quality PE reads were matched with Operational Taxonomic Units (OTUs) at 97% sequence identity collected in the chimera-checked Greengenes database (DeSantis et al., 2006), version 13_5, using the "closed reference" approach. For the OUT-picking the SortMeRNA algorithm (Kopylova et al., 2012) was used. The singleton OTUs, OTUs associated only to a single read, were filtered *in silico* and using the command merge_otu_tables.py the OTU tables obtained from the two independent sequencing runs were merged to obtain an unique OTUs table. Then, from this OTUs pool were *in silico* filtered the OTUs assigned to host- derived sequences, i.e. plastidial or mitochondrial DNA. Through the command summarize_taxa.py, the taxonomy matrix correspondent to the OTUs table was generated. The taxonomy matrix, reporting the number of reads for each identified taxonomy, was finally merged to the OTUs table generating a unique file reporting identified OTUs with correspondent taxonomies and number of reads for each taxonomy in the individual libraries. This file has been used for the statistical analysis performed in R using the R Phyloseq package (McMurdie and Holmes, 2013) as follow described.

The alpha and beta-diversity calculations were performed for two samples sets in parallel, each one composed of all the rhizosphere and water samples plus respectively the first set of rhizoplane replicates (set1) or the second set of replicates (set2). Therefore, two independent OTUs tables were obtained. Firstly, low abundance OTUs were filtered from the datasets, referring to those OTUs observed for less than 25 reads in at least the 20% of samples. This represents a modification of an abundance threshold previously adopted for a comparable sequencing protocol applied to rice (Edwards et al., 2015). The adjustment has been conceived considering the characteristic of the obtained dataset, targeting to discard the poorly reproducible OTUs and retain the ones which mainly describe the microbiota composition. Then, the residual reads were rarefied at the sequencing depth

of 66,000 sequencing reads per sample. After filtering, we obtained 1,906 unique OTUs for the samples set1 and 1901 for the samples set2. For the alpha-diversity calculation, the richness within samples was evaluated through number of Observed OTUs and Chao1 index whereas the evenness was estimated through Shannon index using the function estimate_richness. Data were visualized using the function ggplot from the package ggplot2. For each dataset, the normality of rhizosphere and rhizoplane data distribution was evaluated applying the Shapiro–Wilk test through the function shapiro.test to evaluate the microbial diversity between the two more closely related microhabitats. We imposed the alpha level to infer whether the data tested were normally distributed establishing a p-value <0.01 for the richness parameters, i.e. Observed OTUs and Chao1 index, and a p-value <0.05 for the evenness calculation through Shannon index. For datasets whose Shapiro–Wilk test generated a p-value lower than the established alpha levels, and consequently resulted not normally distributed, a not-parametric analysis of variance was performed through Wilcox test, run by the function wilcox. test, to evaluate the microhabitat effect on the microbial diversity. For the beta-diversity calculation firstly the OTUs counts were transformed to relative abundance using the function transform_sample_counts and then running the function ordinate the distance between samples was calculated using both the Bray-Curtis index, which is sensitive to the OTU relative abundance only, and the weighted UniFrac index, sensitive to OTU relative abundance and also to phylogenetic assignment (Lozupone and Knight, 2005). Distance matrices were represented through principal component analysis (PCoA). In order to evaluate the effect of microhabitat and plant species on the samples distancing, the analysis of variance using the distance matrices was performed through the adonis function of the package Vegan and the p-values were calculated for 5000 permutations. Furthermore, a differential analysis of the count data was executed to identify individual bacteria differentially recruited between the rhizoplane and the rhizosphere of the two studied plants using negative binomial generalized linear models and the package DESeq2 (Love et al., 2014).

For each sample set the OTU count and sample information were collapsed to generate two DESeq objects using the function DESeqDataSetFromMatrix. Then, running the function DESeq the DESeq objects were subjected to the differential analysis to enumerate and identify the OTUs enriched in the rhizoplane of *Phragmites australis* and *Typha latifolia* respect to their rhizosphere.

Through the DESeq function we extracted as rhizoplane enriched OTUs only OTUs whose adjusted p-value in the considered comparison was <0.05 and fold change >0.

After that, we obtained for each plant two sets of enriched OTUs in the rhizoplane from the two samples sets. We considered as rhizoplane enriched OTUs of *Phragmites australis* and *Typha latifolia* only those resulted being enriched respect to the rhizosphere in both the samples sets. Then

we compared the rhizoplane enriched OTUs of the two plant species between each other and we enumerated and identified the conserved ones as the OTUs enriched in the rhizoplane of both plants. Therefore, to compare the proportion of enriched OTUs in the rhizoplane of each and both plants we generated a Venn diagram using the R package VennDiagram. The complete script used to perform the data analysis of the present study and to generate the related figures is available at https://github.com/BulgarelliD-Lab.

3.2.2 PICRUSt predictive analysis of metagenomes

The OTUs table generated using QIIME (software version 1.9.0) as describe above, was also used to perform the PICRUSt analysis (Langille et al., 2013). The OTUs table in biom format was uploaded through the directory “Get Data” into the online platform Galaxy Version 1.1.1 (http://galaxy.morganlangille.com/). Then, the uploaded OTUs table was set as input file into the panel of PICRUSt analysis dedicated to normalization step, thus the command 'Normalize by Copy Number' was run to correct the OTUs table for multiple 16S copy number setting the GreenGenes version “GG 13.5” as reference database. Subsequently, using the normalized OTUs table generated as output from previous step, the 'Predict Metagenome' command was executed to obtain the metagenome prediction, setting the GreenGenes version “GG 13.5” as reference database and the KEGG Ortholog as the type of functional prediction. This module produced a 'virtual' metagenome of KEGG Ortholog abundances for each sample in the given OTUs table. A text file containing accuracy metrics for the predicted metagenome has been also generated (NSTI values). As defined by Langille et al. (2013) NSTI represents the sum of phylogenetic distances of each organism of the OTUs table by the nearest relative from the reference genome. The phylogenetic distance for each organism is measured in term of number of substitutions per site in the 16S rRNA gene, weighted by the frequency of the organism in the OTUs table. Experimentally estimated values for this index defined the NSTI greatest values for phylogenetically diverse hypersaline mat microbiome (mean NSTI = 0.23 ± 0.07 s.d.), lowest values for the well-covered Human Microbiome Project metagenomes (HMP) (mean NSTI = 0.03 ± 0.02 s.d.), mid-range values for the soils samples (mean NSTI = 0.17 ± 0.02 s.d.) and varied for the mammals ones (mean NSTI = 0.14 ± 0.06 s.d.).

Figure 4 summarizes the PICRUSt workflow described here.

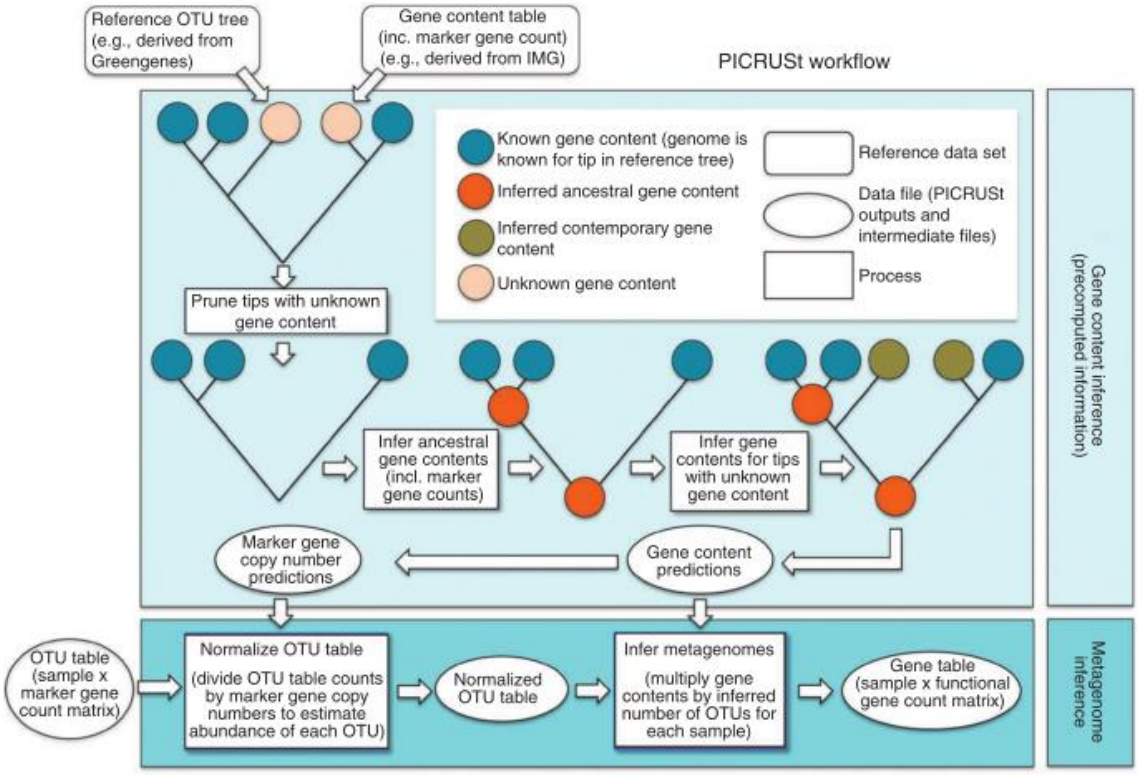

FIGURE 4. PICRUSt workflow. Predictive functional profiling of microbial communities through PICRUSt analysis (from Langille et al., 2013).

Finally, the generated metagenome prediction file was used for analyzing the metagenomic profile through the software package STAMP (Statistical Analysis of Metagenomic Profiles). STAMP permits to statistically analyze data regarding detected functions since it supports statistical hypothesis tests for pairs of samples or groups of samples along with a wide range of exploratory plots (Parks et al., 2014). Therefore, to infer the biological relevance of features in the metagenomic profile the exploration of statistical results and generation of plots were performed.

3.3 Culture-dependent approach

For studied plants a composite sample of five roots was generated pooling one washed root from each root system. The five roots sample was subjected to the ultrasound treatment following the same procedure described above. After the ultrasound treatment, the roots were removed and the rhizoplane suspension was divided in two series of 4 ml, 400 µl, 40 µl and 4µl aliquots. Sterile MilliQ

water was added to the aliquots to reach the final volume of 100 ml. The samples were filtered through sterile mixed esters of cellulose membranes (S-Pak^{TM} Membrane Filters, 47 mm diameter, 0.22 µm pore size, Millipore Corporation, Billerica, United States). The filters obtained were placed on 2% Nutrient (Difco-BD, Sparks, United States) and R2A (Lab M, Lanchashire, United Kingdom) agarised media and plates were incubated for 48 hours at 25°C and 37°C, respectively (Calheiros et al., 2009; Kirzhner et al., 2009). An aliquot of the samples (50 µl) was also directly spread without filtering on the surface of each medium and incubated in the same conditions. Because of the presumed complexity of the investigated microbial community and diverse nutritional requirements of constituting microorganisms, for their isolation two different media commonly used for isolation of microorganisms from water environments were simultaneously used. The Nutrient agar was used as nutrient medium to isolate the majority of nonfastidious microorganisms whereas R2A was used as low nutrient medium to reduce the growth rate of nonfastidious microorganisms permitting also the isolation of oligotrophic microorganisms otherwise overcome in nutrient medium (Mina et al., 2011; Kirzhner et al., 2009; Calheiros et al., 2009).

After incubation the colonies grown on the membrane surface were discriminated on the basis of their morphological characteristics and color. The selected colonies were picked and re-streaked onto 2% agarised TY medium (1,6% Tryptone, 1% yeast extract, 0,5% NaCl) to obtain pure cultures and to confirm the maintenance of distinct colonies aspect regardless to the medium they were isolated on. Then, colonies were inoculated in TY broth at 37°C in shaking condition (rpm 200) and culture aliquots were stored in 20% glycerol stocks at -80 °C originating a collection of rhizoplane isolates for each plant species. The isolates were tested for their ability to form biofilm *in vitro* through a modified Stepanović biofilm formation assay (Stepanović et al., 2004). They were statically grown over-weekend in TY broth at room temperature and subsequently their O.D. was measured using the spectrophotometer UV-1601 (SHIMADZU, Kyoto, Japan) at the wavelength of 600 nm. The cultures were diluted in triplicates to the O.D. of 0.2 in a final volume of 0.2 ml of TY broth in sterile 0.5 ml Eppendorf tubes. Controls were generated in triplicates using 0.2 ml of TY broth only. The replicates of each isolate and also the controls were incubated statically at 37°C for 24, 48 and 72 hours respectively. At the end of each incubation time the culture was removed and the tubes were washed three times with 300 µl of distilled water. The biofilm attached to the tube walls was fixed with 250 µl of methanol. After 15 min the tubes were emptied and dried under the laminar flow hood. The dried tubes were stained with 250 µl of 2% Crystal violet solution from the Gram staining kit (Biolife Italiana srl., Milano, Italy) for 5 min. The excess of stain was removed firstly using a pipette and then rinsing out under flowing tap water. The tubes were dried in upside down position under the laminar

flow hood. Subsequently, 250 µl of 33% (v/v) glacial acetic acid was added to redissolve the dye entered in the biofilm cells. The solution was transferred to a spectrophotometric 2 ml plastic cuvette and 33% (v/v) glacial acetic acid was added to reach the final volume of 1 ml. The O.D. at 570 nm was measured using the spectrophotometer UV-1601 (SHIMADZU, Kyoto, Japan) and compared to the O.D. measured for the controls. According to Stepanović et al. (2004) protocol and classification, the cut-off O.D. (O.D.c) was calculated as three standard deviations above the mean O.D. measured for the negative controls and the rhizoplane isolates were classified as: no biofilm producer when O.D. $\leq$ O.D.c , weak biofilm producer if O.D.c < O.D. < (2 · O.D.c), moderate biofilm producer when (2 · O.D.c) < O.D. < (4 · O.D.c) and strong biofilm producer in case of (4 · O.D.c) < O.D (Stepanović et al., 2004). Some of the isolates which resulted able to form biofilm *in vitro* test were identified. Their 16S rRNA gene was amplified and sequenced through BAct16S protocol at BMR genomics by University of Padova (Clarridge, 2004; Han, 2006). The obtained sequences were matched with the Greengenes database obtaining the identification for each considered isolate.

