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BIOREMEDIATION OF A POLYCHLORINATED BIPHENYL (PCB) POLLUTED SITE: DEGRADING POTENTIAL OF SOIL MICROBIOTA AND EXPLOITATION OF PLANT-BACTERIA INTERACTIONS FOR ENHANCED RHIZOREMEDIATION

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Cover:

Plants growing in greenhouse during a rhizoremediation experiment (pictures originally taken by Simone Anelli).

Contents

Abstract

The release of xenobiotic chemicals into the environment has dramatically increased over the last century following industrialization, with a consequent impact on the ecosystems and human health. Polychlorinated biphenyls (PCB), in particular, are among the twelve chlorinated organic compound families initially listed as persistent organic pollutants (POPs) by the Stockholm Convention on POPs. PCB, due to their chemical properties and high stability, have been widely used by industries in the twentieth century as dielectric and coolant fluids. Despite their production has been banned since the 1970s-1980s, these pollutants contaminate soils and waters and affect the ecosystems worldwide, being widespread global contaminants. Due to their high lipophilicity, PCB are recalcitrant to biodegradation, persist in the environment and bioaccumulate in the lipids of animals and humans, biomagnifying in the food web. It has been proved that PCB have relevant toxic effects on human health, including carcinogenic activity. The remediation of PCB-contaminated soils represents therefore a primary issue for our society; nonetheless, the available physical-chemical technologies have strong environmental and economic impact and are unsuitable for *in situ* soil remediation in extended contaminated areas. Rhizoremediation is a type of phytoremediation that relies on the capability of soil microbes responding to plant biostimulation, to degrade pollutants. This strategy appears as the most suitable for the detoxification of large-scale PCB-polluted soils. Among soil contaminants, rhizoremediation of PCB is specifically relying on the positive interactions between plants and microorganisms in the rhizosphere. In fact, several organic aromatic compounds released through root deposition can promote the activation of the biphenyl catabolic pathway that is responsible for the microbial oxidative PCB metabolism, thereby improving the overall PCB degradation performance in aerobic conditions in soil. Moreover, plant-growth promoting (PGP) microorganisms selected in the rhizosphere can sustain plant growth under stressed conditions typical of polluted soils, in turn enhancing the plant biostimulation. Nevertheless, the efficiency of this biotechnology *in situ* has been poorly assessed in the scientific literature, since the upscaling from laboratory to greenhouse conditions to the field was rarely implemented. Site-specific environmental conditions still represent a major challenge for an efficient *in situ* rhizoremediation intervention, especially when it comes to understand how the pollution fingerprint affects the autochthonous degrading bacterial populations and whether these are able to establish positive interactions with the introduced plant species. This PhD project focused on the Site of National Priority (SIN) Caffaro, a large site located in Northern Italy historically polluted by chlorinated POPs and metals. Aim of the work was to study the phylogenetic and

functional diversity of the soil microbiota, assessing the correlation between diversity and pollutant profiles as a proxy to evaluate the biodegradation potential in the SIN Caffaro soil. A further aim of the thesis was to focus on the plant rhizosphere microbiome in order to setup *in situ* rhizoremediation strategies by evaluating the plant species with the higher potential for biostimulation.

The soil microbiome of three former agricultural fields within the SIN Caffaro was investigated with molecular ecology –16S rRNA metagenomic sequencing and DNA fingerprinting- and biochemical – fluorescein hydrolyses- approaches. The results revealed that the bacterial communities' structure, their phylogenetic diversity and the soil microbial activity were related with the soil physical and chemical parameters, both along the soil depth profile and across the surface of the area of collection. These findings suggest the adaptation of the microbial communities to the high xenobiotics concentrations in the soil, possibly resulting in PCB biodegradation abilities.

To assess the natural attenuation potential of autochthonous rhizosphere bacteria, we studied bacterial communities along a soil gradient from the non-vegetated to the root-associated soils of three different plant species spontaneously established in the most polluted field within the SIN Caffaro. The overall bacterial community structure of the non-vegetated and root-associated soil fractions was described by 16S rRNA metagenomic sequencing, and a collection of rhizobacteria isolates able to use biphenyl as unique carbon source was assayed for plant growth promotion (PGP) traits and bioremediation potential. The three plant species differentially affected the structure of the bacterial communities in the root-associated soil fractions, establishing the well known so-called rhizosphere effect. Nonetheless, the similar phylogenetic composition of the communities in all the soil fractions and the ubiquitous presence of the degrading potential, assessed by the presence of the *bphA* gene in the soil metagenome, leads to speculate that the soil contamination was one of the drivers for the enrichment of populations potentially able to sustain the process of natural attenuation. *In vitro* screening showed that biodegradation and PGP potential were widespread in the rhizosphere cultivable microbiome and the results of *in vivo* test on model plants suggested that two *Arthrobacter* sp. strains could be further investigated as bioenhancers on plant species of interest for rhizoremediation.

To assess the rhizoremediation potential of different plant species and soil treatments, a microcosm-scale experiment was set up with the SIN Caffaro soil in greenhouse conditions, and the biostimulation effect was studied on the soil microbiota at different sampling times for 24 months. Plant species and treatments were identified basing upon an extensive literature screening, aimed

to select the species/treatments that in previous studies showed to be effective in PCB rhizoremediation. The results of bacterial DNA fingerprinting and biochemical analysis of the soil surrounding the plant roots revealed that all the plants, when compared with unplanted control microcosms, significantly changed the bacterial communities' structure and stimulated the overall degrading activity in the soil. The stimulation of the soil microbiota leading to i) a shift in phylogenetic composition and ii) an increase in the organic matter hydrolytic activity, which may contribute to enhance PCB bioavailability and in turn their degradation in the polluted soil, is an indication of a potential positive rhizoremediation effect. The results need nevertheless to be substantiated by chemical analysis which will confirm the effective decrease of pollutants in soil surrounding roots and/or a change in the pollutants fingerprint. From the biostimulated soil a collection of Actinobacteria strains displaying *in vitro* biodegradation and PGP-related traits was also obtained. Three strains belonging to the genus *Rhodococcus* were in particular characterized for their PCB degradation capacity and for the ability to promote *Arabidopsis thaliana* growth and root development under laboratory conditions. Since *A. thaliana* root exudates previously showed to promote PCB degradation by a *Rhodoccoccus* bacterial isolate, these results open future research perspectives on the investigation of plant-bacteria interaction for PCB rhizoremediation.

Overall, the results disclosed the existence of a PCB natural attenuation potential within the autochthonous microbial communities of the SIN Caffaro soil, and pointed out that rhizoremediation could be an effective strategy to enhance soil detoxification. Further research should be focused to better characterise the degrading microbiome inhabiting the soil and to identify the best plant-treatment combination with the support of chemical investigations assessing the rate of PCB removal from soil. Also, *in vivo* test with PCB-degrading and PGP bacterial strains are required to assess their potential as bioaugmentation tools to sustain a rhizoremediation intervention.

Riassunto

Il rilascio nell'ambiente di composti chimici xenobiotici è cresciuto drammaticamente con l'industrializzazione durante lo scorso secolo, con conseguente impatto sugli ecosistemi e sulla salute umana. In particolare i policlorobifenili (PCB) sono una tra le dodici famiglie di composti organici clorurati inizialmente classificate come composti organici persistenti (Persistent Organic Pollutants, POPs) dalla convenzione di Stoccolma sui POPs. I PCB, grazie alle loro caratteristiche chimiche e all'elevata stabilità, sono stati ampiamente utilizzati dalle industrie come fluidi dielettrici e di raffreddamento nel ventesimo secolo e, nonostante la loro produzione sia stata vietata tra gli anni 70 e 80, continuano ad avere un effetto inquinante sugli ecosistemi contaminando suoli e acque a livello globale. A causa della loro idrofobicità i PCB sono recalcitranti alla biodegradazione, persistenti nell'ambiente e bioaccumulano nei lipidi degli organismi, dando origine a biomagnificazione nelle reti trofiche. E' stato dimostrato che i PCB hanno effetti tossici significativi per la salute umana e sono potenziali carcinogeni. La bonifica dei suoli contaminati da PCB rappresenta quindi una questione primaria per la società, nondimeno le tecnologie fisico-chimiche ad oggi disponibili hanno un forte impatto ambientale ed economico e sono quindi insostenibili per la bonifica *in situ* di aree inquinate molto estese. La rizodepurazione è un tipo di fitodepurazione che si basa sulla capacità delle piante di biostimolare la degradazione degli inquinanti da parte dei microrganismi del suolo, e costituisce la strategia più promettente per la bonifica su larga scala di suoli inquinati da PCB. In particolare la rizodepurazione dei PCB si basa sulle relazioni positive tra le piante e i microrganismi della rizosfera; infatti numerosi composti organici aromatici che vengono rilasciati attraverso la rizodeposizione sono in grado di promuovere l'attivazione della via catabolica del bifenile, responsabile della degradazione ossidativa dei PCB, migliorando così il tasso di degradazione aerobica di questi inquinanti nel suolo. Inoltre i microrganismi promotori della crescita vegetale (Plant-Growth Promoting, PGP) selezionati nella rizosfera possono sostenere la crescita della pianta nelle condizioni di stress tipiche dei suoli inquinati, favorendo di conseguenza l'attività di biostimolazione. Tuttavia, l'efficacia di questa biotecnologia *in situ* è stata scarsamente valutata dalla letteratura scientifica, dato che il passaggio da condizioni di laboratorio a prove in campo è stato effettuato raramente. Le peculiari condizioni ambientali di ogni sito costituiscono ancora la sfida maggiore per un efficace intervento di rizodepurazione *in situ*, in particolare quando di tratta di capire come la distribuzione degli inquinanti influenzi le popolazioni microbiche autoctone e se queste siano in grado di stabilire interazioni positive con le specie vegetali introdotte. Questa tesi di dottorato è stata incentrata sul Sito di Interesse Nazionale (SIN) Brescia-Caffaro, una vasta area

dell'Italia settentrionale storicamente inquinata da composi organici clorurati e metalli. Lo scopo del lavoro è stato studiare la diversità filogenetica e funzionale del microbiota del suolo, valutando la presenza di una correlazione tra la diversità e i profili degli inquinanti come indicatore per stimare il potenziale di biodegradazione presente nei suoli del SIN Caffaro. Scopo ulteriore di questa tesi è stato valutare il potenziale di biostimolazione di diverse specie vegetali sul microbiota rizosferico, per lo sviluppo di strategie di rizodepurazione *in situ.* Il microbiota del suolo di tre ex campi agricoli inclusi nel SIN Caffaro è stato studiato con metodi di ecologia molecolare (DNA fingerprinting e sequenziamento metagenomico del gene rRNA 16S) e biochimici (idrolisi della fluoresceina diacetato). I risultati hanno mostrato come la struttura delle comunità batteriche, la loro diversità filogenetica e l'attività microbica nel suolo fossero correlate con i parametri chimico-fisici del suolo, sia secondo la profondità che secondo l'area di raccolta dei campioni. Questi risultati suggeriscono l'adattamento delle comunità microbiche alle alte concentrazioni di xenobiotici nel suolo, e verosimilmente una capacità di degradazione dei PCB. Per valutare il naturale potenziale di attenuazione dell'inquinamento da parte dei batteri rizosferici autoctoni, sono state studiate le comunità batteriche lungo un gradiente esteso dal suolo privo di vegetazione al suolo associato alle radici di tre specie vegetali spontanee cresciute nel campo più inquinato all'interno del SIN Caffaro. La struttura delle comunità batteriche delle frazioni di suolo privo di vegetazione e di quelle associate alle radici delle piante è stata descritta mediante sequenziamento metagenomico del gene rRNA 16S, e una collezione di batteri rizosferici in grado di utilizzare bifenile come unica fonte di carbonio è stata saggiata per attività di promozione della crescita vegetale (PGP) e per il potenziale di biodegradazione. Le tre specie vegetali influivano in modo diverso sulla struttura delle comunità batteriche nelle frazioni di suolo associate alle radici, stabilendo il cosiddetto "effetto rizosfera". Nondimeno, la similarità nella composizione filogenetica delle comunità in tutte le frazioni di suolo e la presenza ubiquitaria del potenziale degradativo, valutata amplificando il gene *bphA* dal metagenoma del suolo, consentono di speculare che la contaminazione del suolo sia uno dei fattori responsabili dell'arricchimento di popolazioni potenzialmente in grado di sostenere un processo di attenuazione naturale dell'inquinamento. Lo screening *in vitro* ha mostrato come il potenziale PGP e di biodegradazione fossero ampiamente diffusi nella frazione coltivabile del microbiota rizosferico e i risultati positivi del test *in vivo* su piante modello suggeriscono che due ceppi appartenenti al genere *Arthrobacter* potrebbero essere ulteriormente saggiati per la promozione della crescita di piante di interesse per la rizodepurazione. Per valutare il potenziale di rizodepurazione di diverse specie vegetali e di diversi trattamenti del suolo, è stato allestito un esperimento in serra in scala di

microcosmo con il suolo proveniente dal SIN Caffaro, e l'effetto di biostimolazione sul microbiota del suolo è stato valutato a diversi tempi di campionamento nell'arco di 18 mesi. Le specie vegetali e i trattamenti del suolo sono stati scelti in seguito a un'estesa ricerca bibliografica, con l'obiettivo di selezionare specie e trattamenti efficaci nella rizodepurazione di PCB secondo studi precendenti. I risultati del fingerprinting del DNA batterico e delle analisi biochimiche del suolo sotto l'influenza dell'apparato radicale hanno rivelato che tutte le specie vegetali modificavano in modo significativo le comunità batteriche e stimolavano l'attività enzimatica nel suolo se confrontate con i rispettivi microcosmi di controllo non piantati. La stimolazione del microbiota del suolo, che porta a i) un cambiamento nella distribuzione filogenetica e ii) un aumento dell'attività idrolitica della materia organica, che potrebbe contribuire ad aumentare la biodisponibilità dei PCB e quindi la loro degradazione nel suolo inquinato, indica un risultato potenzialmente positivo del processo di rizodepurazione. I risultati devono comunque essere corroborati da analisi chimiche per confermare un effettivo decremento dei livelli di inquinamento nel suolo e/o un cambiamento nel profilo degli inquinanti. Inoltre, dal suolo sottoposto a biostimolazione è stata ottenuta una collezione di ceppi appartenenti alla classe *Actinobacteria* che hanno mostrato attività PGP e di biodegradazione *in vitro.* In particolare tre ceppi appartenenti al genere *Rhodococcus* sono stati caratterizzati per la loro capacità di degradare i PCB e di promuovere la crescita e lo sviluppo radicale di *Arabidopsis thaliana* in condizioni di laboratorio. Questi risultati aprono nuove prospettive di ricerca nello studio delle interazioni pianta-batterio per la rizodepurazione di PCB, dato che studi precedenti hanno dimostrato la capacità di essudati radicali di *A. thaliana* di promuovere la degradazione di PCB da parte di un ceppo del genere *Rhodococcus.*

Complessivamente, i risultati ottenuti mostrano l'esistenza di un potenziale naturale di attenuazione da parte delle comunità microbiche autoctone del suolo del SIN Caffaro e indicano che la rizodepurazione potrebbe essere una strategia efficace per la bonifica del suolo. Ulteriori ricerche dovranno essere focalizzate a caratterizzare il microbiota del suolo con capacità degradative e ad indentificare la miglior combinazione di specie vegetali e trattamento del suolo con il supporto di indagini chimiche in grado di stabilire il tasso di riduzione dei PCB. Inoltre, sono necessari test *in vivo* per verificare il potenziale di ceppi batterici PGP e PCB-degradatori per interventi di bioaugmentation volti a sostenere strategie di rizodepurazione.