4. RESULTS

4.1 Composition of the prokaryotic communities associated to *Phragmites australis* and *Typha latifolia*

From the amplicon sequencing of 16S rRNA gene we obtained a total of 6,903,866 high quality reads. After *in silico* depletion of OTUs derived from plant mitochondrial and plastidial DNA the number of reads useful for downstream analysis decreased to 6,766,926 with a retaining percentage of 98%. In total we identified 15,436 operational taxonomic units (OTUs) at 97% sequence identity. To increase the efficiency of amplification of rhizoplane samples, we subjected all our specimens to a nested-PCR approach. However, this approach relies on a high number of cycles which might increase the proportion of PCR biases and spurious amplifications of environmental microbes (i.e., contaminations). To control for this source of variation, we decided to subject to sequencing also four no-template controls (sNTCs) and two sets of technical replicates of the rhizoplane specimens (hereafter, set1 and set2). We reasoned that a sequencing contamination would have been represented by a limited number of OTUs accounting for a large proportion of the sequences recovered from sNTCs samples. Consistent with our assumption, we identified 56 OTUs with a relative abundance equal or greater to 0.01% which accounted for the vast majority of the sNTCs sequencing profiles (>99%). Next, we pruned these OTUs, and their assigned sequences, from the entire dataset. Remarkably, after removal of contaminant OTUs, we were able to retain more than 97% of the initial high quality reads assigned to water, rhizosphere and rhizoplane samples indicating that the occurred contamination had a negligible impact on the profiling of the samples of interest.

4.1.1 Alpha-diversity calculations: the diversity within samples

In order to investigate the microbial diversity within samples the alpha-diversity at OTU level was evaluated considering the two sets of samples, each one composed of rhizosphere and water samples plus respectively the first set of rhizoplane replicates (set1) or the second set of replicates (set2). In detail, the OTUs richness was evaluated through the number of Observed OTUs and Chao1 index whereas the OTUs evenness was evaluated through Shannon index. Since the results obtained for the two samples sets were consistent with each other, only those regarding the first set are treated below; the results obtained for the other samples set are shown in Supplementary Figure 1. Therefore, the statistical analysis was performed comparing the rhizosphere and rhizoplane communities of the

two plants. The rhizoplane communities of *Phragmites australis* and *Typha latifolia* resulted being characterized by a mean value of Observed OTUs of 1,575 and 1,331 respectively; more interestingly, both the OTUs richness (Figure 5A, B) and OTUs evenness (Figure 5C) of the rhizoplane communities resulted significantly different and lower than the rhizosphere communities (Shapiro-Wilcox test with P>0.01 for Observed OTUs and Chao1 index, with P>0.05 for Shannon index). This result suggested clearly the specificity of the rhizoplane microbial community composition respect to the rhizosphere one for both the studied plants and, at the same time, it confirmed that the ultrasound treatment of root samples was effective to isolate selectively the rhizoplane microhabitat and microbial community.

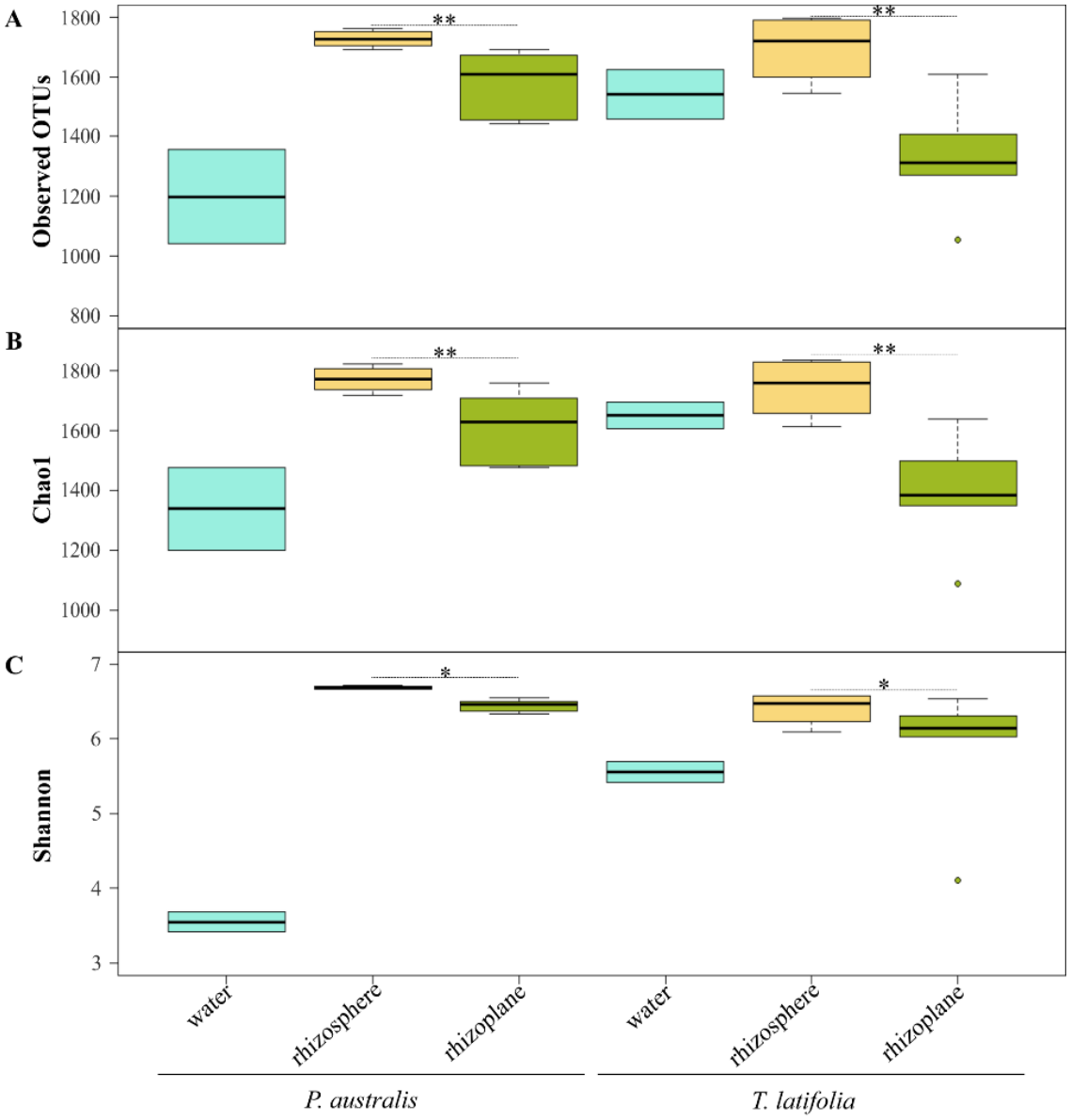

FIGURE 5. Alpha diversity calculation. OTUs richness of water, rhizosphere and rhizoplane microbiotas of *Phragmites australis* and *Typha latifolia* indicated by number of Observed OTUs **(A)** and by Chao1 index **(B)**. The OTUs evenness of the two plants microbiotas is shown by Shannon index **(C)**. Upper and lower edges of the box plots represent the upper and lower quartiles, respectively. The bold line within the box denotes the median. Maximum and minimum observed values are represented by the whiskers. Dots denote outlier observations whose value are 3/2 times greater or smaller than the upper or lower quartiles, respectively. Asterisks denote statistically significant differences between rhizosphere and rhizoplane microhabitats (**P < 0.01, * P < 0.05).

4.1.2 Beta-diversity calculations: the diversity between samples

Subsequently, the diversity between samples was evaluated through calculation of beta-diversity indexes. For both samples sets we generated Bray-Curtis distance matrix to evaluate the diversity on the base of OTUs relative abundance and then we also generated weighted UniFrac distance matrix which is sensitive at the same time to OTU relative abundance and phylogenetic classification. Also for the beta-diversity analysis, only the results regarding one samples set are treated below whereas the results obtained for the other samples set are shown by Supplementary Figure 2. The Bray-Curtis matrix showed a clear effect of species factor which produced the segregation of samples along the axis 2 in two well separated clusters including water, rhizosphere and rhizoplane samples of each plant species; however, the largest variation in OTUs relative abundance was observed along the axis 1 and it was due to the effect of microhabitat factor as confirmed by the values calculated for sources of variation (Figure 6A). Finally, the lower variation resulted being due to the interaction between microhabitat and species (Figure 6A). Instead, the weighted UniFrac distance matrix showed a more pronounced effect of the microhabitat factor and a lower effect of the species compared to the Bray-Curtis distance matrix. Finally, in this case the effect of the interaction between microhabitat and species resulted larger than the effect of species only (Figure 6B). These results suggest that, irrespective of the initial diversity of the starting inoculum, represented by the 'water' microbiota, the *Phragmites*- and *Typha*-associated microbiotas tend to converge towards a similar composition. Yet, the distinct host-associated habitats, the rhizosphere and the rhizoplane, exert their selection pressure on microbes which is further fine-tuned by the individual plant species.

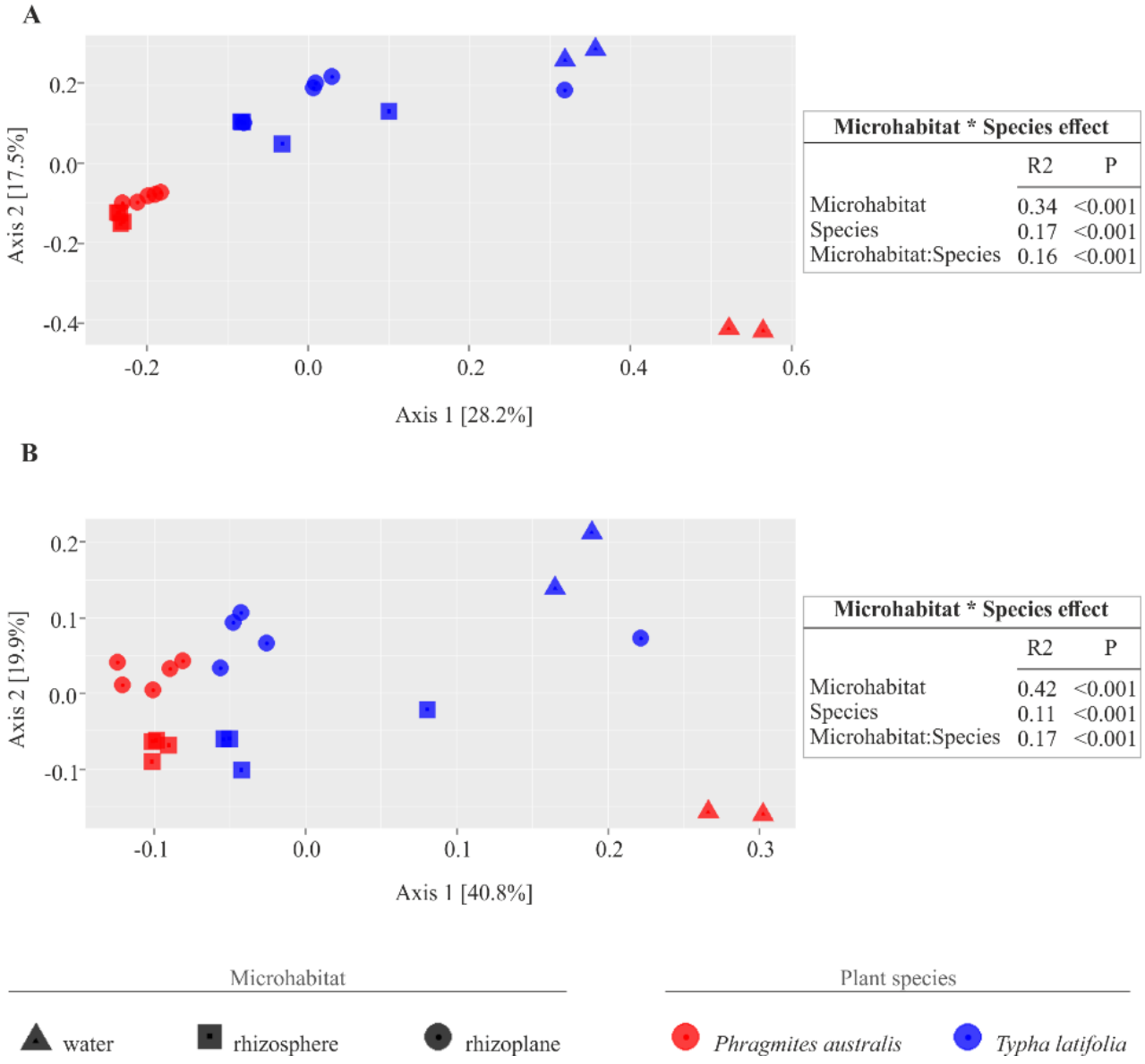

FIGURE 6. Beta-diversity calculation. On the left, the PCoA plots show the distance between samples calculated on the base of Bray-Curtis index sensitive to the OTUs relative abundance **(A, left)** and on the base of weighted UniFrac index sensitive to both OTU relative abundances and taxonomic affiliation **(B, left)**; colors indicate the plant species and shapes the microhabitats whom samples belong to. On the right, the permutational analysis of variances for the indicated sources of variation calculated for the Bray-Curtis **(A, right)** and weighted UniFrac **(B, right)** indexes. The R2 value shows the proportional effect of the indicated factors in the samples distancing and the P-values were calculated for 5,000 permutations.

4.2 The rhizoplane microbiota

Due to their intimate relationships with the host, the rhizoplane communities represented an attractive model to further characterize species-specific signatures of *Phragmites* and *Typha* on the microbiota. In detail, the OTUs significantly enriched in rhizoplane community respect to the rhizosphere were identified as determinants of rhizoplane microbial community diversification. Firstly, for each plant species we determined the number of rhizoplane enriched OTUs as the pool of OTUs shared between the rhizoplane replicates (set1 and set2): a total of 80 enriched OTUs for *Phragmites australis* rhizoplane and 71 for the *Typha latifolia* one were identified (CDS test, FDR<0.05 and log2FC>0) as reported in Supplementary Table 1.

Then, the identified enriched OTUs of *Phragmites australis* and *Typha latifolia* rhizoplanes were compared to each other to point out the OTUs enriched in both rhizoplanes and the differentially enriched ones: 20 OTUs resulted enriched both in *Phragmites australis* and *Typha latifolia* rhizoplane, whereas 60 OTUs were differentially enriched in *Phragmites australis* rhizoplane and 51 in the rhizoplane of *Typha latifolia* (Figure 7 and Supplementary Table 1).

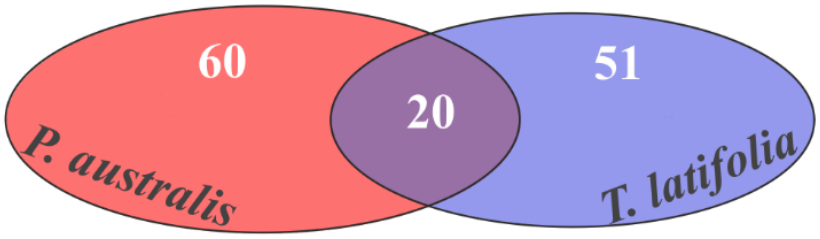

FIGURE 7. Rhizoplane enriched OTUs. Number of OTUs enriched in the rhizoplanes of both studied plants and of those ones resulted as differentially enriched.