Rational and aim of the work

Polychlorinated biphenyls (PCB) are a class of xenobiotics that had been extensively produced and used worldwide during the 20th century for several industrial purposes. After serious concerns about their human toxicity, between the 1970s and the 1980s several countries restricted or banned their use, nonetheless their dispersal in the environment still makes the disposal of these compounds a global problem. PCB were listed as persistent organic pollutants (POPS) by the Stockholm Convention on Persistent Organic Pollutants (2009) and their persistence, bioaccumulation through the food web and toxicity following the contamination of waters and soils have been assessed. Their safe removal from polluted ecosystems represents thereby a priority for our society. Despite that, PCB-remediation is still a challenging task, being available technologies often disruptive, unsustainable for the environment and unfeasible when considering the contamination of extended areas (Gomes et al. 2013). Phytoremediation has now emerged as a promising strategy for *in situ* removal of many organic contaminants through a number of processes (e.g. phytovolatilization, phytodegradation, phytoextraction). Nonetheless, unlike microorganisms, plants lack the enzymes and metabolic pathways that confer the capacity to degrade chemically recalcitrant hydrophobic organic molecules, as are PCB. Moreover, high concentrations of contaminants tend to inhibit plant growth, in part due to oxidative stress, limiting the rate of phytoremediation. Contaminated soils also tend to be nutrient poor and/or lack microbial diversity, which contributes to sub-optimal plant biomass accumulation, impeding as well the rates of remediation (McGuinness and Dowling, 2009; Gerhardt et al. 2009). Rhizoremediation is a phytoremediation technology that relies on complex interactions between the plant and the microbial populations of the rhizosphere soil, and it has been described as an effective strategy for the detoxification of PCB-impacted soils in the sites where degrading microorganisms have been demonstrated to occur. In this case, aromatic compounds released in the soil by root deposition stimulate the proliferation and the activity of rhizosphere bacteria, representing a carbon source and activating as co-metabolites the biphenyl degradation pathway (*bph*) responsible for PCB aerobic degradation (Van Aken et al. 2010). Moreover, bacteria selected in the rhizosphere often display beneficial plant-growth promoting (PGP) capabilities that sustain plant growth and development in phytotoxic soils (Ma et al. 2011).

This PhD thesis focuses on the Site of National Priority (SIN) Caffaro, an aged PCB-contaminated area located in northern Italy nearby the city of Brescia. This site includes more than one hundred hectares of former agricultural fields displaying chlorinated POPs, arsenic and mercury as major contaminants, distributed in variable concentrations and often exceeding the safety values established by the law. The extension of the site makes excavation-based remediation technologies

unsuitable for the SIN Caffaro, which in turn represents a case study to set up *in situ* rhizoremediation strategies for the reclamation of soils affected by PCB pollution. The overall objective of this PhD project was the study of the microbial communities inhabiting the contaminated soils within the SIN Caffaro, to depict their diversity in term of phylogeny and functionality (i.e. degradation potential toward PCB and PGP potential) and to find new microbial resources to setup biotechnological strategies for the enhancement of soil rhizoremediation. Part of this thesis was dedicated to explore the natural attenuation potential of the SIN Caffaro soils, taking advantage of the selective pressure imposed by pollutants on the autochthonous microbiome. Molecular and biochemical tools in microbial ecology were applied to study the microbial diversity and activity in relation to the peculiar chemical-physical soil characteristics of three abandoned agricultural fields within the SIN Caffaro. The taxonomic diversity and functional potential of bacterial communities associated to different spontaneous plant species naturally selected in this historically contaminated environment was investigated, considering the occurrence of the rhizosphere effect. Although rhizoremediation occurs putatively naturally, the introduction of suitable plant species and soil treatments may optimize the biostimulation of the potential PCB-degrading microbiome, in order to increase the contaminant depletion rates. For this reason, this thesis contributed with an extensive literature research to the setup of a rhizoremediation experiment in greenhouse conditions, aiming to select the most promising plant species and soil treatments for the detoxification of polluted soils collected within the SIN Caffaro. The effect of the different treatments applied at pot-scale in greenhouse conditions on the soil microbiome was then evaluated, to assess the effect of biostimulation on the bacterial or fungal communities' structure and on the overall microbial activity in the soil. In addition, this project aimed to establish a wide bacterial collection from the SIN Caffaro polluted soils, selecting strains showing *in vitro* and *in vivo* PGP traits together with PCB aerobic degradation potential to be further exploited in the field to improve and sustain PCB rhizoremediation strategies.

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Introduction

Phyto-rhizoremediation of polychlorinated biphenyl contaminated soils: an outlook on plant-microbe beneficial interactions

1. Introduction

An increasingly industrialized economy over the last century has led to a dramatically elevated release of anthropogenic chemicals into the environment, with a consequent impact on human health and on the ecosystems. Prevalent contaminants include aliphatic and polycyclic aromatic petroleum hydrocarbons, halogenated compounds, pesticides, solvents and metals. Polychlorinated biphenyls (PCB) in particular are among the twelve chlorinated organic compound families initially listed as persistent organic pollutants (POPs) by the Stockholm Convention on Persistent Organic Pollutants (chm.pops.int). PCB are synthetic organic compounds derived by chlorination of a biphenyl molecule. Basing on the number (1 to 10) and the position (meta, ortho and para) of the chlorine substitutes, PCB are classified in 209 different congeners. They are highly soluble in apolar solvents, while their vapour pressure and water solubility are extremely low and decrease with the increasing number of chlorine atoms. PCB, due to their chemical properties and high stability, have been widely used by industries in the twentieth century as dielectric and coolant fluids (Qi et al. 2014). Despite their production has been banned since the 1970s-1980s, these pollutants contaminate soils and water nearby former manufacture sites and also affect the ecosystems worldwide, being widespread global contaminants due to their large use in the past. Due to their high lipophilicity, PCB have high affinity for the organic matter in water sediments, atmospheric particulate and soils, where their degradation is very slow, showing half-life values that can vary from 3 to 37 years and increase with the degree of chlorination (Sinkkonen and Paasivirta, 2000). In addition, PCB bioaccumulate in the lipids of animals and humans, biomagnifying in the food web (Beyer and Biziuk 2009; Bertocchi et al. 2015; Jepson et al. 2016). It has been proved that PCB have relevant toxic effects on human health, being related to neurological, reproductive, cutaneous and endocrine diseases (Quinete et al. 2014). PCB can be divided into two classes: congeners with toxic effects comparable to polychlorinated dibenzodioxins (PCDD) and polychlorinated dibenzofurans (PCDF), named dioxin-like PCB (dl-PCB), for which the World Health Organization (WHO) established toxicity equivalent factors (TEF) (Van den Berg et al. 2006), and congeners which do not share PCDD/F toxic mechanism, named non-dioxin like PCB (ndl-PCB). PCB were included among the so called “endocrine disruptors” and banned after the Stockholm convention. Furthermore, PCB have been recently classified by the International Agency for Research on Cancer as carcinogenic to humans (Group 1) (Qi et al. 2014; IARC 2015). For these reasons, the capability of effectively reducing the concentration of PCB represents an urgent and primary goal in many contaminated environments. In this review, we focus on PCB removal from polluted soils using phytoremediation

and rhizoremediation technologies, based on the ability of plants and soil microorganisms to uptake, transform and degrade these chemicals. These technologies need a long period to be effective, and their success resulted often controversial (Passatore et al. 2014). Phyto- rhizoremediation represents, nevertheless, an *in situ* sustainable alternative to thermal and physical/chemical treatments, which often require the removal of the contaminated soil from quite extended sites, resulting in high costs from both economic and environmental perspectives (Gomes et al. 2013).

2. Phyto- and rhizoremediation of PCB-contaminated soils

Phytoremediation has recently emerged as a promising strategy for *in situ* removal of many organic contaminants through a number of processes performed by different plant species (e.g. phytovolatilization, phytodegradation, phytoextraction) (Sandermann 1994; Schnoor et al. 1995; Cunningham et al. 1995). Nonetheless, being photoautotrophic, plants do not rely on organic molecules as source of energy or carbon. Therefore we can suppose that, unlike microorganisms, during evolution plants were not under selective pressure to develop the capacity to degrade recalcitrant organic molecules like PCB, leading to a much more limited spectrum of chemical structures that they can mineralize (Van Aken 2008). Moreover, high concentrations of contaminants in the soil could significantly inhibit plant growth, including root growth, in part due to oxidative stress, limiting the rate of phytoremediation (Hu et al. 2016; Gerhardt et al. 2009). Other limiting factors for plant growth and phytoremediation efficiency are the lack of nutrients and low microbial diversity often associated with polluted soils (McGuinness and Dowling 2009; Gerhardt et al. 2009). In the case of PCB, biodegradation in soil depends on different factors including pollution age and soil organic carbon content (Passatore et al., 2014). These key factors control PCB bioavailability in soil, which is much reduced by the relevant adsorption to organic carbon resulting from the high hydrophobicity of these molecules. Rhizoremediation is a specific type of phytoremediation that involves both plants and their associated rhizosphere microbes. It relies on the stimulation by the plant of the degrading microbes in its rhizosphere, and it has been described as an effective strategy for the removal and/or the degradation of organic contaminants from impacted soils (Kuiper at al. 2004). Complex interactions involving roots, root exudates, rhizosphere soils and microbial communities can result in the degradation of PCB to non-toxic or less toxic compounds, potentially leading to soil clean-up (Van Aken et al. 2010).

3. Phytoremediation players: PCB removal by plants

3.1 Metabolism of PCB in plants

Plants have the potential to metabolize some organic xenobiotic compounds through a pathway that generally includes three steps: activation, conjugation and compartmentalization. This pathway involves detoxification enzymes such as cytochrome P450, N-glucosyltransferase and glutathione S-transferase, reacting with POP chemicals to make them more hydrophilic and store them into the vacuoles or cell walls. This process presents high similarities with the detoxification systems of mammals, leading to the formulation of the "green liver" concept (Fletcher et al. 1987; Sandermann 1994; Campanella et al. 2002). However, the plant detoxification potential of hydrophobic chemicals such as PCB is heavily affected by a limited uptake capacity, due to the generally strong adsorption of these compounds to the soil organic matter (Schäffner et al. 2002). Nonetheless, it has been reported that some species belonging to the family of *Cucurbitaceae* are able to uptake chlorinated recalcitrant compounds as a consequence of nutrient uptake, mediated by root exudates (Schäffner et al. 2002; Ficko et al. 2011a), and to translocate them into the shoot tissues through the xylem sap (Greenwood et al. 2011). The mechanism of this process has not yet been described for PCB, however Whitfield Åslund and colleagues (2007) formulated two hypotheses based on former studies regarding the uptake and transport of other chlorinated POPs. One of them suggests that the uptake of these compounds could be enhanced by the exudation of high levels of specific low molecular weight organic acids that were reported to cause the release of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) from the soil organic matrix (Luo et al. 2006). The second hypothesis is that root exudates involved in nutrient uptake would bind POP molecules, such as dioxins and furans, making them more hydrophilic and thereby available not only for plant uptake but also for their translocation (Hülster et al. 1994; Campanella and Paul 2000; Campanella et al. 2002). Low and co-authors (2010) suggested that in the case of *Cucurbita pepo* spp. *pepo* it is important to consider the experimental duration, since the quantity of PCB removed from the soil is higher when the plant biomass increases.

Direct evidences of PCB metabolism in plants have been obtained only in very simplified conditions, and further studies are required to better understand the mechanisms and the potential of this process in contaminated environments. The ability of different plant species to metabolize PCB has been tested *in vitro* by using cell cultures. Living cells of *Solanum nigrum* hairy roots showed the capacity to hydroxylate PCB molecules. Three mono-chlorobiphenyls (-CB) (PCB2, PCB3, PCB4) were transformed in monohydroxy-CB and dihydroxy-CB, while 6 di-CB (PCB4, PCB7, PCB8, PCB10, PCB11,

PCB13) were transformed in different monohydroxy-CB (Kucerova et al. 2000). Cell cultures of the same plant were furthermore tested by Rezek and colleagues (2007) for the ability to metabolize di-, tri-, tetra- and penta-CB (PCB 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, 28, 30, 31,38, 40, 52, 77, 80, 81 and 101). *S. nigrum* cells demonstrated a hydroxylation activity producing biphenylol metabolites, without changing the degree of chlorination of the modified PCB congener. The number of metabolites was lower for tri- compared to di-CB and the authors did not observe any metabolite of tetra- and penta-CB, with the exception of PCB 77 (Rezek et al. 2007). In a different study, axenic cell cultures of tobacco (*Nicotiana tabacum* WSC-38) were able to metabolize 6 di-CB, producing three types of metabolites: hydroxy-CB, methoxy-CB and hydroxy-methoxy-CB. The production of methylated compounds could be explained by the action of *O*-methyltransferases present in other metabolic pathways, like those involved in lignin biosynthesis. PCB metabolites display indeed structural similarity with plant compounds acting as lignin precursors (Rezek et al. 2008). Notably, according to these studies, plant metabolism can produce hydroxylated PCB that are even more toxic than the parental compounds.

The knowledge on the metabolic processes involved in PCB metabolism in plants is nevertheless still limited, even though common enzymes like peroxidase and cytochrome P450 are likely involved (Chroma et al. 2002). Zhai and colleagues studied the ability of *Populus deltoids* x *nigra* DN34 to transform chiral PCB mixture (PCB 95 and PCB 136) in an enantioselective way under hydroponic conditions. The results showed that mixtures of PCB 136 remained racemic in most parts of the plant, while PCB 95 was enantioselectively transformed inside poplar tissues, demonstrating that plant activity in PCB modification strictly depend on the congener type (Zhai et al. 2011).

3.2 Plant species tested for PCB uptake and/or degradation

As mentioned, plants are poorly adapted to uptake and metabolize PCB efficiently, with the apparent exception of some species of the *Cucurbitaceae* family. Nonetheless, a large number of plant species, including crops and spontaneous ones, has been tested in PCB phytoremediation experiments. Most of the studies focused, however, mainly on plant ability to stimulate PCB degradation by the soil microbial community (*i.e.* rhizodegradation) rather than to directly remove PCB from soil. Interestingly, in a recent study (Ficko et al. 2010), the ability of different weeds and crop species to extract PCB was described, comparing them with *Cucurbita pepo* ssp. *pepo*. The authors found out that 17 out of the tested 27 species were able to move a similar or greater quantity of contaminants than *Cucurbita pepo* ssp. *pepo* into the shoots, even if the uptake and translocation mechanisms remained undescribed.

The most frequent plant species employed for remediation experiments belong to the genera *Cucurbita* (*C. pepo, C. pepo spp. pepo, C. maxima,* and *C. moschata*), *Brassica* (*B. napus, B. nigra*), *Medicago* (*M. sativa*, *M. polymorpha*), *Nicotiana* (*N. tabacum*), *Festuca* (*F. arundinacea*) and *Panicum* (*P. virgatum, P. variegatum,* and *P. clandestinum*). As shown in Table 1, the vast majority of these works have been conducted in simplified systems using artificially spiked soils, *i.e.* containing freshly added PCB, and in greenhouse conditions. Indeed, PCB bioavailability in soil generally decreases with time due to increased sorption to the soil organic matter (Luthy et al. 1997) that in turns reduces the fraction available for biodegradation. For this reason, biodegradation experiments conducted in laboratory with spiked soil do not reproduce field conditions and could lead to overly optimistic biodegradation rates. This aspect implies a lack of knowledge about plant adaptation and performance *in situ*, where phytoremediation potential is affected by soil biochemical and physical characteristics, uneven, mixed and aged contaminations and variable environmental conditions (Campanella et al. 2002).

Table 1: Plant species and conditions applied in experiments of phytoremediation of PCB-contaminated soils.

	Plant species	environment	soil conditions	duration (days)	Bioagumentation by microbial inoculum	Reference
1	*Pinus nigra, Fraxinus excelsior, Betula pendula, Salix caprea, Robinia pseudoacacia*	Field	environmental polluted soil	nd	no	Leigh et al. 2006
2	*Cucurbita pepo, Festuca arundinacea, Carex normalis*	Field	environmental polluted soil	70	no	Whitfield Åslund et al. 2007
3	*Chrysanthemum leucanthemum* L., Rumex crispus L., Solidago canadensis L.	Field	environmental polluted soil	730	no	Ficko et al. 2011
4	*Zea mays* L., Helianthus annuus, Populus nigra x P. maximowiczii, Salix x smithiana	Field	environmental polluted soil	730	no	Kacálková et al. 2011
5	*Ambrosia artemisiifolia, Daucus carota, Polygonum persicaria, Setaria pumila, Sonchus asper, Vicia cracca, Amaranthus retroflexus, Brassica nigra, Cirsium arvense, Cirsium vulgare, Echinochloa crusgalli, Lythrum salicaria, Solidago canadensis, Symphyotrichum ericoides, Symphyotrichum novae-angliae, Barbarea vulgaris, Capsella bursa - pastoris, Chemopodium album, Chrysantemum leucanthemum, Echium vulgare, Medicago lupulina, Polygonum convolvolus, Sisymbrium officinale, Solanum nigrum, Trifolium pratense, Verbascum thapsus.*	Field	environmental polluted soil	nd	no	Ficko et al. 2010
6	*Pinus nigra L.*	Field	environmental polluted soil	nd	no	Leigh et al. 2007
7	*Medicago sativa*	Field	environmental polluted soil	180	*Glomum caledonium, Rhizobium sp.*	Teng et al. 2010
8	*Medicago sativa*	Field	environmental polluted soil	730	no	Tu et al. 2011
9	*C. pepo ssp. pepo cv. Howden*	Field	environmental polluted soil	70	no	Whitfield Åslund et al. 2008
10	*Medicago sativa*	Field	environmental polluted soil	90	*Rhizobium meliloti Burkholderia xenovorans*	Xu et al. 2010
11	*Panicum virgatum*	Greenhouse	PCB spiked soil	168	LB400	Liang et al. 2014
12	*Medicago sativa, Nicotiana tabacum, Solanum nigrum*	Greenhouse	environmental polluted soil	180	no	Demrenova et al. 2005
13	*Nicotiana tabacum, Solanum nigrum*	Greenhouse	environmental polluted soil	90	*BPseudomonas* sp. L15, *Pseudomonas* sp. JAB1, *Ochrobactrum* sp. KH6.	Kurzawova et al. 2012

14	*Cucurbita pepo ssp. pepo*	Greenhouse	environmental polluted soil	42	no	Low et al. 2010
15	*Brassica nigra*	Greenhouse	PCB spiked soil	63	*Ralstonia eutropha* H850, *Rhodococcus* sp. ACS	Singer et al. 2003
16	*Carex aquatalis, Spartina pectinata, Tripsacum dactyloidetes, Morus rubra, Scirpus fluvialis.*	Greenhouse	PCB spiked sediment	548	no	Smith et al. 2007
17	*Medicago sativa* L., Festuca arundinacea	Greenhouse	environmental polluted soil	150	no	Li et al. 2013
18	*Panicum virgatum, Populus deltoids x nigra DN34*	Greenhouse	PCB spiked soil	224	no	Meggo et al. 2013
19	*Oryza sativa*	Greenhouse	environmental polluted soil	240	no	Chen et al. 2014a
20	*Oryza sativa*	Greenhouse	environmental polluted soil	300	no	Chen et al. 2014b
21	*Cucurbita pepo, Cucurbita maxima, Cucumis sativus, Cucumis melo, Daucus carota, Lycopersicum esculentum*	Greenhouse	vermiculite amended with TCDD	90	no	Campanella et al. 2000
22	*Festuca arundinacea, Phalaris arundinacea, Panicum variegatum, Panicum clandestinum, Medicago sativa, Coronilla varia, Lespedeza cuneata, Lathyrus sylvestris*	Greenhouse	PCB spiked soil	180	no	Dzantor et al. 2000
23	*Lathyrus sylvestris, Phalaris arundinacea, Medicago polymorpha*	Greenhouse	PCB spiked soil	100	no	Dzantor et al. 2001
24	*Cucurbita pepo, Bidens cernua, Chenopodium album, Daucus carota, Plantago major, Rumex crispus*	Greenhouse	environmental polluted soil	92	no	Ficko et al. 2011b
25	*Lupinus luteus L.*	Greenhouse	environmental polluted soil	28	no	Gutiérrez-Ginés et al. 2014
26	*Nicotiana tabacum, Solanum nigrum, Armoracia rusticana, Salix caprea*	Greenhouse	environmental polluted soil	180	no	Ionescu et al. 2009
27	*Brassica napus*	Greenhouse	PCB spiked soil	75	no	Javorská et al. 2009
28	*Cucurbita pepo, Glycine max, Zea mays*	Greenhouse	PCB spiked soil	60	no	Li et al. 2011
29	*Lolium perenne*	Greenhouse	Environmental polluted soil	180	*Glomus caledoniun* L., *Eisenia foetida*	Lu et al. 2014
30	*Cucurbita pepo, Cucurbita moschata, Cucurbita maxima, Cucumis sativus*	Greenhouse	environmental polluted soil	60	Arbuscular mycorrhizal fungi (AMF)	Qin et al.2014
31	*Festuca arundinacea*	Growth chamber	environmental polluted soil	70	*Burkholderia xenovorans LB400*	Secher et al. 2013
32	*Medicago sativa, Lathyrus sylvestris, Lespedera cuneata, Panicum clandestinum, Festuca arundinacea, Panicum virgatum, Phalaris arundinacea*	Growth chamber	PCB spiked soil	120	no	Chekol et al. 2004
33	Panicum virgatum, Populus deltoids	Growth chamber	PCB spiked soil	224	no	Meggo et al. 2013

34	*Phragmites australis, Oryza sativa L. subsp. Indica*	Hydroponic	PCB and DDT amended growth medium	14	no	Chu et al. 2006
35	*Salix alaxensis, Picea glauca*	Microcosm	PCB spiked soil	180	no	Slater et al. 2011
36	*Citrus sinensis, Citrus reticulata, Pinus nigra, Hedera helix*	*in vitro*	PCB-amended growth medium	2	*Pseudomonas stutzeri*	Dudášová et al. 2012
37	*Atropa belladonna, Solanum aviculare, Armoracia rusticana, Solanum nigrum*	*in vitro*	PCB amended growth medium	25	no	Mackova et al. 1997
38	*Solanum nigrum*	*in vitro*	PCB amended growth medium	14	no	Kucerová et al. 2000
39	*Rosa* cv. Paul's Scarlet	*in vitro*	PCB amended growth medium	8	no	Fletcher et al. 1987
40	*Mentha spicata*	*in vitro*	PCB amended growth medium	0,6	*Arthrobacter sp.* strain B1B	Gilbert et al. 1997
41	*Medicago sativa, Nicotiana tabacum, Solanum nigrum*	no info	environmental polluted soil	180	no	Ryslavá et al. 2005

2

4. Rhizoremediation players: PCB-degrading microbes

Bacteria and fungi represent the main agents of PCB degradation in the environment, through two different pathways: anaerobic reductive dechlorination and aerobic oxidative degradation of the biphenyl ring (Furukawa 1994; Furukawa 2000; Field and Sierra Alvarez 2008). Reductive dechlorination is performed by halorespiring bacteria that use chlorinated compounds as electron acceptors for their energetic metabolism, thereby replacing chlorines with hydrogen atoms (Smidt et al. 2000). Currently, only some genera belonging to the phylum *Chloroflexi*, such as *Dehalococcoides,* are known to perform reductive dehalogenation on PCB, and they preferably remove chlorines in *para* and *meta* positions rather than *ortho* ones (Abraham et al. 2002; Furukawa and Fujihara 2008; Zanaroli et al. 2012). This process has been documented in anaerobic environments, like river sediments and flooded paddy fields (Brown et al. 1988; Chen et al. 2014a). The reduction of the number of chlorines makes PCB less toxic and generates dechlorination products (*i.e.* PCB with less than five chlorine atoms) that are more easily degradable by aerobic microorganisms able to attack the biphenyl ring (Demiterpe et al. 2015; Chen et al. 2014a; Master et al. 2002).

Oxidative biodegradation of PCB by aerobic bacteria results from the action of different catabolic enzymes expressed by the *bph* gene clusters. These genes, codifying for different dioxygenases, are spread among both Gram negative and Gram positive bacteria, including members of the genera *Pseudomonas*, *Burkholderia*, *Cupriavidus*, *Pandorea*, *Acidovorax, Sphingobium, Dyella, Achromobacter, Corynebacterium, Rhodococcus* and *Bacillus* and they have been detected on chromosomes, plasmids or transposons (Hu et al. 2015; Field and Sierra-Alvarez 2008; Furukawa and Fujihara 2008; Pieper and Seeger 2008). The finding of PCB degradative gene clusters in mobile genetic elements could support their horizontal spread in bacterial communities. Typically, two clusters of genes are involved in this metabolic pathway. The first one is responsible for the transformation of PCB into chlorobenzoates and chlorinated aliphatic acids (*i.e.* biphenyl upper pathway), and the second one is involved in further mineralization of chlorobenzoates and aliphatic acids (*i.e.* biphenyl lower pathway) (Leewis et al. 2016; Pieper and Seeger 2008). The upper pathway, which is similar for all described aerobic PCB degraders, involves seven genes, grouped into one operon (*bphA1-A4, bphB, bphC* and *bphD*) (Furukawa 1994). Notably, most studies report that biodegradation occurs only on low-chlorine congeners, with less than 6 chlorine substitutes, and on *para* and *meta* substituted molecules rather than *ortho* ones (Borja et al. 2005; Furukawa 1994). Anaerobic dehalorespirers are known to utilize even higher chlorinated molecules as electron

acceptors, and show, as the aerobic degraders, a preference for *para* and *meta*-substituted molecules (Borja et al. 2005; Furukawa 1994). *Burkholderia xenovorans* LB400, a species mainly found in soils and associated to plant rhizosphere, is the most studied bacterial strain responsible for oxidative remediation of PCB. The genome of this bacterium has been sequenced, revealing the occurrence of eleven central and twenty peripheral catabolic pathways for aromatic compounds, and indicating a high metabolic versatility towards different molecules derived from root exudates, root decay and aromatic pollutants (Chain et al. 2006). This bacterium showed the ability to oxidize a broad range of *ortho* and *meta*-substituted PCB with up to six chlorines, through both 2,3-dioxygenation and 3,4-dioxygenation, and its degrading pathway has been characterized (Erickson and Mondello 1992; Gibson et al. 1993). *Bph* genes from the upper pathway of *Pseudomonas pseudoalcaligenes* KF707 were the first to be characterized (Furukawa and Myazaki 1986). Degradation of PCB by this strain occurs through 2,3-dioxygenation, displaying a narrower range of substrate molecules than *B. xenovorans* LB400, but a stronger activity on double *para*-substituted congeners (Furukawa and Fujiara 2008). The study of these two model strains clarified that minor structural differences in bphA α subunit induce major changes in substrate specificity (Ericksonn and Mondello 1992). Biphenyl-metabolizing strains have been grouped as LB400- or KF707-type, according to their degradation pattern (Mondello et al. 1997). The classification of the biphenyl dioxygenase (BPDO) enzyme appears, nevertheless, much more complex. For instance, *Pandoraea pnomenusa* B356 hosts a BPDO that shares 70% of amino acidic sequence with LB400 and KF707, and has a greater PCB transformation capacity (Gomez-Gil et al. 2007). Another model bacterium for the study of PCB degradation is *Rhodococcus* sp. RHA1. This strain is able to transform a wide range of congeners through 2,3-dioxygenation and, unlike LB400 and KF707, shows high activity on both *para* and *ortho*-substituted molecules (Masai et al. 1995). Recent studies shed light on uncultured bacterial communities inhabiting contaminated soils, combining stable isotope probing (SIP) and high-throughput sequencing technologies. Besides known degrading species, several other bacterial taxa belonging to the classes *Proteobacteria, Actinobacteria, Gemmatimonadetes, Nitrospira* and *Acidobacteria* were identified as putative biphenyl and benzoate degraders, extending the range of potential microbial actors for PCB remediation (Uhlik et al. 2012; Leewis et al. 2016).

Research on PCB degradation by fungi has been focused mainly on white rot fungi (Cajthaml et al. 2015). Even if the degradation pathways have not yet been described, apparently these fungi do not possess specific enzymes but rather produce extracellular peroxidases and laccases normally used

to degrade lignin and other aromatic compounds, which could lead also to non-specific degradation abilities (Takagi et al. 2007; Field and Sierra-Alvarez 2008; Hong et al. 2012). Intracellular enzymes such as cytochrome P-450 and dehydrogenases seem also to be involved (Cvancarova et al. 2012). Low chlorinated biphenyls are attacked more easily, but recent studies reported the capacity of white rot fungi to degrade hexa-CB to benzoic acid and to transform recalcitrant coplanar congeners, as well as the ability to suppress the toxicity of complex PCB mixtures (Hong et al. 2012; Kamei at al. 2006; Takagi et al. 2007; Cvancarova et al. 2012).

5. Plant-microbe beneficial interactions for PCB-contaminated soil remediation

Due to beneficial interactions between plants and bacteria, plant-assisted bioremediation seems to be the most effective way for bioremediation of PCB-contaminated soils (Figure 1). In aged polluted soils, degrading bacteria can be found associated to the rhizosphere of native spontaneous plants (Leigh et al. 2006), naturally selected to cope with the toxic effects of contaminants. Plants, due to rhizodeposition, exert a biostimulation activity toward rhizosphere microbial communities, in turn eliciting contaminant degradation when they include degraders (Singer et al. 2003; Musilova et al. 2016).