Subsequently, we evaluated the taxonomical composition at phylum level firstly of all the enriched OTUs in the *Phragmites australis* and *Typha latifolia* rhizoplane microbiotas then of the conserved OTUs only, referring to the OTUs enriched in the rhizoplane of both plant species.

Therefore, among all the enriched OTUs of *Phragmites* and *Typha* rhizoplane a total of 9 and 7 phyla were identified, respectively (Figure 8).

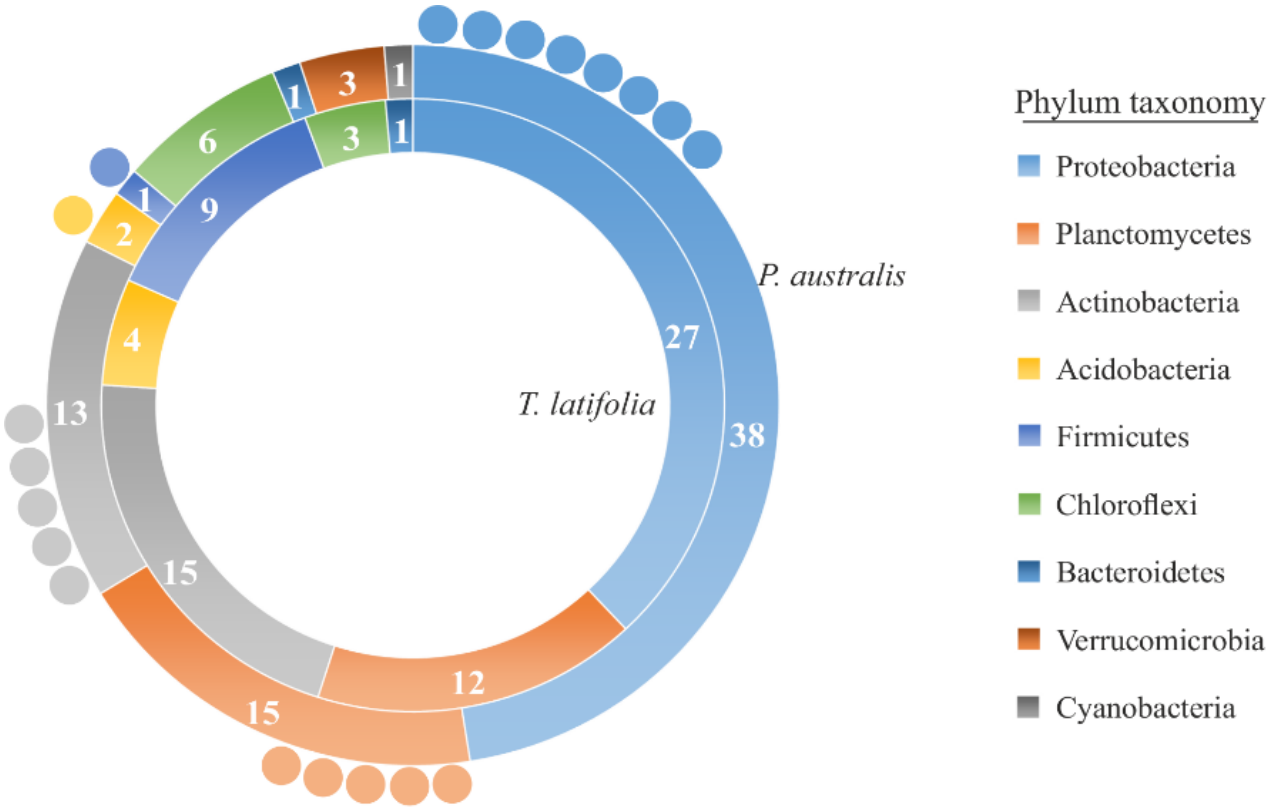

FIGURE 8. Phylum taxonomy of the OTUs enriched in the rhizoplane microbiota. Phyla significantly enriched in rhizoplane of *Phragmites australis* **(outer ring)** and *Typha latifolia* **(inner ring)** respect to the rhizosphere (CDS test, FDR<0.05 and log2FC>0); inside each ring sector is shown the number of OTUs identified for each phylum. The external circles represent the number of phyla enriched in the rhizoplane of both plant species.

The rhizoplane microbiotas resulted domination of the phyla Proteobacteria, Planctomycetes and Actinobacteria, observed at similar proportions in case of both plant species. On the contrary, the phyla of Acidobacteria and Firmicutes were more dominant in the rhizoplane community of *Typha latifolia* as well as the Chloroflexi phylum resulted more abundant in the rhizoplane microbiota of *Phragmites australis*. Only one OTU belonging to the phylum of Bacteroidetes was detected in both the rhizoplanes and the phyla of Verrucomicrobia and Cyanobacteria were differentially detected in the rhizoplane community of *Phragmites australis* (Figure 8). Instead, regarding to the taxonomy of the OTUs enriched in both the rhizoplane microbiotas we verified that these conserved OTUs

belonged mostly to the phyla of Proteobacteria, Planctomycetes and Actinobacteria but also to the phyla of Acidobacteria and Firmicutes although they were represented at really low proportion respect to the other detected phyla (Figure 8). Summarizing, all the results obtained from the taxonomical analysis of the enriched OTUs suggested that the phyla of Proteobacteria, Planctomycetes, Actinobacteria, Acidobacteria and Firmicutes constitute a central core of the rhizoplane microbiota with which other phyla like the Chloroflexi, Bacteroidetes, Verrucomicrobia and Cyanobacteria interact at different level and depending on the plant species.

4.2.1 Proteobacteria

Proceeding deeply to analyze the rhizoplanes composition at lower taxonomic levels, among the phylum of Proteobacteria the differences between two species rhizoplane enriched OTUs are detectable already at class level. Bacteria belong to the class of Deltaproteobacteria result to constitute only the rhizoplane community of *Phragmites australis* whereas they are not observed in *Typha* rhizoplane. At same time, Betaproteobacteria result more enriched in the rhizoplane of *Phragmites* respect to *Typha* one and conversely the Gammaproteobacteria are more represented in *Typha* rhizoplane.

Also at order level differences in rhizoplane composition have been detected. Although the class of Alphaproteobacteria is represented at similar proportion in both rhizoplanes, the orders of Sphingomonadales and Rhodobacterales constitute differentially the rhizoplane of *Phragmites australis* and *Typha latifolia* respectively. Instead, for the bacteria belong to the order of Rhizobiales no differences in their presence are detected between the two plants rhizoplanes. However, some families belonging to this order show a differential enrichment. It is the case of Hyphomicrobiaceae and Phyllobacteriaceae families which result more enriched in *Phragmites* rhizoplane as well as Bradyrhizobiaceae family is observed only in *Typha* rhizoplane. In particular, for the families of Hyphomicrobiaceae and Phyllobacteriaceae more represented in *Phragmites* and even detected in *Typha*, the genera of Hyphomicrobium, Devosia and Rhodoplanes and the genus of Mesorhizobium have been detected only in *Phragmites* rhizoplane whereas no characterization at genus level has been obtained for *Typha* one.

Instead, among the class of Betaproteobacteria the orders of Methylophilales and SC-I-84 are distinctly detected in *Typha latifolia* rhizoplane as well as microorganisms belonging to the order of SBla14 result to constitute only the *Phragmites* rhizoplane. Finally, for the order of Rhodocylales and

the related family of Rhodocyclaceae the analysis highlighted they are larger represented in the *Phragmites* rhizoplane respect to *Typha* one.

Regarding to the Gammaproteobacteria class, the major differences in the rhizoplanes composition are detected at order level. The orders of Xanthomonadales and Legionellales belonging to this class show their selectively association to the rhizoplane of *Phragmites australis* as well as the Pseudomonadales order has been detected only in the *Typha latifolia* rhizoplane. The results are shown in Table 1.

A

PHYLUM	**Proteobacteria**									
P.a.	38									
T.l.	27									
CLASS	**Alphaproteobacteria**									
P.a.	23									
T.l.	19									
ORDER	**Sphingomonadales**	**Rhizobiales**								**Rhodobacterales**
P.a.	1	22								
T.l.		15								4
FAMILY	**Sphingomonadaceae**	**Hyphomicrobiaceae**			**Rhizobiaceae**	**Phyllobacteriaceae**	**Methylocystaceae**		**Bradyrhizobiaceae**	**Rhodobacteraceae**
P.a.	1	5			1	3	3		1	
T.l.		1			1	1	3			4
GENUS	**Novosphingobium**	**Hyphomicrobium**	**Devosia**	**Rhodoplanes**		**Mesorhizobium**	**Pleomorphomonas**	**Methylosinus**		**Rhodobacter**
P.a.	1	1	2	1		1	1	2		
T.l.							1	2		2

B

PHYLUM	**Proteobacteria**			
P.a.	38			
T.l.	27			
CLASS	**Betaproteobacteria**			
P.a.	7			
T.l.	4			
ORDER	**SBla14**	**Rhodocyclales**	**Methylophilales**	**SC-I-84**
P.a.	1	4		
T.l.		1	1	2
FAMILY		**Rhodocyclaceae**	**Methylophilaceae**	
P.a.		4		
T.l.		1	1	

C

PHYLUM	**Proteobacteria**				
P.a.	38				
T.l.	27				
CLASS	**Gammaproteobacteria**				
P.a.	2				
T.l.	4				
ORDER	**Xanthomonadales**	**Legionellales**	**Pseudomonadales**		
P.a.	1	1			
T.l.			4		
FAMILY	**Sinobacteraceae**		**Pseudomonadaceae**	**Moraxellaceae**	
P.a.	1				
T.l.			1	3	
GENUS			**Pseudomonas**	**Acinetobacter**	
P.a.					
T.l.			1	3	
SPECIES			**viridiflava**	**rhizosphaerae**	**johnsonii**
P.a.					
T.l.			1	1	1

D

PHYLUM	**Proteobacteria**			
P.a.	38			
T.l.	27			
CLASS	**Deltaproteobacteria**			
P.a.	6			
T.l.				
ORDER	**Desulfobacterales**		**Myxococcales**	**Desulfuromonadales**
P.a.	3		2	1
T.l.				
FAMILY	**Desulfobulbaceae**	**Desulfobacteraceae**		**Geobacteraceae**
P.a.	2	1		1
T.l.				
GENUS	**Desulfobulbus**			**Geobacter**
P.a.	1			1
T.l.	0			

TABLE 1. Taxonomic classification of microorganisms belonging to Proteobacteria phylum for the portions of Alphaproteobacteria (A), Betaproteobacteria (B), Gammaproteobacteria (C) and Deltaproteobacteria (D). Colored bars represent the proportion of taxonomical ranks in *Phragmites* and *Typha* rhizoplanes.

4.2.2 Planctomycetes

Among the phylum of Planctomycetes the bacterial class named C6 constitutes differentially the *Typha latifolia* rhizoplane. Conversely, although the Planctomycetia class is detected in both rhizoplanes the order of Pirellulales belonging to this class is more represented in the *Phragmites* rhizoplane as well as the related family of Pirellulaceae and the genera of Pirellula and A17 result differentially detected in *Phragmites* microbiota.

Instead, even if the order of Gemmatales is detected at same proportion in both rhizoplanes, the family of Isosphaeraceae belonging to this order constitute only the *Typha latifolia* microbiota. The results are shown in Table 2.

PHYLUM	**Planctomycetes**				
P.a.	15				
T.l.	12				
CLASS	**Planctomycetia**				**C6**
P.a.	15				
T.l.	11				1
ORDER	**Pirellulales**		**Planctomycetales**	**Gemmatales**	
P.a.	7		2	6	
T.l.	2		2	7	
FAMILY	**Pirellulaceae**		**Planctomycetaceae**	**Gemmataceae**	**Isosphaeraceae**
P.a.	7		2	6	
T.l.	2		2	6	1
GENUS	**Pirellula**	**A17**	**Planctomyces**	**Gemmata**	
P.a.	1	2	2	3	
T.l.			2	4	

TABLE 2. Taxonomic classification of microorganisms belonging to Planctomycetes phylum. Colored bars represent the proportion of taxonomical ranks in *Phragmites* and *Typha* rhizoplanes.

4.2.3 Actinobacteria

Going deeply inside the characterization of Actinobacteria phylum, at class level the analysis shows that while the Actinobacteria class is detected in both rhizoplane communities at similar proportion, the classes of MB-A2-108 and Thermoleophilia are preferentially associated to the *Phragmites australis* roots and conversely the Acidimicrobiia class is more represented in the *Typha latifolia* rhizoplane.

Among the Acidimicrobiia class the only order detected, the order of Acidimicrobiales, shows the same trend of the class it belong to, but more differences are verified at family level between the two rhizoplanes. More precisely, microorganisms of Microthrixaceae and EB1017 families are

differentially associated to the roots of *Typha latifolia* whereas those ones belonging to the C111 family are detected in both rhizoplanes but in larger proportion in the *Typha* one.

Similarly, regarding the Actinobacteria class, the order of Actinomycetales is detected at similar proportion in both rhizoplanes as it is verified for the class distribution. However, the families of Streptomycetaceae and Mycobacteriaceae associated to this order are observed only on *Typha* roots, the family of Intrasporangiaceae only on *Phragmites* roots and finally the Nocardioidaceae family constitutes both the rhizoplanes but shows a larger presence in the *Phragmites australis* rhizoplane community. Moreover, among the Nocardioidaceae family the genus of Nocardioides is observed only as constituent of *Phragmites* rhizoplane.

Also for the class of MB-A2-108, the only associated order, identified as 0319-7L14, shows the same trend of the class level, since microorganisms of this order result more abundant in *Phragmites* rhizoplane than in *Typha* one.

Finally, for the class of Thermoleophilia also the related order of Gaiellale and the genus of Gaiellaceae result to constitute both rhizoplanes but being more represented in the *Phragmites australis* one. Instead, the other order detected among this class, the order of Solirubrobacterales, results associated differentially to *Phragmites* roots since no detection has been obtained in *Typha* rhizoplane microbiota. The results are shown in Table 3.

PHYLUM	Actinobacteria														
P.a.	13														
T.l.	15														
CLASS	Acidimicrobiia			Actinobacteria							MB-A2-108	Thermoleophilia			
P.a.	3			5							2	3			
T.l.	7			6							1	1			
ORDER	Acidimicrobiales			Actinomycetales							0319-7L14	Gaiellales	Solirubrobacterales		
P.a.	3			4							2	2	1		
T.l.	7			6							1	1			
FAMILY	C111	Microthrixaceae	EB1017	Intrasporangiaceae	Nocardioidaceae		Streptomycetaceae		Mycobacteriaceae			Gaiellaceae			
P.a.	2			1	2							2			
T.l.	4	2	1		1		1		2			1			
GENUS					Nocardioides				Mycobacterium						
P.a.					1										
T.l.									2						

TABLE 3. Taxonomic classification of microorganisms belonging to Actinobacteria phylum. Colored bars represent the proportion of taxonomical ranks in *Phragmites* and *Typha* rhizoplanes.