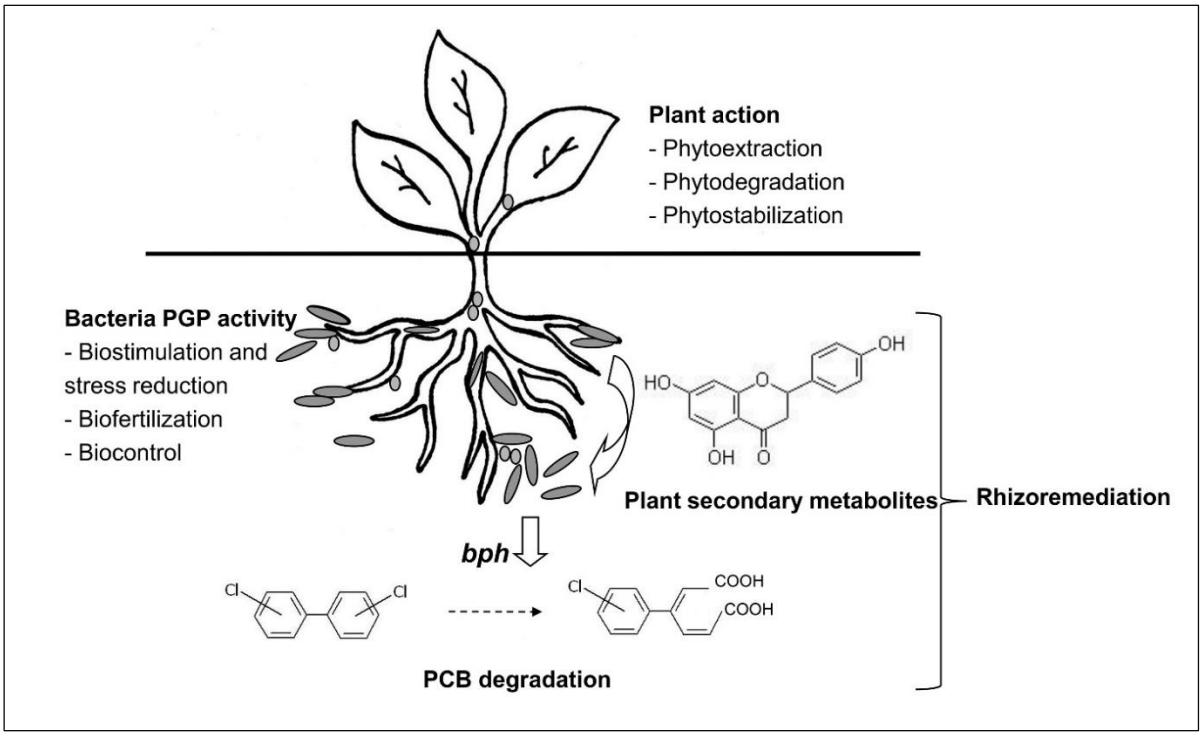

Figure 1. Plant – bacteria interactions in PCB-polluted soils. Plant growth promoting (PGP) bacteria play an important role by helping the plant to thrive in polluted soils. In the same time root exudates sustain bacteria

growth and plant's secondary metabolites (PSM) induce *bph* gene expression, due to PCB structure similarity, thereby enhancing biodegradation in the rhizosphere.

A pivotal aspect of rhizoremediation is represented by the occurrence of Plant Growth Promoting (PGP) bacteria associated to plants growing in polluted soils (Figure 2).

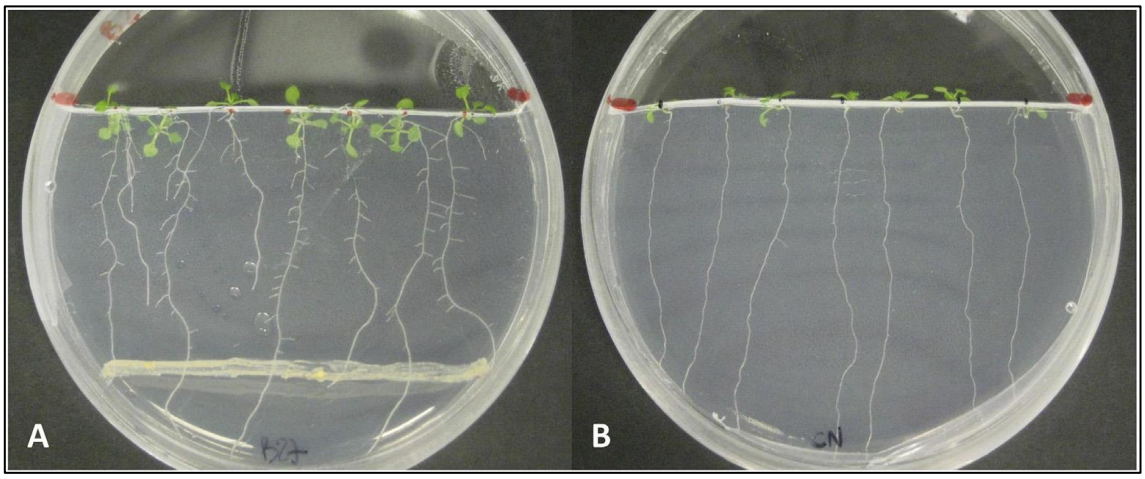

Figure 2. PGP potential of rhizobacteria associated to PCB-polluted soils. Illustrative example of *Arabidopsis thaliana* growth promotion carried out by a bacterial strain, streaked on the agarized medium (A) compared to control plants growing on sterile medium (B). The bacterium was isolated from the rhizosphere of plants growing on a PCB-contaminated soil.

PGP bacteria can boost growth and provide various benefits to plants through an array of direct and indirect activities. These include: i) the synthesis of compounds that protect the plants by decreasing stress hormone levels, ii) chelators for delivering key plant nutrients, iii) protection against plant pathogens and iv) degradation of contaminants before they can negatively impact the plants (Balloi et al. 2010; Ma et al. 2011). In particular, plants growing in contaminated soils often have to cope with the combined stresses of nutrient deficiency and chemical phytotoxicity (Mapelli et al. 2012). In stressful conditions, the biosynthesis of ethylene hormone leads to plant growth inhibition and decreased biomass, therefore threatening the phytoremediation potential. A successful strategy for overcoming the challenge of plant stress in phytoremediation may therefore come from PGP rhizobacteria that express 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Glick 2010). This enzyme hydrolyses the immediate precursor of ethylene in plants, thereby lowering the rate of ethylene biosynthesis. Remarkably, the effect of PGP bacteria is only related to a reduction in the level of deleterious ethylene, without affecting its small burst, which is believed to activate key plant defence responses (Hardoim et al. 2008).

On their side, plants provide root microbes with carbon and photosynthetic products, offering them a nutritionally rich and stable environmental niche. Furthermore, root growth promotes soil aeration, which can enhance oxidative degradation of recalcitrant organic compounds (Van Aken et al. 2010). In addition to these general effects, the wide set of root exudates produced by plants includes complex aromatic compounds, like for example salicylates. Notably, there is little accumulation of these compounds in soil, since they are promptly consumed by soil microbiome as a source of carbon and nitrogen (Walker et al. 2003). It is well known that bacteria can degrade PCB in co-metabolism with biphenyl, a molecule that is not common in the environment (Donnelly et al. 1994). In soil, aromatic compounds from root exudates and root turnover are structurally similar to many organic contaminants such as PCB, and they can therefore stimulate the proliferation and activity of PCB-degrading bacteria, acting as cometabolites and activating the expression of *bph* genes (Federici et al. 2012; Sylvestre 2013; Jha et al. 2015). For instance, crushed fine roots from *Salix alaxensis* proved to influence the microbial community and to enhance PCB depletion in a polluted soil, for which a reduced toxicity was also observed (Slater et al. 2011). Pagé and colleagues set up a microcosm experiment with a mixed-contaminated soil to study the effect of *Salix purpurea* on the expression level of genes related to the detoxification of aromatic compounds (alkane 1-monooxygenases, cytochrome P450 monooxygenases, laccase/polyphenol oxidases, and biphenyl-2,3-dioxygenase small subunits). Metatranscriptomic analysis revealed that the transcripts of these genes were significantly more abundant in the rhizosphere of *Salix purpurea* compared to the bulk microcosm soil, demonstrating the positive role of plant rhizodeposition in pollutant detoxification (Pagé et al. 2015). Raw root exudates have been also demonstrated to enhance the desorption of a hydrophobic contaminant such as naphthalene, probably by containing surface-active compounds (*i.e.* biosurfactants) (LeFevre et al. 2013). This effect may even be increased in case of higher molecular weight molecules like PCB, making them more available to the degrading microorganisms. The production of biosurfactants, including rhamnolipids, threalose lipids and surfactin that favor pollutant desorption and bioavailability, is also widespread among different soil and rhizosphere bacterial genera, such as *Pseudomonas, Rhodococcus, Arthrobacter* and *Bacillus* (Christofi and Ivshina, 2002). These bacteria thereby represent natural resources potentially exploitable for PCB remediation approaches, even though *in situ* studies are lacking (Abraham et al. 2002; Berg et al. 1990; Robinson et al. 1996).

Several evidences support therefore the concept that phytoremediation improvement could be achieved by exploiting the abovementioned natural positive interactions between plants and

bacteria. In the following section, several studies in which selected species were planted on PCB-contaminated soils to stimulate biodegradation by the indigenous soil microbial community are described.

5.1 PCB rhizoremediation: experimental trials

Javorskà and coauthors (2009) set up a greenhouse experiment in which *Brassica napus* was planted on two different soils spiked with a mixture of seven PCB congeners, showing a positive effect on PCB degradation in plant rhizosphere. PCB depletion was significantly higher for low-chlorinated molecules in the soil with lower concentrations of organic matter compared to the negative control. Alfalfa (*Medicago sativa*) and tall fescue (*Festuca arundinacea*) were shown to enhance bacterial growth in the rhizosphere, as well as contaminant degradation, in greenhouse tests performed with soil derived from a storage site of PCB containing equipment (Li et al. 2013). The co-presence of the two plant species favoured the degrading microbial community, as shown by the higher number of *bphA* gene copies and the recorded increase in dehydrogenase activity. Similar results were obtained planting alfalfa alone during *in situ* phytoremediation trials on agricultural soil contaminated by more than 20 PCB congeners (Tu et al. 2011). At the end of the two-year experiment, the planted soil showed higher dehydrogenase and esterase activities in parallel with the removal of about 80% of total PCB compared to 31% of the control. The authors observed a major decrease of di CB compared to higher chlorinated compounds, confirming that these congeners are more easily attacked (Tu et al. 2011).

Plant-bacteria association seems to have the most efficient remediation potential in environments subjected to alternate redox conditions, since complete PCB decontamination requires the combination of reductive dechlorination, the only metabolism active on highly chlorinated congeners, and aerobic oxidation of the lower chlorinated congeners produced and the biphenyl ring (Meggo and Schnoor 2013). Oxic-anoxic conditions are typical of paddy fields, recently studied by Chen and co-workers (2014b). The authors set up a rhizobox experiment with PCB-contaminated soils and rice plants subjected to sequential flooding and drying periods and analysed three different compartments: bulk soil, rhizosphere soil and soil-water interface. The experiment proved that paddy-field simulated conditions favoured PCB dechlorination during the flooding period and allowed aerobic degradation of their lower chlorinated products when the soil was subsequently directly exposed to the air. The results also showed that these processes were accelerated and enhanced in the rhizosphere compared to the other soil niches. This finding was supported by

phospholipids fatty acids (PFLAs) analysis, revealing that the rhizosphere selected a specific microbial community, which structure was significantly correlated to the PCB degradation rate (Chen et al. 2014b). This work sustains the hypothesis that root exudates have a positive effect on the enrichment and activity of a degrading microbial community. Regarding the reductive dehalogenation, positive results were also obtained with other plant species able to grow in flooded soil. In the experiment performed by Meggo et al. (2013), soil spiked with a mixture of three PCB congeners (52, 77 and 153) was planted with poplar (*Populus deltoids x nigra*) and switchgrass (*Panicum virgatum*) and exposed to flooding cycles for 32 weeks. Both the planted treatments showed a depletion rate over 50% for each of the three congeners in the root associated soil, significantly higher than the unplanted control. In addition, high yields of low-chlorinated compounds resulting from PCB dechlorination were detected in the planted soils, an interesting result since aerobic degraders could subsequently more easily attack these products. The authors then studied the impact of PCB spiking, flooding conditions and switchgrass planting on the structure of soil microbial community after 24 weeks of incubation. Analysis through quantitative PCR (qPCR) revealed that putative dechlorinating bacteria belonging to the phylum *Chloroflexi* were enriched by the redox cycling and that *Geobacter* sp., a bacterium known to be implicated in reductive dehalogenation processes (Merlino et al. 2015), was favoured in planted soils (Liang et al. 2015). However, since a significant PCB depletion was observed in soil microcosms, and these strains together represented only about 1% of the total bacteria, other microbial groups were also probably involved in the halorespiration activity (Liang et al. 2015).

Another explored tool to improve PCB rhizoremediation is the inoculation of selected plant species with PGP, and/or PCB-degrading bacteria or fungi as individual strains alone or in consortia (bioaugmentation). For instance, alfalfa has been reported to enhance PCB removal in field conditions when inoculated with the PGP nitrogen-fixing symbiont *Rhizobium melioti* (Xu et al. 2010). The application of this non degrading PGP bacterium to the plants displayed a positive effect in increasing the number of total and degrading bacteria in the soil, data that was significantly correlated to the PCB depletion. Possibly, the increased root exudation due to the higher plant biomass induced by the PGP activity of *R. meliloti* may have stimulated the proliferation of the PCB-degrading microbial community (Xu et al. 2010). The inoculation of the same plant species with the arbuscular mycorrhizal fungus *Glomus caledonium* together with *R. melioti* further enhanced the rate of PCB depletion (Teng et al. 2010). Liang and colleagues (2014), during a 24 weeks microcosm experiment, inoculated the switchgrass *Panicum virgatum* with the well-known degrader

Burkholderia xenovorans LB400 in the presence of PCB 52, 77 and 153 congeners, obtaining enhanced removal rates comparing planted and inoculated treatments to the controls (*i.e.* untreated, non-inoculated plant, unplanted bioaugmented soil). In particular, the association of *P. virgatum* and *B. xenovorans* LB400 led to a depletion of 47% of total PCB, compared to 40% of non-inoculated plants. The less chlorinated congener PCB 52 was the one most subjected to degradation, likely because it is less recalcitrant and more available to microbial cells than PCB 77 and 153. The authors also observed that *bphA* expression levels were not promoted by the presence of the plant, possibly because of inhibition by the presence of other carbon sources in the rhizosphere, while it was increased only in the bioaugmented pots. To overcome bioaugmentation failure, by ensuring the survival of exogenous bacteria in the soil, the inoculation of *B. xenovorans* LB400 was performed once a month (Liang et al. 2014). The bioaugmentation approach indeed results often unsuccessful, and different factors have to be taken into account since exogenously added "invader" bacteria may not successfully colonize plant roots or establish in the soil due to competition with the autochthonous microbiome (Rolli et al. 2015). This ecological dynamic represents a known limitation common to bioaugmentation strategies even in different environmental matrices, including polluted marine sediments (Fodelianakis et al. 2015). As mentioned, the bacteria may not efficiently degrade the target pollutant also due to its low availability and preferably metabolize other molecules more available in the soil (Passatore et al. 2014; Secher et al. 2013). The strain *B. xenovorans* LB400 was used for a bioaugmentation experiment in association with *Festuca arundinacea*, in comparison with non-planted and non-inoculated treatments (Secher et al., 2013). The pot experiment lasted 70 days and was conducted using an aged contaminated soil with a mixed contamination of PCB, polyaromatic hydrocarbons (PAHs) and heavy metals, Zn and Pb. The quantitative data obtained applying qPCR showed that LB400 was in this case able to establish among the indigenous bacteria population. Moreover, plant root biomass was increased compared to the non-inoculated control, even if the effective PGP potential of this model degrading strain has still to be demonstrated. Nonetheless, while the autochthonous soil microbiome resulted capable of PCB degradation, this ability was not significantly different between the treatments and the control non-planted or non-inoculated. As the authors hypothesized, this could be due to the low availability of PCB in aged contaminated soils, the preferential use of other molecules by *B. xenovorans* (i.e. PAHs), or to the too short experimental duration.

5.2 The potential of endophytes

Plant growth promoting endophytes are bacteria that successfully colonize the internal tissues of the plant and spend there at least part of their life cycle, determining drastic changes in plant physiology and growth (Hardoim et al. 2008). The beneficial effect of these bacteria is enhanced when the plant is subjected to stress conditions (Fernandez et al. 2012; Cherif et al. 2015), so their role may be of great interest in case of plants growing in polluted soils. Moreover, some endophytic strains could degrade the organic pollutants uptaken by the roots and then translocated to the aerial part (Eevers et al. 2016; Gutierréz-Ginés et al. 2014). To be effective, endophyte-assisted phytoremediation requires that (i) selected plant species are able to uptake the target pollutant, (ii) bacteria have the capacity to colonize the roots and the xylem of the plant (iii) bacteria are able to degrade the pollutant once established inside the plant. This field is still poorly studied and there are no reports of exploitation of plant endophytic communities for phytoremediation of PCB, nonetheless some interesting results have been obtained with other chlorinated organic compounds (Eevers et al. 2016; Becerra-Castro et al. 2013). For instance, the trichloroethylene (TCE)-degrading strain *Pseudomonas putida* W619-TCE is able to colonize poplar cuttings roots and stems, and to promote plant growth in hydroponic conditions. Moreover, significantly lower levels of TCE were detected in the leaves of plants treated with TCE and inoculated with this strain, in both hydroponic and microcosm condition, compared to the untreated controls, resulting in decreased evapotranspiration of the contaminant (Weyenes et al. 2010). These results suggest that endophyte-mediated phytoremediation of chlorinated POPs is a promising technology. More studies are nevertheless needed based on this approach, especially at field scale. In particular, the potential of plants and the application of PCB-degrading endophytic bacteria remains unexplored and constitutes a big challenge due to their hydrophobic and recalcitrant chemical nature.

6. PCB-degrading bacteria: understanding the role of plant secondary metabolites

Given the evidence of the so-called “rhizosphere effect” and its role in shaping soil microbial community, in turn potentially stimulating its degradation abilities, it is of great interest for rhizoremediation to understand which plant secondary metabolites (PSMs) contained in root exudates are actually involved in such promotion and what is their mechanism of action. Some studies focused on plant terpenes, such as carvone and limonene, reporting that these compounds effectively foster PCB degradation by soil bacteria (Hernandez et al. 1997; Gilbert and Crowley, 1997; Dudasdova et al., 2012). Donnelly and colleagues demonstrated the ability of three PCB-degrading species (*Burkholderia xenovorans* LB400, *Cupriavidus necator* H850 and *Corynebacterium*

sp. MB1) to grow on different plant phenolic compounds, demonstrating that some of these molecules fostered the capacity of the three strains to metabolize PCB, and that each strain preferred a different compound (Donnelly et al. 1994). The PCB-degrading bacterium *Burkholderia xenovorans* LB400 is able to grow on phenolic compounds exuded by the roots of *Morus sp.* provided as sole carbon source. The growth rates, in particular, were comparable when the strain was cultivated on exudates and biphenyl carbon sources (Leigh et al. 2002). However, none of these studies clarified whether PSMs actually induced *bph* genes expression.

Toussaint et al. (2012) tried to elucidate this mechanism under *in vitro* conditions, reporting that the strain *Rhodococcus erythropolis* U23A was able to grow on a mix of root exudates extracted from *Arabidopsis thaliana* as sole carbon source and to co-metabolize trichlorobiphenyl producing chlorobenzoates in higher quantity than when growing in LB medium. The authors concluded that *Arabidopsis* exudates might act as co-metabolites by enhancing the expression of the *bph* genes, chromosomally harbored by the strain. A further evidence of the role of plant exudates as PCB co-metabolites was that biphenyl dioxygenase was involved in the degradation of flavanone, one of the main components of *A. thaliana* root extract, and that this compound triggered PCB degradation in the presence of sodium acetate. A recent study also identified in *A. thaliana* root exudates other flavonoids that, according to qPCR results, induced the expression of *bphA* genes in *R. erythropolis* U23A being co-metabolized with sugar substrates (Pham et al. 2015). The phenylpropanoids and the co-substrate tested in this study improved the degradation ability and the induction of the biphenyl catabolic pathway. In particular, flavone and isoflavone were identified as the best inducers compared to biphenyl, and sucrose was the best co-substrate. Moreover, the authors demonstrated that isoflavone induced the *bph* pathway of the well-known PCB degrader *Pandoraea pnomenusa* B356, while flavone, isoflavone or flavanone did not show this effect on the catabolic operon of *B. xenovorans* LB400 (Pham et al. 2012).

Recently, Uhlik et al. (2013) set up a microcosm experiment to investigate the effect of three plant secondary metabolites, namely naringin, limonene and caffeic acid, on a soil artificially contaminated with a commercial PCB mixture. 16S rRNA pyrosequencing analysis showed that the application of each PSMs induced a specific shift in the structure of soil microbial community after 8 weeks incubation, and all the treatments resulted in a decreased diversity compared to the negative control. Moreover, stable isotope probing (SIP) was applied to identify bacteria involved in 4-chlorobiphenyl degradation, showing that all of the treatments selected different microbial populations able to use PSMs as growth substrate. Naringin and caffeic acid showed the best effect

on PCB removal, promoting the growth of bacterial genera like *Hydrogenophaga, Burkholderia* and *Pseudoxanthomonas*.

The evidences that some PSMs actually serve as carbon sources and PCB degradation inducers for the rhizosphere microbial community open new perspectives for their possible use in rhizoremediation treatments. PSMs could be exploited both for the isolation of degrading bacteria and as amendments to polluted soil (by means of extracted PSMs or PSM producing plants) to foster microbial activity and PCB removal. PSMs represent in fact a natural alternative to biphenyl to induce co-metabolic PCB degradation, which is unsuitable for field application because expensive, scarcely soluble in water and mildly toxic (Donnelly et al. 1994; Federici et al. 2012). In this perspective, also other plant derivatives may be effective for the biostimulation of the microbial community in PCB-contaminated soils. For instance, maize stalks were used, alone or in combination with soybean oil as biosurfactants, for the amendment of contaminated soil microcosms (Federici et al. 2012). Even though soybean oil proved to be ineffective in this case, maize stalks showed positive effects on microbial diversity and on the abundance of functional genes (i.e. biphenyl-2,3-dioxygenase and catechol-2,3-dioxygenase). Moreover, enhanced dechlorination and depletion of highly chlorinated PCB congeners could be observed with the combination of the two treatments.

7. New tools for the investigation of PCB-degrading microbes

Metagenomics and other "omics" approaches represent a fundamental tool to study unculturable microorganisms and discover new enzymes involved in contaminant degradation (Ufarté et al. 2015). Considering the limited number of isolated strains able to utilize PCB, these molecular methods could be particularly useful to mine new degrading strains and to better understand their functional role in the environment. Uhlik et al. (2012) applied stable isotope probing (SIP) and 16S rRNA gene pyrosequencing to an aged PCB-polluted soil in microcosms amended with ^{13}C-labelled biphenyl, benzoate and naphthalene, finding a prevalence of *Proteobacteria* sequences among the taxa that metabolized the labelled substrates. In particular, sequences related to the genera *Rhodanobacter, Burkholderia, Dyella* and *Pandoraea* represented the majority of the bacterial community after 14 days in biphenyl-spiked soil, while *Pseudomonas* dominated in the microcosms amended with benzoate (Uhlik et al. 2012). In the attempt to identify which bacterial communities performed the upper and/or the lower *bph* pathway, Leewis and colleagues (2016) applied the SIP method to ^{13}C biphenyl and ^{13}C benzoate spiked microcosms, collecting samples at early and late incubation time. They detected Operational Taxonomic Units (OTUs) belonging to the phyla

Acidobacteria, Actinobacteria, Gemmatimonadetes and *Nitrospira* in all the microcosms. *Actinobacteri*a were the prevalent group among the ^{13}C labelled biphenyl utilizers, whereas *Rhizobiales* was the most abundant among the ^{13}C benzoate utilizers after one day since substrate addition, and *Burkholderi*a and *Actinomycetales* became prevalent after four days. As previously demonstrated, shared OTUs between biphenyl and benzoate supplemented microcosms were mostly belonging to *Proteobacteria* (Leewis et al. 2016), suggesting a predominant role of this phylum in the frame of PCB-polluted soil remediation. In addition, the analysis of transcriptomic profiles may contribute to assess the effectiveness of rhizoremediation, for instance allowing the comparison of *bph* gene transcripts in rhizosphere and bulk soils, as discussed in section 5 (Pagé et al. 2015). The nucleotide sequence diversity of the *bphA* gene has been adopted to describe the functional potential of soil microbial communities. Recently, Iwai et al. (2010) applied gene-targeted metagenomics studies of the *bph*A α subunit to a PCB-polluted soil, designing a new primer set (BPHD-f3 and BPHD-r1) to obtain the genes from soil metagenomes by PCR amplification. Interestingly, after sequence validation, 95% and 41% of the *bphA* sequences were assigned to novel clusters and revealed a great diversity of dioxygenases gene belonging to different families. The same primer set was used to study the *bphA*-harboring bacterial community of different type of Brazilian soils (de Lima Brossi et al. 2014), obtaining sequences related to aromatic hydrocarbon dioxygenases both from known and uncultivated bacteria, that significantly differed according to the soil of provenience. Although based on a low-throughput method (*i.e. bphA* clone libraries), a recent study showed further hints of distribution patterns of the bacteria potentially involved in PCB and PAH degradation according to the pollutant profile in the environment (Hoostal et al. 2016). Overall, these results open new perspectives on the evolution, ecology and functional diversity of aromatic dioxygenases (Iwai et al. 2010).

An additional strategy to study PCB-degrading bacteria is the exploitation of resuscitation promoting factor active on those bacteria that cannot be cultivated *in vitro* due to their occurrence in the soil in a viable but not culturable (VBNC) state in response to stress conditions, , like high pollution levels. The resuscitation promoting factor (Rpf, a cytokine from the bacterium *Micrococcus luteus*) was applied on an enrichment culture established from PCB-contaminated soil, supplying biphenyl as unique carbon source (Su et al. 2013), suggesting its potential for the isolation of novel degrading strains. Indeed, the results of Denaturing Gradient Gel Electrophoresis (DGGE) fingerprinting applied on the soil metagenome and phylogenetic analysis of the isolates highlighted that the treatment with Rpf increased the overall soil bacterial diversity. Moreover, the treated enrichment culture

showed an enhanced performance in biphenyl degradation compared to the control one, a result that opens interesting perspectives on innovative solutions for the enrichment of novel and effective microbial resources for PCB-contaminated soil remediation. Recently, the application of the Rpf factor and the reversal of stress conditions proved the efficacy for cultivability recovery on *Rhodococcus* strains isolated on PCB-contaminated soils (Su et al. 2016).

8. Concluding remarks

In vitro and microcosm-scale studies showed that rhizoremediation could be an effective way to overcome traditional phytoremediation limitations when it comes to PCB removal, taking advantage also of the degrading ability of soil microorganisms. Nonetheless, some issues make difficult to apply rhizoremediation in the field and there is a general lack of research in this direction. The main reasons are the requirement of prolonged experimental time and the complexity of aged polluted sites, such as mixed and uneven contaminant distribution and low bioavailability of the pollutants bounded to soil organic matter. Further research is thereby needed to address the potential of rhizoremediation under field conditions, a crucial issue to evaluate the concrete feasibility of this technology. The contributes of endophytic microbial populations in PCB degradation and of plant organic exudates in priming the degradative pathways constitute promising and open field of investigation. In this perspective, “omic” approaches can be powerful tools to unveil taxonomic and functional diversity of microorganisms, overcoming their limited cultivability, allowing the identification of the best plant-microbe combination involved in the remediation of PCB-contaminated soils under different environmental conditions. Besides identification, a main challenge remains the cultivation of new PCB-degrading microbes to design, in the future, effective remediation strategies based on bioaugmentation.

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Chapter 1.

Microbial profiling of soils from the SIN Caffaro, a case study for PCB rhizoremediation

INTRODUCTION

The SIN Caffaro is a polluted site of national priority located in the municipality of Brescia (Italy), originated by the activities of the former Caffaro s.p.a. chemical plant that produced PCB and PCB mixtures until the ban on the production of these compounds in 1984. The site includes more than 100 hectares of former agricultural soils that present a mixed contamination of halogenated Persistent Organic Pollutants (POPs) with high concentration of PCB and metals and metalloids, particularly arsenic (As) and mercury (Hg) often exceeding the law limits and uneven distributed in the area (Di Guardo et al. 2017; Turrio-Baldassarri et al. 2007). Given the extent of the contamination, physical and chemical treatments would be too expensive and ecologically unsustainable (Gomes et al. 2013), so no remediation intervention has been implemented since 1984. The SIN Caffaro represents thereby a case study for the remediation of aged and heterogeneously PCB-polluted areas and for the development of *in situ* sustainable strategies that can preserve the soil functions, based on the natural biodegradation potential of the soil itself. To setup a successful strategy, it is essential to consider if and how the soil microbial community, which is primarily involved in the biodegradation process, responds to the uneven distribution of the contaminants and to the variation of other environmental factors, such as soil pH and organic matter content. In fact, all these factors may affect soil microbial activity and degradation ability together with the community structure (Mukherjee et al. 2014; Quero et al. 2015; Sun et al. 2015).

In this study, we applied the Automated Ribosomal Intergenic Spacer Analysis (ARISA) fingerprinting and the Fluorescein DiAcetate (FDA) hydrolysis test to characterize the bacterial community structure and activity in soil samples collected to a depth of one meter from three different former agricultural areas located in the SIN Caffaro, with the aim to correlate the soil physical-chemical profiling with the bacterial community structure and the soil hydrolytic activity. Our overall goal was to unravel the drivers of the bacterial microbiota diversity in the soil of the SIN Caffaro.