4.2.4 Acidobacteria

Among the phylum of Acidobacteria the analysis revealed that microorganisms belonging to the class of Chloracidobacteria constitute both rhizoplane microbiotas at same proportion, whereas those ones belonging to the class of Acidobacteria-6 are more enriched in *Typha latifolia* rhizoplane and finally, the bacterial class named Sva0725 results being associated only to *Typha* roots. However, although the class of Chloracidobacteria is represented at same proportion among the rhizoplane microbiotas, at order level those microorganisms show a differential recruitment by plant species. More precisely, the order of RB41 has been detected only in the *Phragmites* microbiota, as the order of DS-100 has been in *Typha* rhizoplane.

Instead, the only order detected among the class of Acidobacteria-6, i.e. the order of iii1-15, maintains the same trend of the class it belong to, being more enriched in *Typha latifolia* rhizoplane respect to *Phragmites australis* one. The results are shown in Table 4.

PHYLUM	**Acidobacteria**			
P.a.	2			
T.l.	4			
CLASS	**Chloracidobacteria**		**Acidobacteria-6**	**Sva0725**
P.a.	1		1	
T.l.	1		2	1
ORDER	**RB41**	**DS-100**	**iii1-15**	
P.a.	1		1	
T.l.		1	2	
SPECIES	**Ellin6075**			
P.a.	1			
T.l.				

TABLE 4. Taxonomic classification of microorganisms belonging to Acidobacteria phylum. Colored bars represent the proportion of taxonomical ranks in *Phragmites* and *Typha* rhizoplanes.

4.2.5 Firmicutes

The phylum of Firmicutes, as previous specified, is larger enriched in *Typha latifolia* rhizoplane respect to the *Phragmites* one and it is constituted by microorganisms belonging to two different classes, i.e the class of Bacilli, which has been detected for both rhizoplanes, and the bacteria grouped by Clostridia class which result being associated differentially to *Typha* roots. Interestingly,

among the class of Bacilli the analysis has detected only the bacterial order of Bacillales which show the same trend of the related class resulting as constituent of both rhizoplanes but more represented in *Typha* one. Yet, the family taxonomy of this order highlights that all the families detected, i.e. the Alicyclobacillaceae, Bacillaceae, Exiguobacteraceae and Planococcaceae families, are identifiable differentially in the *Typha* rhizoplane, whereas no classification at family level is obtained regarding this bacterial order for *Phragmites australis* rhizoplane. The results are shown in Table 5.

PHYLUM	**Firmicutes**				
P.a.	1				
T.l.	9				
CLASS	**Bacilli**				**Clostridia**
P.a.	1				
T.l.	8				1
ORDER	**Bacillales**				**Clostridiales**
P.a.	1				
T.l.	8				1
FAMILY	**Alicyclobacillaceae**	**Bacillaceae**	**Exiguobacteraceae**	**Planococcaceae**	**Peptostreptococcaceae**
P.a.					
T.l.	1	1	2	1	1
GENUS	**Alicyclobacillus**	**Bacillus**	**Exiguobacterium**		
P.a.					
T.l.	1	1	2		

TABLE 5. Taxonomic classification of microorganisms belonging to Firmicutes phylum. Colored bars represent the proportion of taxonomical ranks in *Phragmites* and *Typha* rhizoplanes.

4.2.6 Chloroflexi

For the Chloroflexi phylum which resulted more enriched in *Phragmites australis* rhizoplane than in *Typha* one, the analysis highlighted a major difference between two rhizoplane microbiotas at class level. More precisely, bacteria belonging to the classes of Thermomicrobia and Ellin6529 are selectively associated to the *Phragmites australis* roots as well as the classes of Anaerolineae and Chloroflexi are detected only in *Typha latifolia* rhizoplane. The results are shown in Table 6.

PHYLUM	Chloroflexi			
P.a.	6			
T.l.	3			
CLASS	**Thermomicrobia**	**Ellin6529**	**Anaerolineae**	**Chloroflexi**
P.a.	1	5		
T.l.			1	2
ORDER	**JG30-KF-CM45**		**Caldilineales**	**Roseiflexales**
P.a.	1			
T.l.			1	2
FAMILY			**Caldilineaceae**	**Kouleothrixaceae**
P.a.				
T.l.			1	2
GENUS			**Caldilinea**	
P.a.				
T.l.			1	

TABLE 6. Taxonomic classification of microorganisms belonging to Chloroflexi phylum. Colored bars represent the proportion of taxonomical ranks in *Phragmites* and *Typha* rhizoplanes.

4.2.7 Bacteroidetes

As previous specified the phylum of Bacteroidetes has been detected as similarly enriched in both rhizoplanes. The same trend is shown by the related class of Saprospirae and order of Saprospirales. Differences between the two rhizoplanes are revealed at family level, since the detected families of Saprospiraceae and Chitinophagaceae are associated selectively to the *Phragmites* and *Typha* roots respectively. The results are shown in Table 7.

PHYLUM	Bacteroidetes	
P.a.	1	
T.l.	1	
CLASS	**Saprospirae**	
P.a.	1	
T.l.	1	
ORDER	**Saprospirales**	
P.a.	1	
T.l.	1	
FAMILY	**Saprospiraceae**	**Chitinophagaceae**
P.a.	1	
T.l.		1

TABLE 7. Taxonomic classification of microorganisms belonging to Bacteroidetes phylum. Colored bars represent the proportion of taxonomical ranks in *Phragmites* and *Typha* rhizoplanes.

4.2.8 Verrucomicrobia

Among the phylum of Verrucomicrobia constituting only the *Phragmites australis* rhizoplane, microorganisms group into two different classes each one taxonomically characterized up to the genus level as reported in Table 8.

PHYLUM	**Verrucomicrobia**	
P.a.	3	
T.l.		
CLASS	**Verrucomicrobiae**	**Spartobacteria**
P.a.	2	1
T.l.		
ORDER	**Verrucomicrobiales**	**Chthoniobacterales**
P.a.	2	1
T.l.		
FAMILY	**Verrucomicrobiaceae**	**Chthoniobacteraceae**
P.a.	2	1
T.l.		
GENUS	**Luteolibacter**	**CandidatusXiphinematobacter**
P.a.	1	1
T.l.		

TABLE 8. Taxonomic classification of microorganisms belonging to Verrucomicrobia phylum. Colored bars represent the proportion of taxonomical ranks in *Phragmites* and *Typha* rhizoplanes.

4.2.9 Cyanobacteria

The phylum of Cyanobacteria resulted being associated only to *Phragmites australis* rhizoplane has been taxonomically classified only up to the class level: as shown in Table 9 all microorganisms identified for this phylum belong to the class of YS2.

PHYLUM	**Cyanobacteria**
P.a.	1
T.l.	
CLASS	**YS2**
P.a.	1
T.l.	

TABLE 9. Taxonomic classification of microorganisms belonging to Cyanobacteria phylum. Colored bars represent the proportion of taxonomical ranks in *Phragmites* and *Typha* rhizoplanes.

4.3 Conserved members of the rhizoplane communities

As it is shown in summarizing Table 10, going to analyze the conserved portion of microbiota between the two rhizoplanes we can observe that both rhizoplanes are constituted by microorganisms belonging mainly to the classes of Alphaproteobacteria and Planctomycetia followed by those ones grouping into the classes of Actinobacteria, Chloracidobacteria and Saprospirae. These microbial classes are represented in both rhizoplanes at the same proportion. This finding supports even more the hypothesis that the defined core of bacteria is the building block for structuring the rhizoplane microbiota of both plants. With exception for the Saprospirae class we obtained the taxonomical classification of OTUs until the genus level and thus we identified the genera of Gemmata, Methylosinus, Planctomyces and Pleomorphomonas as constituents of both rhizoplane microbiotas.

Moreover, analyzing more deeply the characteristics of the two rhizoplanes enriched genera, we can observe as the larger part of the identified OTUs of the microbiota of both plants belong to Gram negative and rod shaped bacteria (Table 11). In the case of *Phragmites australis* rhizoplane we counted gram negative bacteria for the 95% of the rhizoplane identified genera, and for 59% these bacteria are characterized by rod-shape. Although at lower rate, similarly the *Typha latifolia* rhizoplane is more enriched for gram negative (82%) and rod-shaped (45%) bacteria. Interestingly, also all the genera reveled as enriched at similar proportion in both rhizoplanes belong to gram negative bacteria (100%), but more variable is the cell shape.

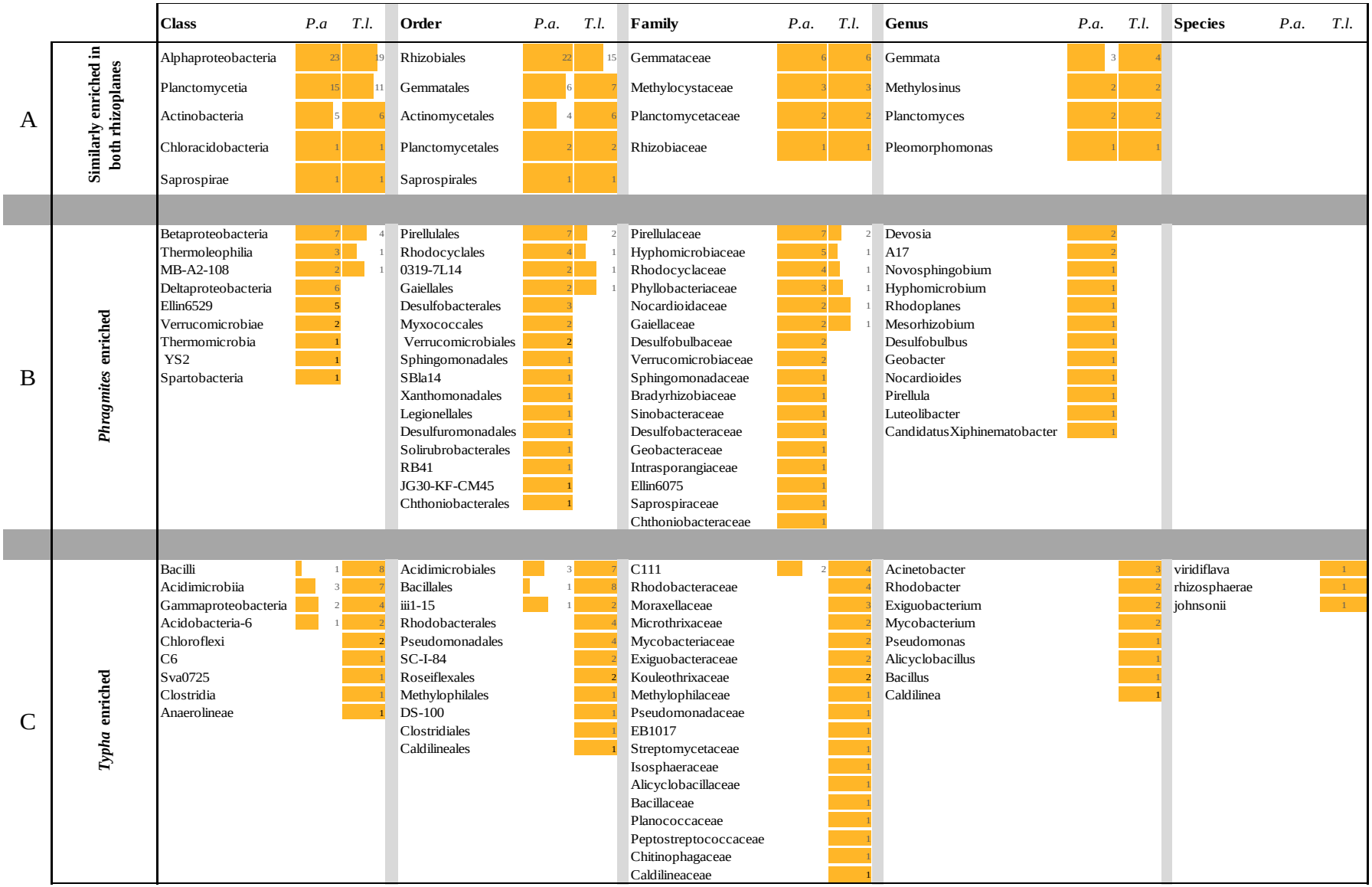

TABLE 10. Taxonomic classification up to the species level of OTUs resulted being similarly enriched in both rhizoplanes (A) or differentially enriched in the rhizoplane of *Phragmites australis* (B) and *Typha latifolia* (C). Colored bars represent the proportion of taxonomical ranks in *Phragmites* and *Typha* rhizoplanes.

	Genus	*P.a.*	*T.l.*	Gram classification	Cell shape
OTUs enriched at same proportion in both rhizoplanes	Gemmata	3	4	negative	spherical
	Methylosinus	2	2	negative	pyriform/vibrioid
	Planctomyces	2	2	negative	ovoid
	Pleomorphomonas	1	1	negative	rod
OTUs differentially enriched in *Phragmites* rhizoplane	Devosia	2		negative	rod
	A17	2		negative	rod
	Novosphingobium	1		negative	rod
	Hyphomicrobium	1		negative	rod
	Rhodoplanes	1		negative	rod
	Mesorhizobium	1		negative	rod
	Desulfobulbus	1		negative	rod/ovoid
	Geobacter	1		negative	rod
	Nocardioides	1		positive	coccoid
	Pirellula	1		negative	ovoid/ellipsoidal/pear
	Luteolibacter	1		negative	rod
	CandidatusXiphinematobacter	1		negative	rod
OTUs differentially enriched in *Typha* rhizoplane	Acinetobacter		3	negative	in pair coccoid
	Rhodobacter		2	negative	rod/ovoid
	Exiguobacterium		2	positive	rod
	Mycobacterium		2	variable	rod
	Pseudomonas		1	negative	rod
	Alicyclobacillus		1	positive	rod
	Bacillus		1	positive	rod
	Caldilinea		1	negative	filamentous

TABLE 11. Microbial genera enriched in each and both rhizoplanes. Colored bars represent the proportion of detected genera in *Phragmites* and *Typha* rhizoplanes. For each genus the Gram classification and associated cell shape are reported.

4.4 Metagenomic analysis through PICRUSt

The metabolic functions putatively encoded by the rhizosphere and rhizoplane microbiomes of both plant species have been predicted using the PICRUSt software. We reconstructed the pool of putative functions associated to 16S rRNA OTUs of each studied microhabitat performing firstly the normalization of OTUs table and finally the metagenome prediction steps as explained in the material and method section. At the same time, the accuracy of analysis was evaluated through calculation of NSTI index, using the default weighted metric for all samples, and values are reported in Table 12.