MATERIALS AND METHODS

Study site and soil sampling

Three former agricultural fields (named A, R and T) located south-west of the Caffaro plant at about 2 Km from the centre of the city of Brescia were chosen for sampling. The first soil sampling was conducted along a gradient of soil depth: nine stations were identified in the three areas (A1, A2, A3, R1, R2, R3, T1, T2, T3) and soil samples were collected from each station by core drilling to a depth of

100 cm (Figure 1A). Cores were then divided in fractions corresponding to seven intervals of depth (0-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-60 cm, 60-80 cm, 80-100 cm) resulting in 63 soil samples. During a second independent soil sampling, 64 soil cores were collected to a depth of 40 cm, corresponding to the layer of soil influenced by the former agricultural practices, in order to cover the whole surface of the three sampling areas A (A1-A17), R (R1-R19) and T (T1-T28) (figure 1B). An accurate homogenization procedure was applied to obtain a homogeneous sample representative of the layer of interest (0-40 cm). Every soil sample was stored at 4°C for FDA analysis and at -20°C for molecular fingerprinting and chemical analyses.

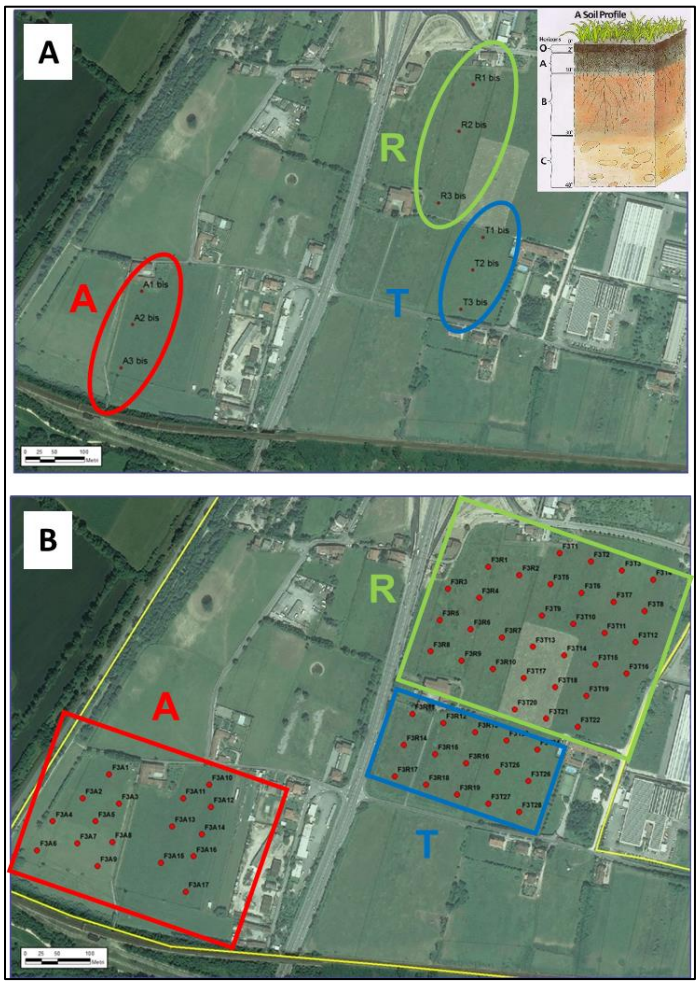

Figure 1. Map of the areas and stations of sampling in the SIN Caffaro. **A.** Collection of 63 samples along a depth gradient (0-100 cm) in three stations for each of the three areas A, R and T. **B.** Collection of 64 samples at a depth of 0-40 cm covering the whole surface of the three areas A, R and T.

Chemical analyses

Soil chemical analyses were performed outsource by Theolab S.p.A. (Rho, Italy). PCB concentration are presented for the soil samples collected along the depth gradient and across the surface of the three areas in Supplementary Table 1 and Table 1 as: Total PCB (expressed as the sum of the 79 PCB congeners measured in the soil); PCB ≤ 5Cl and PCB >5Cl. The threshold of 5 chlorine atoms have been chosen to categorize the different congeners corresponding to the higher limit for a possible PCB aerobic degradation by microbes.

Table 1: Average value of the chemical parameters (pH, organic carbon, As, Hg and PCB concentrations) measured in the soil samples collected across the surface (0-40 cm) of sampling areas A, R and T. Org.=organic¸ Tot.=total.

Area	pH	Org. C (g/Kg)	As (mg/Kg)	Hg (mg/Kg)	PCB ≤ 5 Cl (µg/Kg)	PCB > 5 Cl (µg/Kg)	Tot. PCB (µg/Kg)
A	7,669	0,837	89,256	3,543	304,062	1675,777	1979,839
R	7,474	0,738	43,333	2,517	161,765	1224,098	1385,863
T	7,346	0,717	53,748	3,330	174,840	605,874	780,714

Evaluation of the soil microbial hydrolytic activity

To assess microbial hydrolytic activity, the hydrolysis of fluorescein diacetate (FDA) was measured following the protocol described by Green et al. (2006). Briefly, 1 g of soil was placed into 250 ml serum bottles together with 50 ml of Na_3PO_4 buffer (pH 7.6) and 0.5 ml of 0.02% FDA solution in acetone. One blank sample without soil was also established. Moreover, for each soil sample, a control containing 1 g of soil and acetone instead of FDA was established. The bottles were incubated at 37°C for 3 hours without shaking, then 30 ml of soil suspension were centrifuged at 8000 rpm for 5 minutes and the supernatant filtered in glass tubes using a glass funnel with Whatman filter paper N°2. 1 ml of the solution was transferred into cuvettes and the OD at 490 nm was measured using a spectrophotometer. The quantity of fluorescein released by each sample was calculated from a calibration curve obtained with known fluorescein concentration (0.2, 0.6, 1, 2, 6 and 10 µg/ml).

DNA extraction and ARISA molecular fingerprinting

Total DNA extraction was performed from 0.25 grams of each of the soil samples using the PowerSoil DNA kit (MoBio) according to the manufacturer's protocol. ARISA-PCR was conducted on a standard amount of DNA on each sample by using the primer set ITSF (5'-GTC GTA ACA AGG TAG GCC GTA-3')

and ITSReub (5'-GCC AAG GCA TCC ACC-3'), as described by Cardinale et al. (2004). ARISA fragments were separated using the ABI3730XL genetic analyser applying the internal standard 1200-LIZ (Macrogen, Rep. of Korea) and ARISA fingerprints were analyzed using the Peak Scanner Software (Applied Biosystems). The output peak matrix was transferred to Microsoft Excel for further analysis. Peaks showing a height value < 50 fluorescence units were removed from the output peak matrix before statistical analyses. To account for variability in size associated with standards, ARISA fingerprints were binned at ±1 bp from 100 to 300 bp, ±3 bp from 300 to 600 bp and ±10 bp from 600 to 1200 bp. ARISA matrix was normalized and then transformed on a logarithmic scale for multivariate analysis as described by Ciccazzo et al. (2014).

Statistical analysis were performed as follows (Mapelli et al. 2013). Principal Coordinate Analysis (PCoA) was performed on the ARISA data (quantitative matrix of the OTUs within each sample) to visualize the similarity among the samples. Chemical data was used in the distance-based multivariate analysis for a linear model (DistLM) to determine which significant variables explained the distribution of the samples observed basing on the biotic dataset. The contribution of different chemical variable was assessed, firstly using "marginal tests" to assess the statistical significance and percentage contribution of each variable taken alone, and secondly a "sequential test" was employed to evaluate the cumulative effect of the variables explaining biotic similarity. A distance-based redundancy analysis (dbRDA) was used for a graphical visualization of the DistLM results. Significant differences in bacterial community composition were investigated by permutational analysis of variance (PERMANOVA), considering the area or the depth of sampling as fixed and orthogonal factor.

Analysis of the bacterial taxonomic diversity

Illumina tag screening of the V3-V4 hypervariable regions of the 16S rRNA gene was applied on DNA extracted from soils samples collected along the depth gradient profile at 0-10 and 20-30 cm using the primers 341F and 785R (Klindworth et al., 2013). The obtained sequences were analyzed using a combination of the UPARSE v8 (Edgar, 2013) and the QIIME v1.8 (Caporaso et al., 2010a) softwares. Briefly, raw forward and reverse reads for each sample were assembled into paired-end reads considering a minimum overlapping of 50 nucleotides and a maximum of one mismatch within the region using the fastq-join algorithm (https://code.google.com/p/ea-utils/wiki/FastqJoin). The paired reads were then quality filtered, the primer sequences were removed and the individual sample files were merged in a single fasta file. This file was imported in UPARSE where operational taxonomic units

(OTUs) of 97% sequence similarity were formed and chimeras were removed using both de-novo and reference-based detection. For reference chimera detection, the "Gold" database containing the chimera-checked reference database in the Broad Microbiome Utilities (http://microbiomeutil.sourceforge.net/) was used. Taxonomy was assigned to the representative sequences of the OTUs in QIIME using UClust (Edgar et al., 2010) and searching against the latest version of the Greengenes database (McDonald et al., 2012). The OTU table was calculated with FastTree2 (Price et al., 2010) using default parameters and the PyNast-aligned (Caporaso et al., 2010b) representative sequences as an input. Bray-Curtis distance matrix on the log transformed OTU table was used to perform a Principal Coordinates Analysis (PCoA) and to conduct a permutational multivariate analyses of variance (PERMANOVA). Statistical analyses were conducted in PRIMER v. 6.1, PERMANOVA+ for PRIMER routines (Anderson et al., 2008) to test differences in bacterial community composition among the two depths and the areas three areas of soil collection**.** Chemical data was used in the distance-based multivariate analysis for a linear model (DistLM) as to determine which significant variables explained the distribution of the OTU observed basing on the biotic dataset and a distance-based redundancy analysis (dbRDA) was used for a graphical visualization of the results.

RESULTS

The detailed chemical analyses performed on the samples collected along the soil horizon at depth comprised between 0-100 cm showed that the distribution of pollutants, as well as the total organic carbon (TOC) content, deeply changed along the vertical profile, showing higher concentrations of PCB, As and Hg in the upper soil layer (Supplementary table 1; Figure 2)

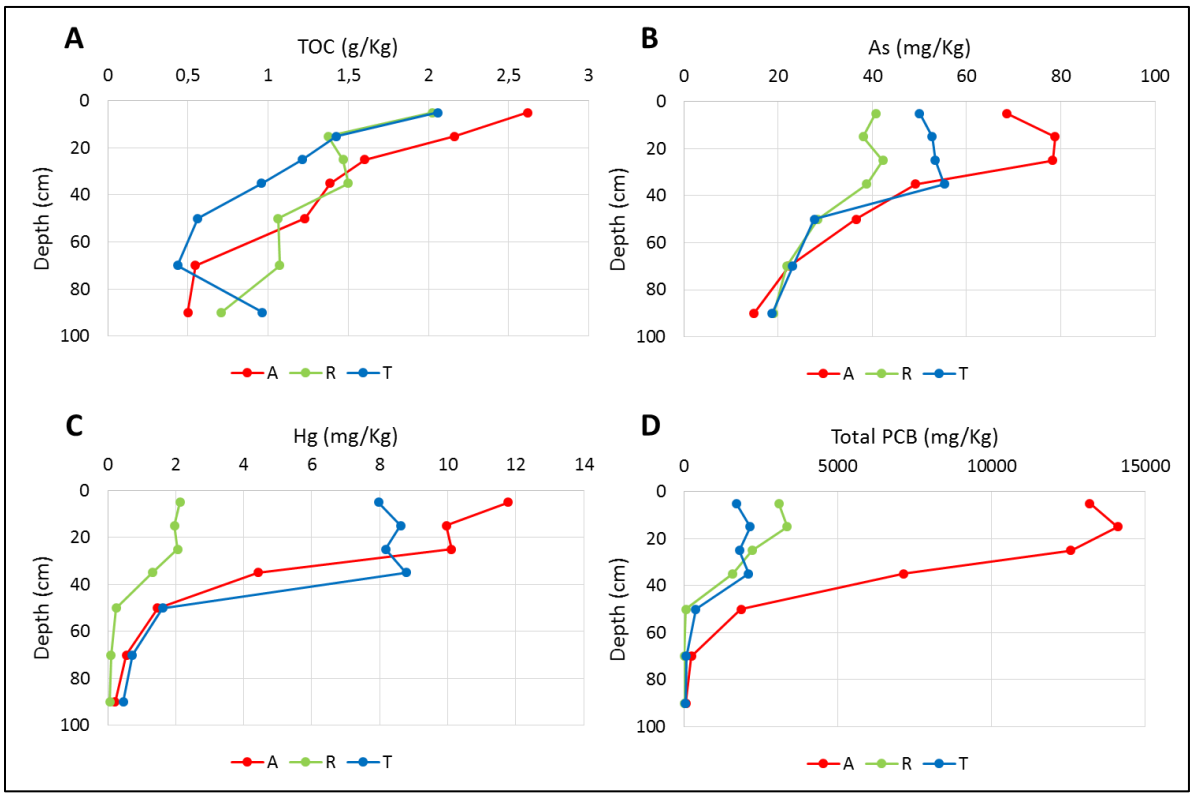

Figure 2. Chemical profiling of soil samples collected along the depth gradient. Mean values of the data obtained from the three stations of each area (Supplementary table 1) are represented. **A.** Total organic carbon content (TOC); **B.** Arsenic (As) concentration; **C.** Mercury (Hg) concentration; **D.** Total polychlorinated biphenyl content (Total PCB).

Pollutants concentration was also different between the three areas according to the chemical analysis of the samples collected in the first 40 cm of soil layer. In particular, soil samples collected in the area A showed the highest PCB, arsenic and mercury concentration. The soils of area T showed a lower concentration of PCB than areas A and R while samples from area R resulted the less polluted in terms of arsenic and mercury concentration (Table 1). These results also showed how, for almost all of the samples collected across the surface of the three areas the total PCB, As ad Hg concentration exceed the limits established by the Italian law for residential and green areas (Table 2; Dlgs 152 06/06/2012, http://www.bresciacaffaro.it).

Table 2. Number of samples (%) exceeding the Italian law limits of the main contaminants of the SIN Caffaro according to the intended use of the polluted area. The tables refers to the samples collected across the surface (0-40 cm) of the three areas A, R and T.

Intended use of the area	As (mg/Kg)		Hg (mg/Kg)		Tot. PCB (µg/Kg)	
	Law limit	Samples exceeding the law limit	Law limit	Samples exceeding the law limit	Law limit	Samples exceeding the law limit
Residential/ green	20	100%	1	95%	60	98%
Industrial/ commercial	50	57%	5	15%	5000	5%

The soils collected in the three areas down to 100 cm depth showed diminished hydrolytic activity with the increase of soil depth for all the sampling stations (Figure 3, Supplementary table 2).

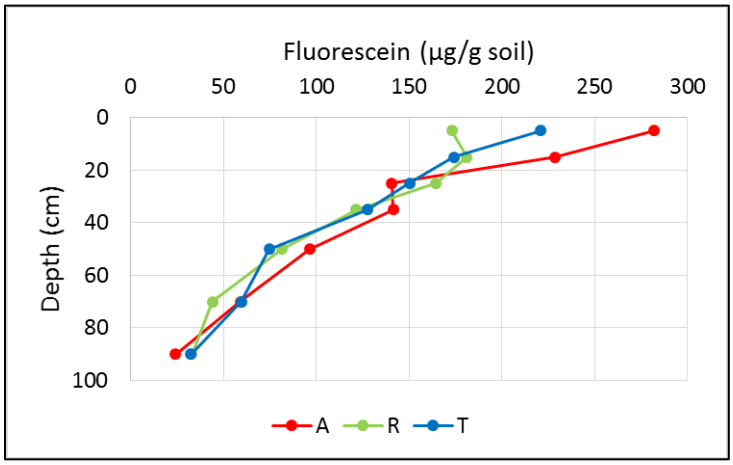

Figure 3. Fluorescein diacetate (FDA) hydrolysis test. The graphic reports the quantity of fluorescein produced by 1 g from soils collected along the depth gradient for each area of sampling A, R and T. Mean values were calculated for the three sampling stations of each area.

Statistical analysis proved that soil samples collected at increasing depth displayed significantly different values of the hydrolytic activity (PERMANOVA: $F[6,62]=14.414$, $p=0.0001$). FDA test and the applied statistic analysis also showed that the soils collected in the surface layer comprised between 0-40 cm were significantly different according to the area of collection (PERMANOVA; Table 3).

Table 3: Statistical analysis (pairwise test, PERMANOVA) comparing the results of the FDA test between soil samples collected at 0-40 cm depth from the three different sampling areas A, R and T.

Groups	t	P(perm)	perms	p(MC)
A, R	4,8583	0,0001	9950	**0,0001**
A, T	2,1218	0,0356	9932	**0,038**
R, T	4,0882	0,0001	9942	**0,0003**

Principal coordinate analysis (PCoA) on ARISA fingerprinting of the soils collected in the three areas along the depth gradient showed that the bacterial communities of the shallower soil layers (0-40 cm) clustered separately from those of the deeper layers (40-100 cm; Figure 4A), as confirmed by statistical analysis (PERMANOVA: F[6,58]=2.288; p = 0.0001).

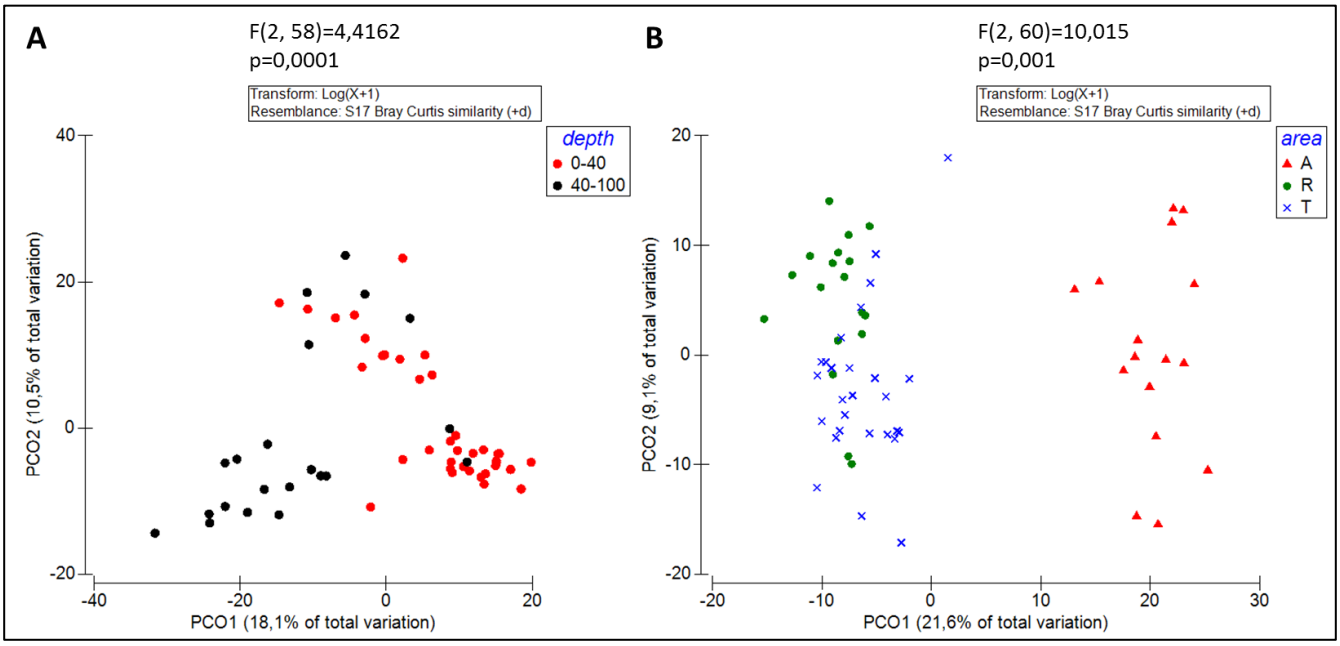

Figure 4. Distribution of soil samples (Principal Coordinate Analysis, PCoA) according to ARISA profiles obtained (**A**) along the soil depth profile and (**B**) across the surface (0-40 cm) of sampling area A, R and T.

Bacterial communities of 0-40 cm surface soils grouped according to the area of collection (Figure 4B) and were significantly different in the three areas (PERMANOVA; p = 0.0001). Statistical significant correlation of the soil physico-chemical parameters and bacterial populations profiling according to ARISA was proven along the depth gradient (Figure 5A, Supplementary table 3) and according to the area of collection (Figure 5B, Supplementary table 4).

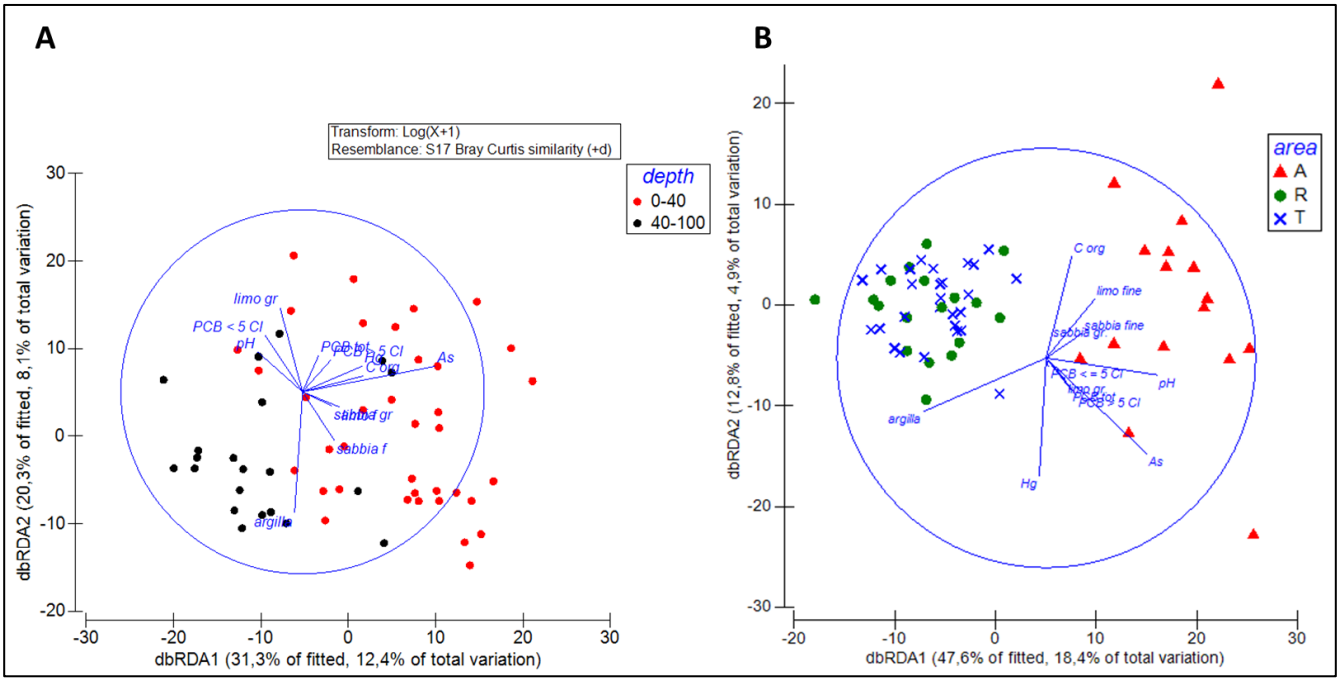

Figure 5. Correlation between ARISA profiling of the bacterial community structure and soil chemical and physical parameters in samples collected along the soil depth gradient (**A**) and across the surface (0-40 cm) of the three sampling areas (**B**) according to distance-based multivariate analysis (DistLM).

The taxonomic analysis of bacterial community composition revealed a similar distribution of the main taxa among all the samples collected along the depth gradient (Figure 6).

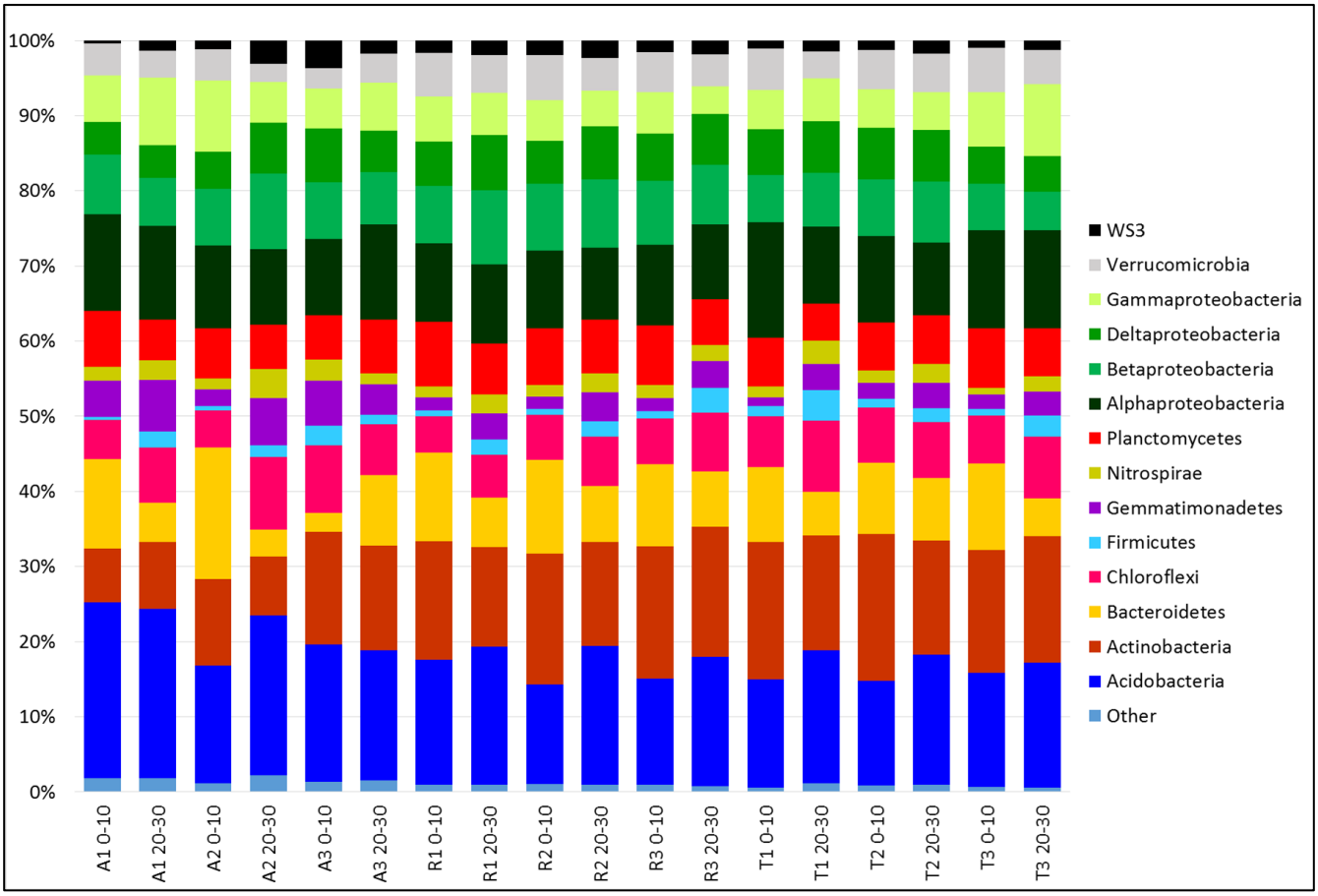

Figure 6. Taxonomic affiliation of bacterial taxa detected by high-throughput 16S rRNA sequencing in soil samples collected along the depth gradient in the three sampling areas at 0-10 and 20-30 cm depth. The bar chart represents the relative abundance of different bacterial phyla/class.

Proteobacteria were the most abundant phyla, representing between the 29,1% and the 32,8% of the total diversity. Within this taxa, 11% of the sequences were on average affiliated to *Alphaproteobacteria,* 7,5% to *Betaproteobacteria,* 5,9% to *Deltaproteobacteria* and 6% to *Gammaproteobacteria.* Other abundant phyla were *Acidobacteria* (from 13% to 22%), *Actinobacteria* (7,5%-19,1%), *Bacteroidetes* (2,4%-16,9%) and *Chloroflexi* (4,7%-9,3%) (Supplementary table 5). No trend of distribution could be either identified at the order level, in which the main identified taxa were *Rhizobiales* (3,9%-7,3%), *Xanthomonadales* (3,4-7%), *Actynomicetales* (1,2%-6,5%), *Saprospirales* (1,2%-6,4%) and *Gaiellales* (1,1%-4,8%). However, PCoA analysis showed that OTU_{97} of soil samples clustered separately according to the depth (Figure 7A) and were significantly different (PERMANOVA; p=0.012). Considering the area of collection (Figure 7B), the OTU_{97} distribution of samples belonging to

area A resulted significantly different from those of area R and T, as confirmed by pairwise statistical comparisons (PERMANOVA; A-R: p=0.007; A-T: p=0.019; R-T p=0.162).