Plant species	Microhabitat	Sample	NSTI Value
Phragmites australis	Rhizoplane	*P*1a	0,16
		*P*2a	0,16
		*P*3a	0,17
		*P*4a	0,18
		*P*5a	0,17
	Rhizosphere	*P*r1	0,16
		*P*r2	0,17
		*P*r3	0,19
		*P*r4	0,18
Typha latifolia	Rhizoplane	*T*1a	0,16
		*T*2a	0,17
		*T*3a	0,11
		*T*4a	0,16
		*T*5a	0,16
	Rhizosphere	*T*r1	0,16
		*T*r2	0,18
		*T*r3	0,17
		*T*r4	0,17

TABLE 12. Accuracy evaluation for PICRUSt analysis. The NSTI index values calculated for all samples are reported as measure of prediction accuracy.

The average of NSTI values for all considered samples resulted 0,17 ± 0,02 s.d., a mid-range value of accuracy and congruent with the value obtained testing soils samples through PICRUSt (Langille et al., 2013). This suggests that, despite the prediction of functions can be considered representative of the investigated environments, caution should be exerted in interpreting these results.

Next, by using the software STAMP the detected pool of putative functions associated to rhizospheres and rhizoplanes of both plants was subjected to the ANOVA analysis followed by a Tukey's *post-hoc* test to identify functions significantly enriched to a given combination microhabitat * species a $p < 0.05$ (FDR corrected). This analysis identified 13 functions fulfilling these criteria (Table 13).

Detected functions	rhizoplaneP		rhizosphereP		rhizoplaneT		rhizosphereT		p-values	p-values (corrected)	Effect size
	mean rel, freq, (%)	std, dev, (%)	mean rel, freq, (%)	std, dev, (%)	mean rel, freq, (%)	std, dev, (%)	mean rel, freq, (%)	std, dev, (%)			
Bacterial invasion of epithelial cells	0,000486	0,000226	0,000363	0,000118	0,000016	0,000013	0,000031	0,000031	0,000421	0,016658	0,714455
Neuroactive ligand-receptor interaction	0	0	0	0	0,000051	0,000019	0,000023	0,000025	0,001294	0,025590	0,663312
Sesquiterpenoid biosynthesis	0	0	0	0	0,000101	0,000037	0,000038	0,000039	0,000415	0,016658	0,715051
Melanogenesis	0,000004	0,000002	0,000009	0,000016	0,000205	0,000085	0,000076	0,000051	0,000304	0,016658	0,727810
1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) degradation	0,000969	0,000317	0,001583	0,000312	0,000372	0,000132	0,000517	0,000163	0,000074	0,013160	0,778267
Indole alkaloid biosynthesis	0,006970	0,000283	0,005701	0,000299	0,005533	0,000645	0,004865	0,000650	0,000548	0,018568	0,703256
Betalain biosynthesis	0,007056	0,000278	0,006023	0,000218	0,006126	0,000712	0,005224	0,000561	0,002235	0,040799	0,635039
Bile secretion	0,004267	0,001051	0,004759	0,000641	0,004668	0,000717	0,007367	0,001238	0,003217	0,049919	0,614811
Ether lipid metabolism	0,018761	0,001378	0,021803	0,000298	0,015628	0,001291	0,016994	0,001596	0,000111	0,013160	0,764979
Non-homologous end-joining	0,070843	0,002336	0,069769	0,001791	0,064055	0,003132	0,062150	0,002686	0,001098	0,025291	0,671368
Ascorbate and aldarate metabolism	0,168207	0,003165	0,183854	0,004277	0,177432	0,004480	0,180584	0,004465	0,001043	0,025291	0,673842
Phosphotransferase system (PTS)	0,086014	0,006291	0,083574	0,007062	0,104807	0,009855	0,080692	0,006765	0,003365	0,049919	0,612229
Germination	0,000573	0,000323	0,000029	0,000017	0,018810	0,010629	0,002390	0,001719	0,001172	0,025291	0,668172
Histidine metabolism	0,549407	0,006072	0,565318	0,002043	0,575924	0,014769	0,549941	0,006025	0,002940	0,049845	0,619914
Transcription factors	1,372890	0,034958	1,382570	0,017388	1,449102	0,016119	1,479891	0,030775	0,000223	0,016658	0,739838
Transporters	5,386262	0,269037	5,178518	0,099888	5,617257	0,085644	5,923238	0,209267	0,001099	0,025291	0,671312

TABLE 13. Enriched functions in *Phragmites* and *Typha* rhizoplane and rhizosphere microbiomes. The color coding from light to dark blue depicts the proportion of each function in the analyzed microbiomes, from lower to higher proportion respectively.

This analysis revealed that the majority of the predicted function (11/13) occurred at a very low abundance, i.e., below 0.5%. Conversely, the functions “Transporters” and “Transcription factors” together represented more than 6% of the overall predicted metagenome. No common trend could be observed for these two functions.

For the “Transporters” function we notice a similar presence between rhizosphere and rhizoplane microhabitats for both plants (Figure 9, Tukey's *post hoc* test). Interestingly, although the presence of the function in the rhizosphere is significantly different between the studied plants (Tukey's *post hoc* test, with $P<0.001$), i.e. significantly greater in *Typha* respect to *Phragmites*, we found a similar number of transporters in both rhizoplanes (Figure 9, Tukey's *post hoc* test). This result suggests a functional specialization of transporters in the rhizoplane compartment. This would mirror the selective enrichment of certain bacteria at the root surface in response to molecular stimula peculiar of rhizoplane compartment and plant species- independent. Furthermore, this finding can be thought as a confirmation that a core-microbiota is recruited at the root surface despite the microbial composition of the community around roots.

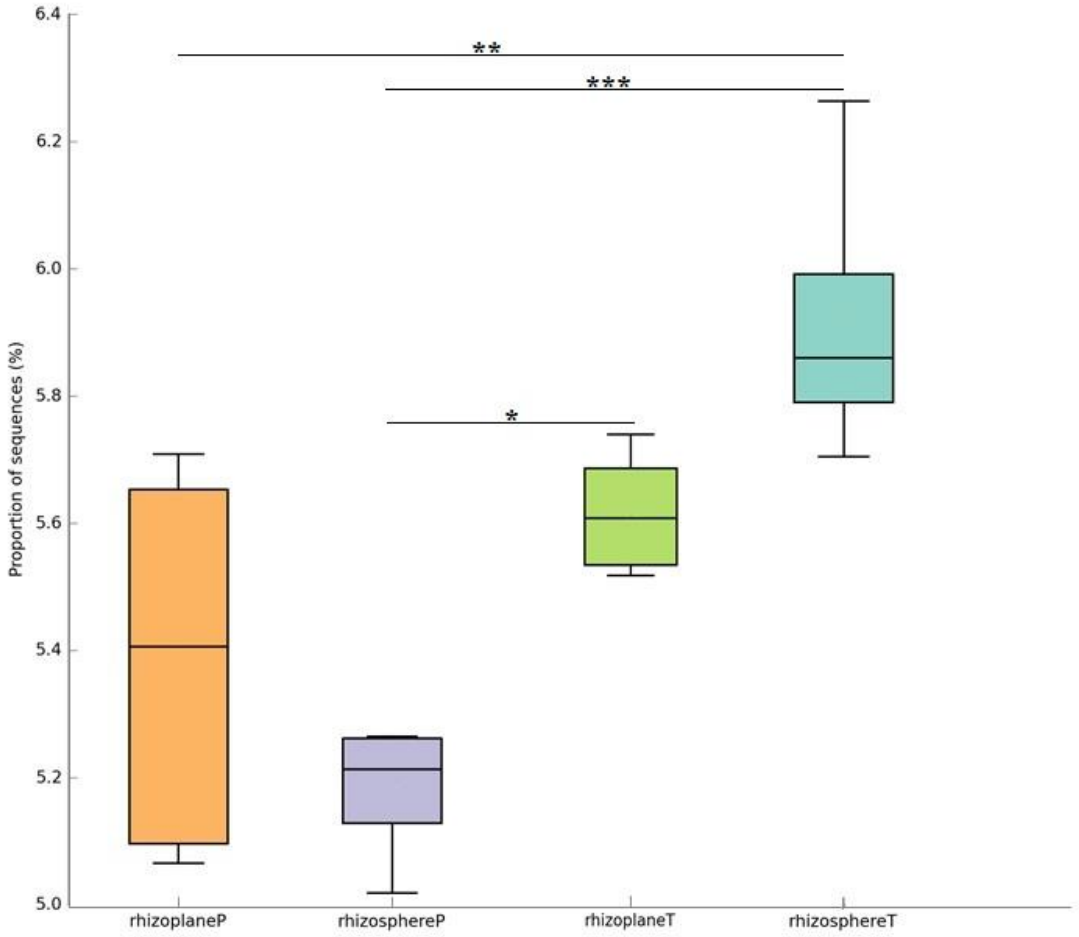

FIGURE 9. "Transporters" enrichment in rhizoplane and rhizosphere microbiomes of studied plants. Upper and lower edges of the box plots represent the upper and lower quartiles, respectively. The bold line within the box denotes the median. Maximum and minimum observed values are represented by the whiskers. Asterisks denote statistically significant differences between rhizosphere and rhizoplane microhabitats (***P < 0.001, **P < 0.01, * P < 0.05).

Conversely, for the function 'Transcription factors' we observe a species-specificity (Figure10).

A similar amount of transcription factors can be observed between the rhizosphere and rhizoplane compartments of each plant (Tukey's *post hoc* test). However, the proportion of this function results significantly higher in the rhizosphere and rhizoplane of *Typha* respect to the same compartments of *Phragmites* (Tukey's *post hoc* test, with P<0.01).

This evidence suggests that the transcription factors of bacteria constituting the microbiotas colonizing roots and their surrounding are responsive to species-specificity molecular signals more abundantly produced and diffused in the wetland ecosystem by *Typha latifolia* roots.

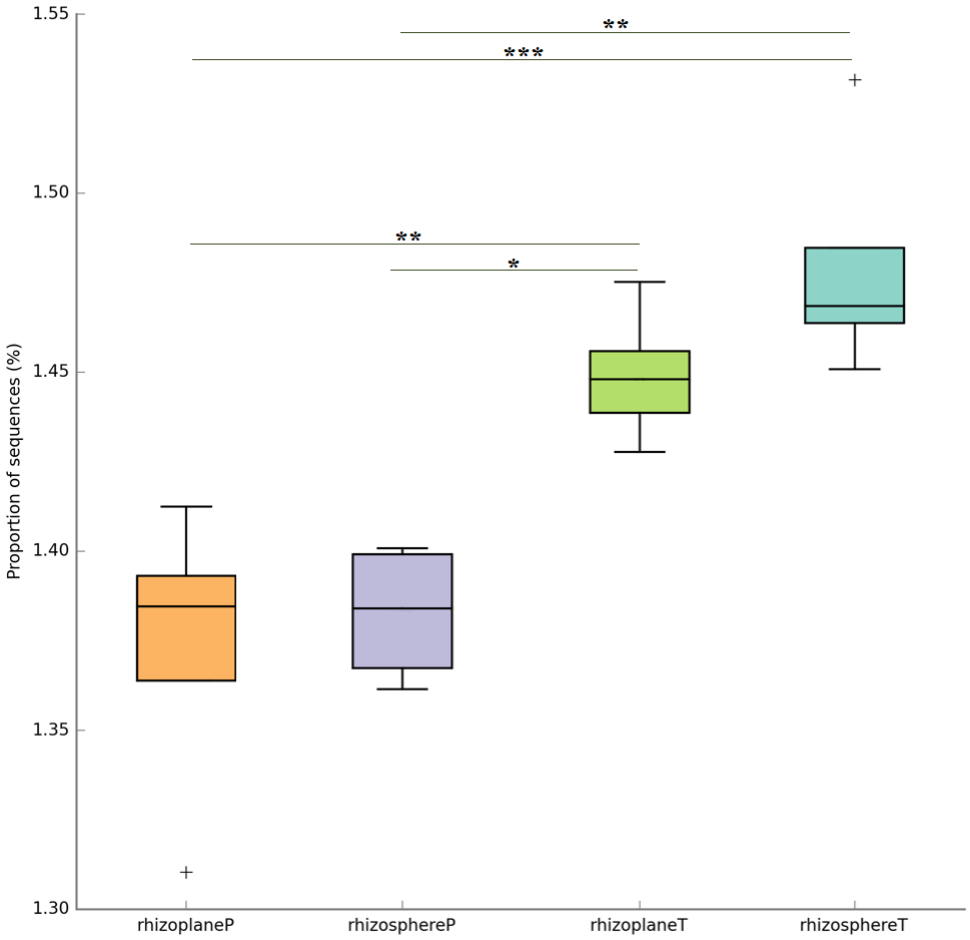

FIGURE 10. "Transcription factors" enrichment in rhizoplane and rhizosphere microbiomes of studied plants. Upper and lower edges of the box plots represent the upper and lower quartiles, respectively. The bold line within the box denotes the median. Maximum and minimum observed values are represented by the whiskers. Plus symbols denote outlier observations whose value are 3/2 times greater or smaller than the upper or lower quartiles, respectively. Asterisks denote statistically significant differences between rhizosphere and rhizoplane microhabitats (***P < 0.001, **P < 0.01, * P < 0.05).

Together, this analysis suggests that *Typha* and *Phragmites* microbiotas define functionally congruent units whose metabolic adaptation is to a given microhabitat and/or host species is mediated, at least in part, by a transcriptional reprogramming. However, it is important to mention that other or additional functions (not accurately predicted by PICRUSt) may contribute to microbiota diversification at the *Typha* and *Phragmites* root-soil interface.

4.5 The rhizoplane of *P. australis* and *T. latifolia* is a site for microbial colonization

To gain further insights into the spatial organization of the *P. australis* and *T. latifolia* rhizoplane microbiota, root specimens were subjected to the Scanning Electron Microscopy (SEM) analysis. SEM micrographs of washed roots revealed the presence of microbial-like structures and assembles colonizing the root surface of both studied plants (Figure 11B, F). Intriguingly, these microbial-like assemblages appeared more compacted and developed on the rhizoplane of *P. australis* (Figure 11B), since we repeatedly identified areas of *T. latifolia* rhizoplane uncovered by this microbial-like matrix (Figure 11F). SEM micrographs of the roots subjected to the sonication pre-treatment confirmed a progressive dislodgment of the microbial-like aggregates from the rhizoplane in both tested species (Figure 11C, G). This phenomenon was more evident on the specimens subjected to the second ultrasound treatment (Figure 11D, H). Collectively, these observations suggest that the rhizoplane of *P. australis* and *T. latifolia* is a site for microbial proliferation whose anchoring to the host cells is sufficiently robust to withstand water streams at the root surface, such as the ones occurring at the sites where these two plants develop. Likewise, these observations directly corroborate our assumption that ultrasound treatments would have enriched the sonication buffer for rhizoplane-colonizing microbes.

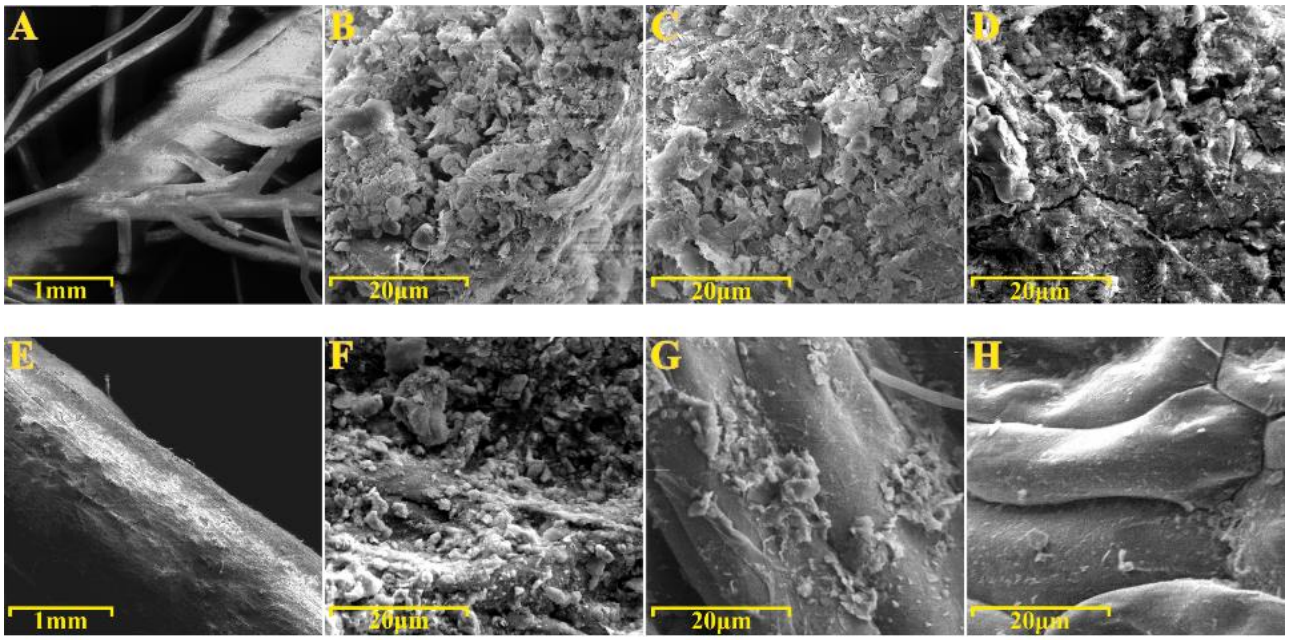

FIGURE 11. Scanning Electron Microscopy of roots specimens before and after ultrasound treatments. SEM micrographs of (A to D) *Phragmites australis* and (E to H) *Typha latifolia* roots. A, B; E, F root specimens before

ultrasound treatments. C, G rhizoplane of specimens subjected to the first ultrasound treatment. D, H rhizoplane of specimens subjected to the second ultrasound treatment. Note the bacterial-like structure proliferating on the rhizoplane of both species.

4.6 Root-isolated bacteria have distinct biofilm-forming capabilities

The observation of that tightly-associated rhizoplane communities proliferate in the tested plants (see Figure 11) motivated us to investigate the biofilm-forming capabilities of *P. australis* and *T. latifolia* root-associated microbiota. We therefore decided to isolate individual members of the root microbiota on complex media. In total, we retrieved 20 morphologically distinct colony forming units (CFUs) from *P. australis* and 31 from *T. latifolia* (Figure 12) and tested their biofilm forming capabilities (Figure 13).

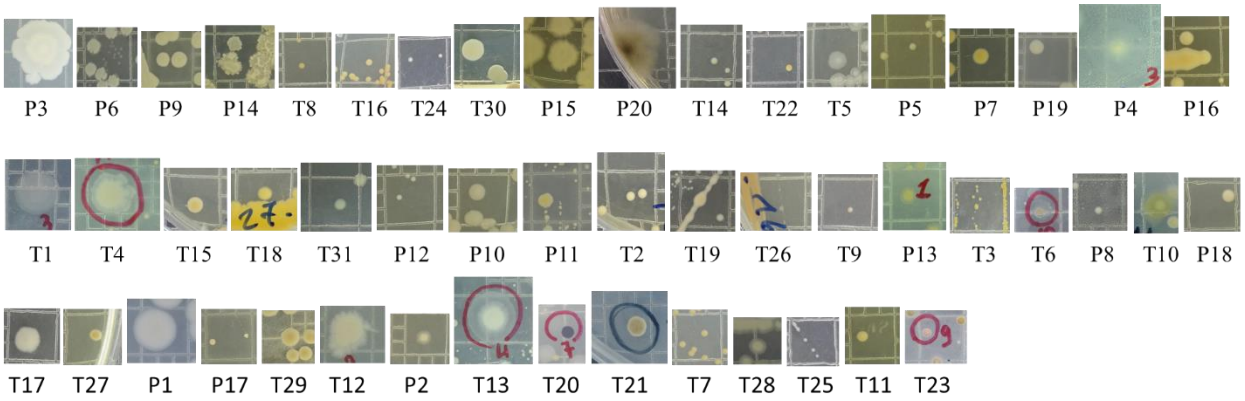

FIGURE 12. Rhizoplane isolates. The rhizoplane isolates obtained from *Phragmites australis* (P) and *Typha latifolia* (T) root specimens.

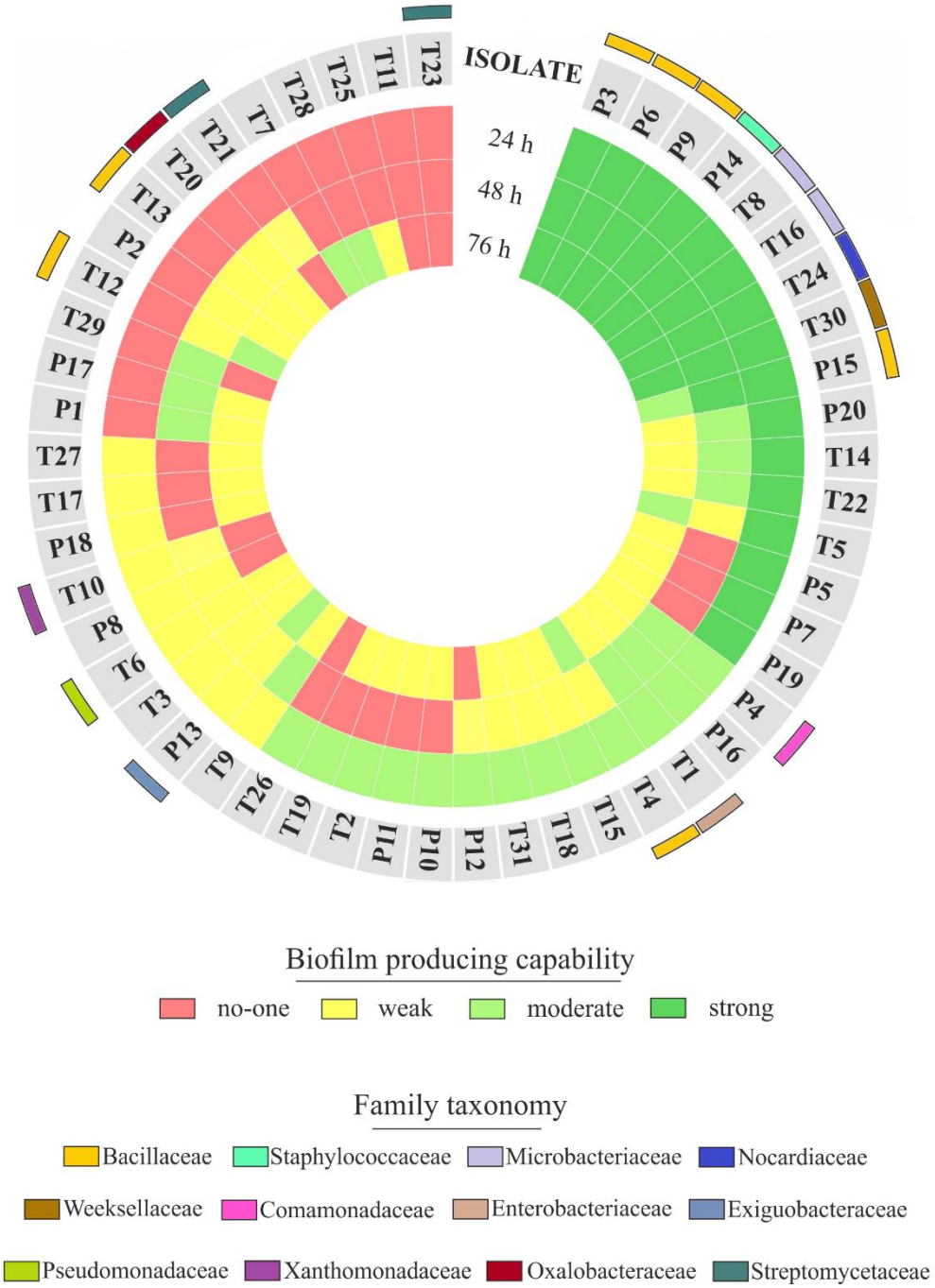

FIGURE 13. Rhizoplane isolates, their biofilm formation capability and their taxonomical classification. The biofilm formation capability of the indicated rhizoplane isolates is represented by the three inner rings which report the results of the biofilm formation assay for each isolate obtained during the indicated incubation times. The different colors exemplify the classification of those isolates as not, weak, moderate or strong biofilm producers. The outer colored bars show the family affiliation of the identified isolates.

Our assay revealed that 38 isolates were able of producing a biofilm already after 24 hours of incubation, 8 isolates after 48 hours and 3 others after 76 hours. More interestingly, of all screened strains 24 resulted biofilm producers at all the tested incubation times and among them the isolates designated P3, P6, P9, P14, T8, T16, T24 and T30 maintained a strong ability to produce biofilm until the last incubation time (Figure 13). Therefore, these strong biofilm producers alongside with other 12 'control' isolates with different trends of biofilm formation were subjected to taxonomic identification using a 16S rRNA gene sequencing approach (Clarridge, 2004; Han, 2006). In detail, the T8, T16, T21 and T20 strains resulted taxonomically classified at species level, the isolates T12, T13 and T4 and the isolate T10, P4 and T1 were classified at family level whereas at genus level all the other tested isolates (Supplementary Table 2).

Since all the isolates resulted classified at least at family level, we estimated their relevance into the different studied microhabitats comparing the abundance of the families they belonged to in water, rhizosphere and rhizoplane microbiotas of each studied plant defined, as described above, through the Illumina sequencing approach. Firstly, we observed as the presumed biofilm isolates grouped by a total of 12 different families: the Bacillaceae family resulted the most represented one including 7 rhizoplane isolates (P6, P9, P3, P15, T12, T13 and T4); following the families of Microbacteriaceae and Streptomycetaceae were represented by 2 isolates each, respectively the isolates T8, T16 and T23 and T21. Finally, the families of Staphylococcaceae, Nocardiaceae, Weeksellaceae, Comamonadaceae, Enterobacteriaceae, Exiguobacteraceae, Pseudomonadaceae, Xanthomonadaceae and Oxalobacteraceae were respectively represented from the isolates P14, T24, T30, P4, T1, P13, T6, T10 and T20 (Figure 13). Therefore, we identified an overlap between the higher rank taxonomies of the bacteria isolated from roots and bacteria identified in our sequencing survey as enriched in the rhizoplane microbiota of *Phragmites australis* and *Typha latifolia* (compare Figure 8 with Figure 13). Moreover, at family level only the Nocardiaceae and Staphylococcaceae families did not match with the families identified in the rhizoplane microbiotas but all the other identified families resulted effectively part of the rhizoplane microbiota of the two studied plants (Table 14).

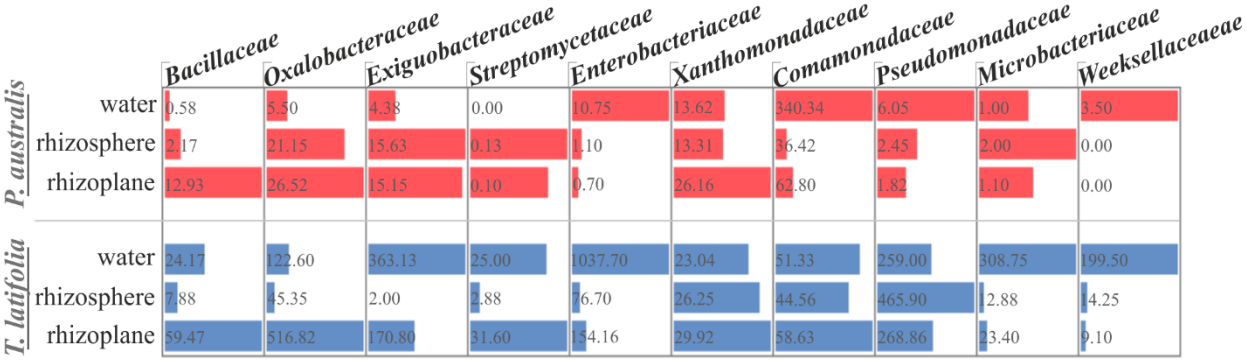

TABLE 14. Proportional abundance of the microbial families whom the identified biofilm formers belong to, in water, rhizosphere and rhizoplane microbiotas of *P. australis* and *T. latifolia*. Bars and inner numbers indicate the average of reads observed for the microbial families in each microhabitat.

More interestingly, the families of Bacillaceae for both the studied plants and the family of Oxalobacteraceae only for *Typha latifolia* showed a clear enrichment in the rhizoplane community respect to the water and rhizosphere microbiotas suggesting that these microbial families are strongly recruited at the root surface of both or at least one of the studied plants. Furthermore, the Exiguobacteraceae and Streptomycetaceae families resulted being enriched only respect to the rhizosphere microbiota for *Typha latifolia* and only respect to the water microbiota for *Phragmites australis* since, in this case, we detected a similar level of their presence in the rhizoplane and rhizosphere microbial communities. These results showed that despite to their presence in the initial inoculum of water, microorganisms belong to the families of Exiguobacteraceae and Streptomycetaceae are more attracted to the root surface of *Typha latifolia* than to the root surface of *Phragmites australis*. Instead, the family of Enterobacteriaceae showed the largest abundance in the water compartment for both the plant species and only in *Typha latifolia* a sensitive enrichment in the rhizoplane microbiota respect to the rhizosphere one. This suggested that the interaction of microorganisms of the Enterobacteriaceae family with the root surface of *Typha latifolia* has an advantaging effect for their surviving in a not-preferential microhabitat as the root zone is, whereas the interaction with the *Phragmites australis* roots did not show the same effect. Furthermore, we found that microorganisms of the Xanthomonadaceae family increased in the rhizoplane microbiota of *Phragmites australis* respect to the water and rhizosphere communities; instead, they were represented at similar level in the water, rhizosphere and rhizoplane microbiotas of *Typha latifolia* although their presence in all the microhabitats associated to *Typha latifolia* was comparable to the

one observed in the rhizoplane community of *Phragmites australis*. These results suggested that the microorganisms belonged to the Xanthomonadaceae family are able to colonize the root surface of both the studied plants, but in the case of *Phragmites australis* they show a greater preference for the colonization of the root surface respect to the other investigated microhabitats. Finally, for the microorganism of the Comamonadaceae family, although they showed the largest abundance in the water microbiota of *Phragmites australis* they also resulted being increased in the rhizoplane respect to the rhizosphere; on the contrary, they were found at similar abundance in water, rhizosphere and rhizoplane of *Typha latifolia*. This suggested that in the root zone those microorganisms are advantaged by a more tightly interaction with the root surface of *Phragmites australis* whereas no particular advantages are shown by the closeness of the *Typha latifolia* root surface. Concluding, our data strongly suggest that the biofilm-forming bacteria isolated from root preparations are phylogenetically-related to members of the rhizoplane microbiota. This is particular evident for members of 2 bacterial families, namely Bacillaceae and Oxalobacteraceae, whose enrichment in the rhizoplane significantly discriminate this compartment from the microbiota surrounding *Phragmites* and *Typha* roots (Table 14).

Chapter 5

5. DISCUSSIONS

The data presented here provide new insights into the composition and functional significance of bacterial microbiota associated to *Phragmites australis* and *Typha latifolia*, two macrophytes commonly present in natural and artificial wetlands. Despite a consistent part of scientific literature is focused on the role of macrophytes and rhizosphere microorganisms in phytodepuration process, very little is known about the microbial communities which more tightly interact with root surface in the rhizoplane (Dhote and Dixit, 2009; Sharma et al., 2013; Shelef et al., 2013). In attempt to unravel the composition and recruitment cues of the rhizoplane microbiota of the two plants we used a combined culture independent and dependent-approach. Through a 16S rRNA gene Illumina MiSeq sequencing survey we characterized the rhizoplane communities of the two plants and compared them to the microbial communities of the surrounding water and rhizosphere. Alpha diversity (i.e., within sample diversity) calculation indicated that, regardless of the tested plant species, the rhizoplane bacterial microbiota of the two plants was significantly different from the one retrieved from rhizosphere specimens (Figure 5). In particular, both richness, i.e. Observed OTUs and Chao1, and evenness, i.e., Shannon, indexes suggested that not all rhizosphere bacteria have the capacity to thrive on the rhizoplane. This is reminiscent of the selective pressure exerted by roots of land plants on the soil biota (Edwards et al., 2015). We therefore wondered whether this selection pressure was mainly driven by the microhabitat (i.e., rhizoplane or rhizosphere) or by the host species. Strikingly, beta diversity (i.e., between sample diversity) analysis computed with both Bray-Curtis, sensitive to taxa abundances, and weighted UniFrac, sensitive to taxa abundances and relatedness, revealed that impact of microhabitat on the microbiota exceeded the one of the species (Figure 6). In particular, rhizosphere and rhizoplane communities retrieved from both species appeared more similar than the community identified in the water surrounding the two plants. This observation concur with the recent findings of Bowen and colleagues who described how phylogenetically-related *P. australis* lineages assemble taxonomically congruent rhizosphere microbiota when grown in common garden experiments (Bowen et al. 2017). To gain further insights into the recruitment cues of the *P. australis* and *T. latifolia* bacterial microbiota we focused our attention on the bacterial taxa underpinning the observed microhabitat diversification. In particular, we decided to focus our attention on the bacteria significantly enriched in and differentiating rhizoplane communities from the surrounding rhizosphere biota. We identified a subset of bacteria whose enrichment in the rhizoplane appears host-species specific, while another subset of bacteria, with a bias for Actinobacteria, Planctomycetes and Proteobacteria, appears to be enriched in a microhabitat-responsive (Figures 7 and 8). Our data suggest that the taxonomic diversity of *Phragmites* and *Typha* root microbiota is broader than previously observed by Li and co-workers (Li et al., 2013). However, owing to the fact that this latter

study and our study focused on geographically separated wetlands (China and Italy, respectively) and the different techniques used in the two investigations (Low- and High-resolution, respectively), caution should be exerted when comparing these results.

Going further than the phylum level inside the taxonomical classification up to the species level, we deeply characterized bacteria which constitute the rhizoplane of both plant species. Regardless to the different environmental factors which act in the different sites of rooting of the two species, we found that both rhizoplanes are constituted by microorganisms belonging to the classes of Alphaproteobacteria, Planctomycetia, Actinobacteria, Chloracidobacteria and Saprospirae, to the orders of Rhizobiales, Gemmatales, Actinomycetales, Planctomycetales and Saprospirales, to the families of Gemmataceae, Methylocystaceae, Planctomycetaceae and Rhizobiaceae and to the genera of Gemmata, Methylosinus, Planctomyces and Pleomorphomonas (Table 10A). This group of microorganisms can be thought as responsive to signals produced by roots of both plant species which attract these bacteria at the root surface and stabilize their interaction with the plants. All the genera identified or the families they belong to have been previous detected in aquatic ecosystems. Planctomyces phylum whom the genera of Gemmata and Planctomyces belong to has been detected in lake systems (Pollet et al., 2014) and the genus Gemmata has been reveled associated also to maize and other Poaceae roots (Bouffaud et al., 2014). Instead, the genera of Methylosinus and Pleomorphomonas have been identified from roots of *Oryza sativa,* another emergent macrophyte of Poaceae family (Bao et al., 2016; Xie and Yokota, 2005; Edwards et al., 2015). More interestingly, Pleomorphomonas genus has been also previously identified from root specimens of both the plants which are object of this study, i. e. *Phragmites australis* and *Typha latifolia* (Li et al., 2013).

Instead, microorganisms characterized by the taxonomy shown in Table 10B, C are those bacteria whose presence in the rhizoplane is influenced by plant species and thus colonize preferentially or uniquely the rhizoplane of *Phragmites australis* or *Typha latifolia*. Therefore, it can be argued that these two bacterial sub-communities of *Phragmites* and *Typha* rhizoplanes are recruited at the root surface of one or the other plant by signals which are plant-species-specific and determine the establishment around roots of advantaging conditions for their selectively survival or interaction with the other conserved members of the rhizoplane community. In addition, considering the different rooting sites of the two plant species, also physic-chemical properties of soils around roots can influence the selection at root surface for specific microbes directly creating conditions that benefit certain types of microbes or indirectly influencing the availability of plant root exudates and their up-taking by microbes (Lareen et al., 2016). In such a model, the soil property, the species-

specificity of roots exudates in association with the kind of adaptation of some microbes to the root immune system are together mechanisms which can determine the differences among the two plant rhizoplanes (Lebeis et al. 2015, Bever et al., 2012; Bakker et al., 2013; Bulgarelli et al., 2013; Philippot et al., 2013).

Then, regarding to the bacterial genera revealed in both rhizoplanes we found that *Phragmites* and *Typha* rhizoplanes are constituted for the majority by genera of Gram negative bacteria, as this is interestingly also for the totality of genera shared between the two rhizoplanes (Table 11). Similarly, analyzing the shape of bacteria detected in rhizoplane microbiotas it resulted that prevalently rod shape bacteria constitute both rhizoplanes. These results confirm that the recruitment of bacterial cells at the root surface is not randomly performed, yet fine targeted to the selection of bacteria cells with specific traits. More likely, the composition of bacterial membrane could permit the establishment of physiochemical and electrostatic interaction between the root surface and the bacterial envelope, fundamental process required in the first step of biofilm formation. Then, to stabilize the attachment, bacterial cells have developed a series of surface adhesins promoting specific or non- specific adhesion under various environmental conditions and numerous types of fimbrial, non-fimbrial and discrete polysaccharide adhesins have been detected in gram-negative bacteria (Berne et al., 2015). Together these findings prospect a possible scenario, where specific bacteria, mostly belonging to the Gram negative group and characterized by rod shape, are attracted by species-specified and not specific- signals from the surrounding at the root surface of the two plants. Firstly, physiochemical and electrostatic forces permit the tight interaction between bacteria and root surface and then the interaction is stabilized by specific matching of bacterial adhesion molecules to root receptors. On this assumptions, genera of Gemmata, Methylosinus, Planctomyces and Pleomorphomonas detected in both rhizoplanes are characterized on their envelope by adhesion molecules which join receptors present on the root surface of both studied plants. Instead, the genera reveled as differentially enriched in the two rhizoplanes perform their attachment recognizing distinct receptors on the root surface subjected to a plant species-specific diversification.

The metagenomic analysis conducted through PICRUSt software revealed that the prevalent functions associated to the detected OTUs in rhizoplane and also in rhizosphere of both plants are the “Transporters” and “Transcription factors” functions (Table 13). Although an important caveat, which is the uncertainty of the prediction, is invariably associated to this type of investigation our results are congruent with the ecological role played by bacteria in wetlands. For instance, as transporters regulate the influx of nutrients, efflux of toxic compounds from bacterial cells (López-

Guerrero et al., 2013; Blanco et al., 2016) and the secretion of molecules involved in biofilm formation (Berne et al., 2015), it is legitimate to hypothesize that this category of genes is required for the colonization of the root-soil interface in wetlands. Interestingly, our analysis did not reveal functions implicated in the antimicrobial biosynthesis and degradation, which emerged as a distinctive feature in a recent predictive metagenomic survey of *Phragmites* (Bowen et al., 2017). Yet, a closer examination of the published dataset revealed that these functions are not significantly different among plants adapted to a given environment. Considering that our investigation focused on an established natural ecosystem and the plants have been adapted to this environment for years, this scenario can explain the apparent discrepancy between our and the aforementioned Bowen et al. study. This observation, combined with the differential enrichment of 'Transcription factors' function suggest that *Typha* and *Phragmites* microbiotas define functionally congruent units whose metabolic adaptation is to a given microhabitat and/or host species is mediated, at least in part, by a transcriptional reprogramming.

To gain further insights on the spatial organization of the rhizoplane microbiota we subjected root specimens to Scanning Electron Microscopy analysis. We directly observed the rhizoplane microbiota on the root specimens. Two major findings emerged from this analysis. First, we observed microbial matrix-like assemblages on the rhizoplane of both plant species (Figure 11B,F) whose adherence to the rhizoplane could be compromised only by two consecutive ultrasound treatments (Figure 11C,D; G,H). Second, we observed that the strength of such adhesion to the rhizoplane was influenced by the host species: microbial matrix-like assemblages detected on *P. australis* appeared more stable and resistant to ultrasound treatments than the ones detected on the roots of *T. latifolia* (compare Figure 11D with 11H). Although the technique used prevented us to generate an accurate enumeration of the bacteria proliferating on the rhizoplane, our results appear in contrast with the findings of Faußer et al. (2012), which showed a greater number of bacterial cells dislocated on root surface of *Typha latifolia* respect to *Phragmites australis* plants. This suggests that environmental factors contribute to shape the rhizoplane communities organization in wetland plants. An important prediction of this analysis is the fact that SEM micrographs confirmed that the ultrasound treatment produced, in both tested species, an effective dislodgment of the microbial-like aggregates from the rhizoplane, allowing us to enrich for and characterize members of the rhizoplane microbiota.

The evidence that our specimens were enriched effectively for microbial cells from the root surface motivated us to investigate the culturable portion of communities and their potential ability to form biofilm during three different incubation times. In detail, we isolated 20 morphologically

distinct colonies forming units (CFUs) from *P. australis* and 31 from *T. latifolia* rhizoplane specimens (Figure 12), and only two of them resulted unable to form biofilm in any tested incubation times *in vitro* (Figure 13). The majority of them resulted biofilm formers at least in one of the tested incubation times. In particular, 8 of them maintained a strong ability to form biofilm until later time points. Strikingly, taxonomic identification of 20 individual strains performed using 16S rRNA gene sequencing revealed an overlap between the higher taxonomic ranks (i.e., family and phylum level) of the rhizoplane-enriched microbiota and bacteria identified in the isolation-assay (compare taxonomic assignments of Figure 8 with the ones of Figure 13). For instance, among the rhizoplane isolates, the Bacillaceae family resulted the most represented one followed by Microbacteriaceae and Streptomycetaceae and finally by Weeksellaceae, Comamonadaceae, Enterobacteriaceae, Exiguobacteraceae, Pseudomonadaceae, Xanthomonadaceae and Oxalobacteraceae. Therefore, our data indicate that a) the biofilm-forming capacity is a distinctive feature of the bacteria isolated from rhizoplane preparations and b) these bacteria are phylogenetically-related to members of the rhizoplane microbiota identified in the Illumina sequencing survey. This becomes evident when looking at members of the Bacillaceae and Oxalobacteraceae families, whose enrichment in the rhizoplane significantly discriminate this compartment from the microbiota surrounding *Phragmites* and *Typha* roots (Table 14).

Together, our results suggest that plant-mediated mechanisms and the metabolic potential of individual bacteria thriving in water sculpt the microbiota developing at the *Phragmites* and *Typha* root-soil interface.

Chapter 6

6. CONCLUSIONS AND PERSPECTIVES

The research presented in this doctoral thesis fill some of the gaps of knowledge about the rhizoplane microbiota of the two most common aquatic plants in natural and artificial wetlands, i.e. *Phragmites australis* and *Typha latifolia*.

In detail, it has found that the rhizoplane microbiota of both plants results significantly different from the microbial communities of the microhabitats surrounding roots and this clearly show that roots create specific micro-conditions which influence and/or enhance their interaction with specific microorganisms, different respect to the ones populate rhizosphere or water. However, regardless to the initial inoculum represented from water, *Phragmites australis* and *Typha latifolia* shape at their root surface a microbiota which converge to a similar composition and this evidence suggest that the shaping of rhizoplane microbiota in wetland ecosystem is influenced larger by the microhabitat factor and only less from the plant species. In detail the two plants are able to interact at their root surface with the same core of bacterial phyla constitute by Proteobacteria, Planctomycetes, Actinobacteria, Acidobacteria and Firmicutes and this finding could be relevant in view of a possible manipulation of the rhizoplane microbiota for the improvement of phytodepuration in those systems which contain both the plants species. Moreover, this research has permitted to more deeply characterize some of the bacterial components of the rhizoplane community. The isolation of bacterial strains able to form biofilm *in vitro* is another important finding of the research. These microorganisms could be able to transform ad remove particular pollutants from water or sediment and at the same time they could presumably act as biofilm formers also on the root surface, thus they could perform their beneficial action and stabilize their interaction with roots forming biofilm and withstanding to the water flow. In addition, also the Scanning Electron Microscopy analysis of roots confirmed that the rhizoplane microbial community characterized by Illumina sequencing and whose culturable members have been isolated is organized on the root surface forming biofilm-like assemblages.

Our findings will set the stage for the development of Synthetic Communities (SynComs) of the tested plant species. This, together with the generated knowledge, represent a fundamental pre-requisite for a) formulate and test novel hypotheses regarding the ecological significance of the wetland plants microbiota and b) the rational manipulation of plant-microbiota interactions for the phytodepuration improvement.

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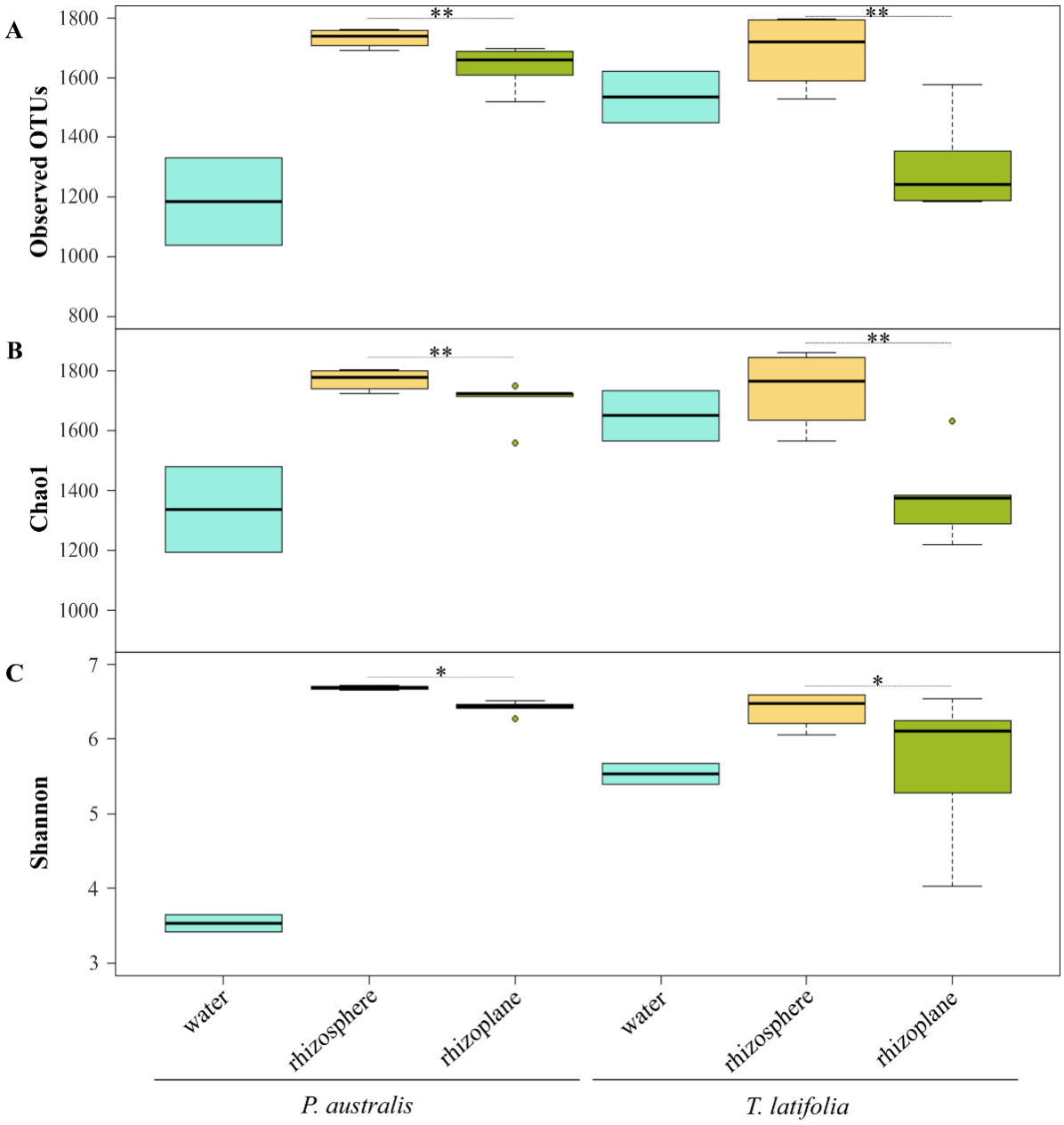
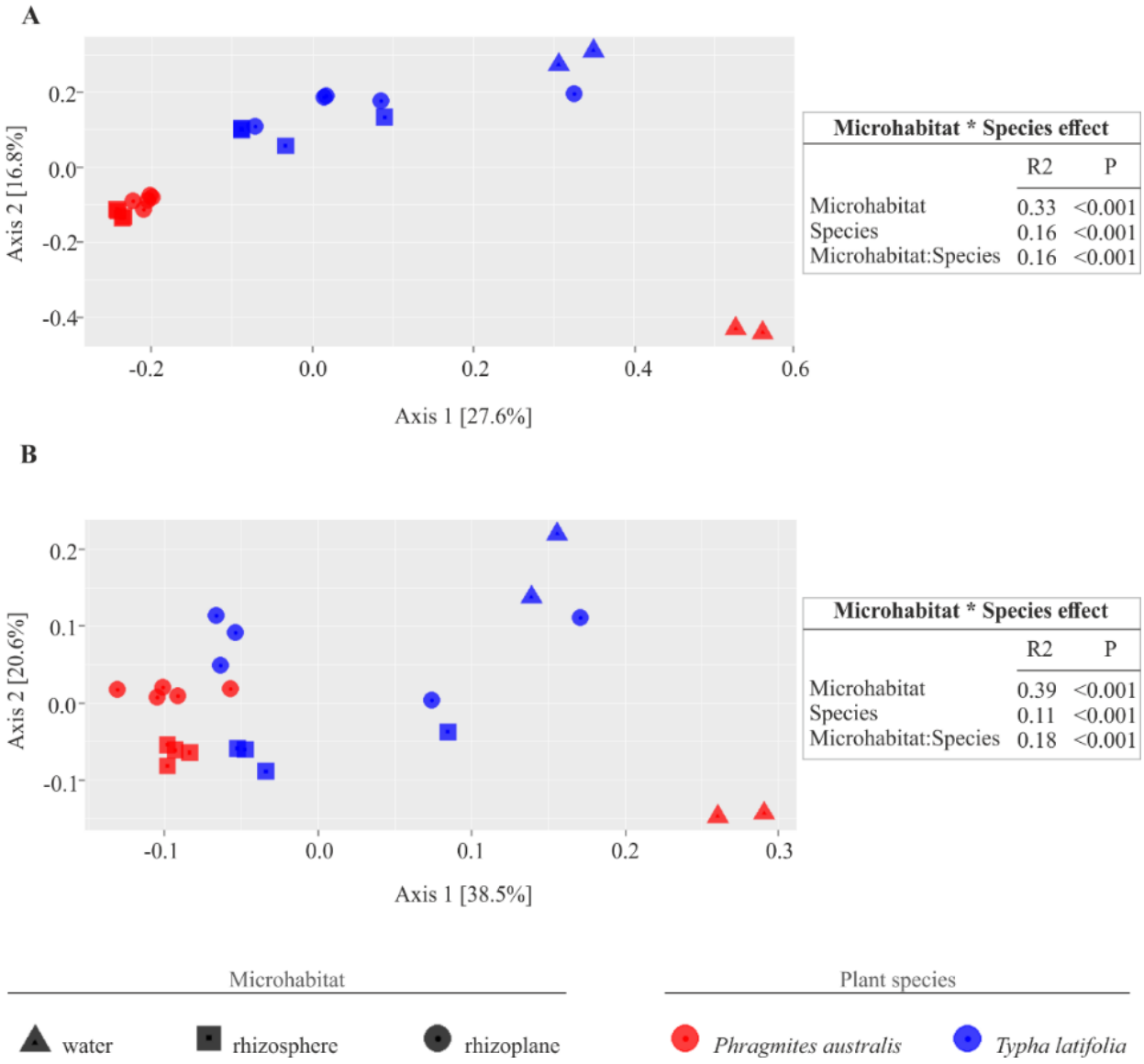
SUPPLEMENTARY MATERIAL

Supplementary figures



SUPPLEMENTARY FIGURE 1. Alpha diversity calculation for samples set2. OTUs richness of water, rhizosphere and rhizoplane microbiotas of *Phragmites australis* and *Typha latifolia* indicated by number of Observed OTUs **(A)** and by Chao1 index **(B)**. The OTUs eveness of the two plants microbiotas is shown by Shannon index **(C)**. Upper and lower edges of the box plots represent the upper and lower quartiles, respectively. The bold line within the box denotes the median. Maximum and minimum observed values are represented by the whiskers. Dots denote outlier observations whose value are 3/2 times greater or smaller than

the upper or lower quartiles, respectively. Asterisks denote statistically significant differences between rhizosphere and rhizoplane microhabitats (**P < 0.01, * P < 0.05).



SUPPLEMENTARY FIGURE 2. Beta-diversity calculation of samples set2. On the left, the PCoA plots show the distance between samples calculated on the base of Bray-Curtis index sensitive to the OTUs relative abundance **(A, left)** and on the base of weighted UniFrac index sensitive to both OTU relative abundances and taxonomic affiliation **(B, left)**; colors indicate the plant species and shapes the microhabitats whom samples belong to. On the right, the permutational analysis of variances for the indicated sources of variation calculated for the Bray-Curtis **(A, right)** and weighted UniFrac **(B, right)** indexes. The R2 value shows the proportional effect of the indicated factors in the samples distancing and the P-values were calculated for 5,000 permutations.

Supplementary tables

SUPPLEMENTARY TABLE 1. Rhizoplane enriched OTUs. Taxonomical classification of OTUs differentially enriched in the rhizoplane microbiota of each and both plants respect to the rhizosphere.

ENRICHED OTUs IN THE RHIZOPLANE MICROBIOTA OF						
Phragmites australis						
KINGDOM	PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES
Bacteria	Acidobacteria	[Chloracidobacteria]	RB41	Ellin6075		
Bacteria	Acidobacteria	Acidobacteria-6	iii1-15			
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales			
Bacteria	Actinobacteria	Actinobacteria				
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales			
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae		

Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	
Bacteria	Actinobacteria	MB-A2-108	0319-7L14		
Bacteria	Actinobacteria	MB-A2-108	0319-7L14		
Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	
Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	
Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales		
Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Saprospiraceae	
Bacteria	Chloroflexi	Thermomicrobia	JG30-KF-CM45		
Bacteria	Chloroflexi	Ellin6529			
Bacteria	Chloroflexi	Ellin6529			
Bacteria	Chloroflexi	Ellin6529			
Bacteria	Chloroflexi	Ellin6529			
Bacteria	Chloroflexi	Ellin6529			

Bacteria	Cyanobacteria	4C0d-2	YS2		
Bacteria	Firmicutes	Bacilli	Bacillales		
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	Pirellula
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	A17
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	A17
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	
Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces
Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	

Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Steroidobacter
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	SBla14		
Bacteria	Proteobacteria	Betaproteobacteria			
Bacteria	Proteobacteria	Betaproteobacteria			
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales		
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	

Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Pleomorphomonas
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Methylosinus

Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Methylosinus
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobulbaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobulbaceae	Desulfobulbus
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales		
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales		
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	Geobacter
Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Luteolibacter

Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae		
Bacteria	Verrucomicrobia	[Spartobacteria]	[Chthoniobacterales]	[Chthoniobacteraceae]	Candidatus Xiphinematobacter	
Typha latifolia						
KINGDOM	PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES
Bacteria	Acidobacteria	[Chloracidobacteria]	DS-100			
Bacteria	Acidobacteria	Acidobacteria-6	iii1-15			
Bacteria	Acidobacteria	Acidobacteria-6	iii1-15			
Bacteria	Acidobacteria	Sva0725	Sva0725			
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Microthrixaceae		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Microthrixaceae		

Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	EB1017	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales		
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales		
Bacteria	Actinobacteria	MB-A2-108	0319-7L14		
Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	
Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	
Bacteria	Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae	Caldilinea
Bacteria	Chloroflexi	Chloroflexi	[Roseiflexales]	[Kouleothrixaceae]	
Bacteria	Chloroflexi	Chloroflexi	[Roseiflexales]	[Kouleothrixaceae]	
Bacteria	Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae	Alicyclobacillus

Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	selenatarsenatis
Bacteria	Firmicutes	Bacilli	Bacillales			
Bacteria	Firmicutes	Bacilli	Bacillales			
Bacteria	Firmicutes	Bacilli	Bacillales			
Bacteria	Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	Exiguobacterium	
Bacteria	Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	Exiguobacterium	
Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae		
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae		
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae		
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae		
Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces	
Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces	
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae		
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae		

Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata	
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata	
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata	
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata	
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Isosphaeraceae		
Bacteria	Planctomycetes	C6	d113			
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae		
Bacteria	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae		
Bacteria	Proteobacteria	Betaproteobacteria	SC-I-84			
Bacteria	Proteobacteria	Betaproteobacteria	SC-I-84			
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	viridiflava
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	rhizosphaerae
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	johnsonii
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	

Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Pleomorphomonas
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Methylosinus
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Methylosinus
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		

Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales			
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter	

Phragmites australis and ***Typha latifolia***						
KINGDOM	PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES
Bacteria	Acidobacteria	Acidobacteria-6	iii1-15			
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111		
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae		
Bacteria	Actinobacteria	MB-A2-108	0319-7L14			
Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae		

Bacteria	Firmicutes	Bacilli	Bacillales		
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	
Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Pleomorphomonas
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Methylosinus
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Methylosinus
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		

SUPPLEMENTARY TABLE 2. Taxonomy of rhizoplane isolates. Taxonomical classification of rhizoplane isolates with the correspondent OTU code from Greengenes database.

ISOLATE	GREENGENES OTUs	TAXONOMICAL CLASSIFICATION						
		Kingdom	Phylum	Class	Order	Family	Genus	Species
P3	580010	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	
P6; P9; P15	357169							
T12; T4	2034540	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae		
T13	3599421							
P14	412145	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	
T8	4458776	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	chocolatum
T16	535932							
T24	3017908	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	
T30	1129906	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Wautersiella	
P4	341259	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		
T1	681779	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		

P13	1108343	Bacteria	Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	Exiguobacterium	
T6	536390	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	
T10	750541	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae		
T20	237591	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	lividum
T21	920852	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Streptomyces	mirabilis
T23	582591	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Streptomyces	

"We cannot fathom the marvelous complexity of an organic being; but on the hypothesis here advanced this complexity is much increased. Each living creature must be looked at as a microcosm – a little universe, formed of a host of self-propagating organisms, inconceivably minute and as numerous as the stars in heaven."

Charles Darwin