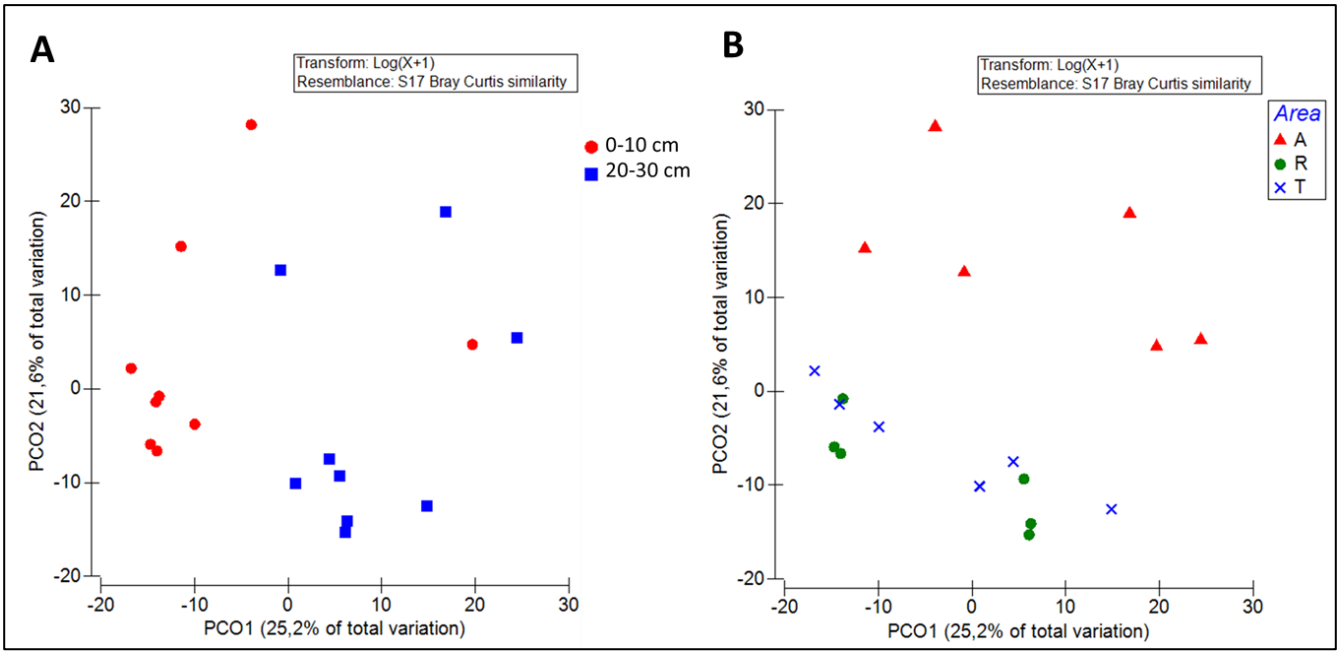

Figure 7. Distribution of OTU_{97} (Principal Coordinate Analysis, PCoA) of soil samples collected along the soil depth gradient according to (**A**) the depth of collection (0-10 or 20-30 cm) and (**B**) the sampling area of provenience (A, R or T).

Also, distLM analysis proved a significant correlation between bacterial species distribution and the main pollutants of the SIN Caffaro (As, Hg and PCB) (Figure 8 and Table 4).

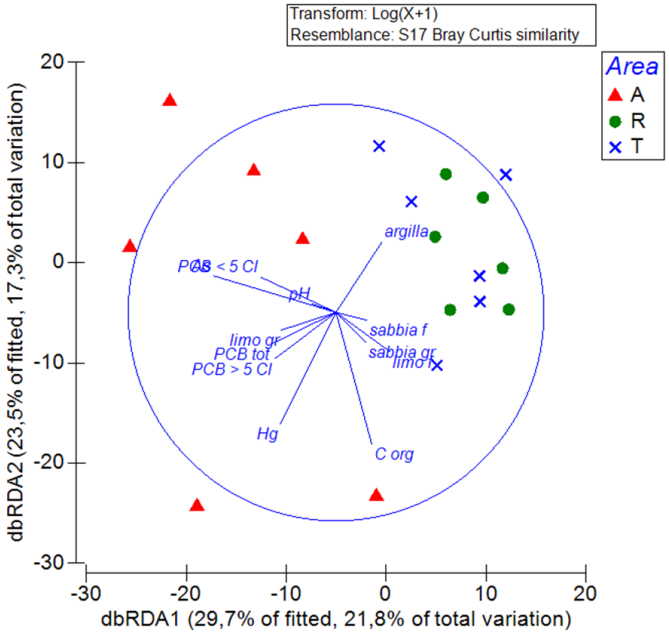

Figure 8. Correlation between OTU_{97} distribution and soil chemical and physical parameters of soil samples collected along the depth gradient at 0-10 and 20-30 cm according to distance-based multivariate analysis (DistLM).

Table 4. Statistical analysis (distLM) showing the correlations between the OTU_{97} distribution and the soil physico-chemical parameters for soil samples collected along the depth gradient at 0-10 and 20-30 cm. Significant values ($p<0.05$) are marked in bold.

Marginal test

Variable	SS(trace)	Pseudo-F	P	Prop.
clay	1101,9	1,6588	0,0726	9,39E-02
fine loam	794,15	1,1619	0,2736	6,77E-02
large loam	1057,2	1,5849	0,0926	9,01E-02
fine sand	845,93	1,2435	0,2175	7,21E-02
large sand	667,5	0,96542	0,4277	5,69E-02
pH	839,97	1,2341	0,2311	7,16E-02
TOC	1318,2	2,0257	0,0259	0,11238
As	1996,7	3,2822	**0,0004**	0,17022
Hg	1663,6	2,6441	**0,0047**	0,14182
Tot PCB	1813,5	2,926	**0,0011**	0,1546
PCB ≤ 5 Cl	1820	2,9383	**0,0005**	0,15515
PCB > 5 Cl	1805,7	2,9111	**0,0015**	0,15393

DISCUSSION

The measured chemical data allowed to clarify the complexity of pollutants' distribution in the three studied fields and to create a map of pollutants' distribution in the SIN Caffaro. The results clearly showed that the distribution of pollutants deeply changed along the vertical profile, presenting the highest pollutants' concentration in the shallower soil layers, up to 40 cm depth.

A DNA based fingerprinting was applied to investigate the soil dwelling bacterial communities showing that their overall structure was significantly different according to area and depth of collection. Soil depth is a known driver of microbial community structure in agricultural soils, regardless of the presence of xenobiotic contaminants, since it is a major player in changing edaphic conditions (Zhang et al. 2017; Fierer et al. 2003). However, by means of statistical analyses, we demonstrated that the concentration of different classes of pollutants was significantly related to the pattern of bacterial population assemblages in the analysed soils, considering both the depth gradient and the area of collection. These findings were also confirmed by the analysis of the taxonomic bacterial diversity of samples collected from the depth profile, with the most polluted area A showing a different species distribution compared to R and T. Moreover, species diversity was significantly related to the concentrations of As, Hg and PCB that represent the main contaminants of the SIN Caffaro. Although remediation potential cannot be generalized at the level of phylum or class, we retrieve as dominant taxa *Acidobacteria, Actinobacteria, Proteobacteria* and *Chloroflexi*, previously detected in heavily PCB-polluted soils (Ridl et al. 2016) and related to PCB metabolism (Uhlik et al. 2012; Leewis et al. 2016). Contaminant distribution may thereby be considered a major driver for bacterial community's structure in the historically-polluted agricultural areas of the SIN Caffaro, leading to the selection of different populations adapted to peculiar pollution levels and possibly capable of biodegradation activities, as suggested by Quero and co-authors (2017) studying the bacterial communities of PCB-contaminated marine sediments. Besides bacterial communities' composition, the depth and area of sampling also influenced FDA hydrolytic activity, which is an indicator of the total soil enzymatic activity since a broad range of enzymes like lipases, esterases and proteases can hydrolyse this molecule (Schnurer and Rosswall 1982). As expected, we observed a decrease in the hydrolytic activity from the surface (0-10 cm) to the deeper soil layers (80-100 cm), together with organic matter content. Total organic carbon (TOC) and other environmental factors that change with soil depth (such as vegetation effect, temperature, nutrients availability and water content) differentially affect soil enzymatic activity, and

it has been reported that a decrease of the hydrolytic activity measured in the subsurface should also be ascribed to the lower microbial biomass (Caldwell 2005; Blume et al. 2002). As reported in previous studies (Mukherjee et al. 2014; Margesin et al. 2003), the trend of microbial activity in our samples followed the pollutants distribution. This suggests a higher natural attenuation potential in the shallower soil layers, where the higher organic matter turnover could result in a mobilization of organic contaminants making them more available for the degrading microbial populations.

CONCLUSION

In this work, we highlighted through a multidisciplinary approach the occurrence of distribution patterns in bacterial populations related to gradients in soil pollution. Analysis of ARISA fingerprinting and 16S rRNA gene sequencing of the soil metagenome indicated that the bacterial communities' structure and species distribution was significantly different according both the area and depth of collection and it was significantly related to the concentration of the main classes of pollutants occurring in the SIN Caffaro. Moreover, a significant relationship occurred between the soil hydrolytic activity and the depth and area of samples' collection. This allows hypothesizing that the high level of pollution constitutes a driving force for the composition of the soil microbiome in the SIN Caffaro and that different phylogenetic groups including PCB degraders were selected in the more contaminated, shallower soil layers. Overall these data imply the possibility to sustain a remediation process in a large long-term polluted area by biostimulation, to be further elucidated investigating the PCB degradation potential of the soil microbiome.

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Chapter 2.

Bacteria associated to plants naturally selected in a historical PCB polluted soil show potential to sustain natural attenuation

INTRODUCTION

Polychlorinated biphenyls (PCB) are highly stable, hydrophobic and persistent organic pollutants. Due to their lipophilic nature, they bioaccumulate and biomagnify through the food web and can have a broad range of toxic effects on humans (Turrio-Baldassarri et al., 2009; Letcher et al., 2012; Quinete et al., 2014; IARC, 2015). Physico-chemical remediation techniques of PCB polluted soils are not sustainable environmentally and economically for extended contaminations and the properties of these molecules make them recalcitrant to biodegradation, impairing the efficacy of bioremediation technologies. However, PCB pollution is a worldwide problem associated to their past production and utilization in industrial facilities (Passatore et al., 2014). PCB strongly bind to the soil organic matter, resulting into low bioavailability both for plant uptake and microorganism metabolism (Sinkkonen and Paasivirta, 2000). Nonetheless, several studies showed that PCB-polluted soils host bacterial communities endowed with aerobic degradation abilities and harboring the biphenyl dioxygenase (*bph*) operon, the most studied and widespread pathway sustaining the aerobic biodegradation ability (Kurzawova et al., 2012; Leigh et al., 2006; Leigh et al., 2007; Abraham et al., 2002). Plants establish mutual beneficial interactions with selected bacterial populations, which promote nutrient uptake and enhance stress tolerance against pollutants, such as PCB, in turn decreasing their phytotoxicity (Abilash et al., 2016; Ma et al., 2011). Root exudates, besides creating a selective ecological niche for specific bacteria, may contain molecules that can induce the expression of genes involved in the PCB degradation pathways, such as the *bph* operon, in turn stimulating PCB degradation in the rhizosphere (Pham et al., 2015; Toussaint et al. 2012). Rhizoremediation, the exploitation of such positive interactions between plants and bacteria for reclamation of polluted soils, represents a sustainable alternative for clean-up of recalcitrant organic pollutants in extended areas (Vergani et al., 2017). Soil natural attenuation potential therefore relies on the presence, diversity and activity of the resident microbiota, driven by the selecting activity of autochthonous plants.

In this work, we studied the phylogenetic and functional diversity of the microbiota associated to the root-soil fractions of the three spontaneous plant species *Medicago sativa, Centaurea nigrescens* and *Dactilys glomerata*, naturally established in a former agricultural field located within the heavily polluted SIN (National Priority Sites) site Caffaro in north Italy. This is a large area polluted by the activity of the former Caffaro chemical factory and includes more than 100 ha of former agricultural areas (Di Guardo et al., 2017). It presents a mixed and uneven contamination of

chlorinated persistent organic pollutants, mainly polychlorinated biphenyls (PCB), heavy metals and metalloids and several other contaminants including dioxins, furans, tetra-chloromethane (Di Guardo et al., 2017; Turrio Baldassarri et al., 2007). PCB were produced by Caffaro for 46 years and banned in Italy in 1984. To avoid pollutant exposure to humans, economic activities, including agriculture, have been banned in 2002 in the SIN Caffaro area. In the last 12 years only natural attenuation processes occurred, with the establishment of spontaneous plants resisting phytotoxic effects. Remediation strategies for such a large and complex site represent a challenge raising the interest for the soil self-depuration potential, which could be exploited and boosted by phyto-rhizoremediation approaches. A basic question is whether PCB degradation potential is present in the bacterial communities enriched by plants able to grow in the polluted soil, mediated by the selection force determined by the rhizosphere effect. We compared the bacterial communities in the root-associated soil fractions with that of the non-vegetated soil, through 16S rRNA gene phylogenomics and the amplification of the *bphA* gene, encoding for the α sub-unit of the biphenyl dioxygenase enzyme, a proxy for the PCB degradation ability (Iwai et al., 2010; Sylvestre 2013; Vergani et al., 2017). By looking to the culturable bacterial fraction, we assessed whether bacteria represent a potentially exploitable resource for rhizoremediation purposes, by assessing their PGP capacity and PCB biodegradation potential.

MATERIALS AND METHODS

Site description, plants and soil sampling

Non-vegetated and root-associated soil fractions were collected in the SIN Caffaro, a site located in Northern Italy (Brescia municipality), within the sampling area A, a former agricultural grassland field that was previously chemically characterized and contained PCB and other chlorinated pollutants in concentrations often exceeding the safety limits (Di Guardo et al., 2017). The root system of three spontaneous plant species was collected from triplicate specimens for each plant species. Plants were identified basing on their morphological traits as *Medicago sativa* L. (MS)*, Centaurea nigrescens* Willd. (CN) and *Dactylis glomerata* L. (DG). These plant species were among the most widely widespread in the meadows present in the agricultural areas of the SIN Caffaro, according to a recent survey (Armiraglio et al. 2009). Non-vegetated bulk soil (B) was collected in triplicate in the same sampling station. Samples were transferred to the laboratories and the different soil fractions (as defined below) were separated within 4 hours from collection as

described in Marasco et al. (2012). Soil fractions were stored at -20 °C for molecular analyses and at 4°C for bacterial isolation.

DNA extraction and analyses of the bacterial community structure

Total DNA extraction was performed from 0.25 grams of each of the three non-vegetated bulk soil (B), root surrounding soil (S) and rhizosphere soil (R) replicates, using the PowerSoil DNA kit (MoBio) according to the manufacturer's protocol. Illumina tag screening of the V3-V4 hypervariable regions of the 16S rRNA gene was applied on DNA of R, S and B triplicate samples using the primers 341F and 785R (Klindworth et al., 2013). The obtained sequences were analyzed using a combination of the UPARSE v8 (Edgar, 2013) and the QIIME v1.8 (Caporaso et al., 2010a) softwares. Briefly, raw forward and reverse reads for each sample were assembled into paired-end reads considering a minimum overlapping of 50 nucleotides and a maximum of one mismatch within the region using the fastq-join algorithm (https://code.google.com/p/ea-utils/wiki/FastqJoin). The paired reads were then quality filtered, the primer sequences were removed and the individual sample files were merged in a single fasta file. This file was imported in UPARSE where operational taxonomic units (OTUs) of 97% sequence similarity were formed and chimeras were removed using both de-novo and reference-based detection. For reference chimera detection, the "Gold" database containing the chimera-checked reference database in the Broad Microbiome Utilities (http://microbiomeutil.sourceforge.net/) was used. Taxonomy was assigned to the representative sequences of the OTUs in QIIME using UClust (Edgar et al., 2010) and searching against the latest version of the Greengenes database (McDonald et al., 2012). After the removal of reads affiliated to chloroplast, Archaea and unassigned sequences, a total of 1319653 high-quality merged paired-end reads with an average length of 450 bp were obtained. All the samples analyzed presented Good's coverage values ranging from 90 to 99 capturing sufficient diversity with an adequate sequencing depth (Supplementary Table 1).

The OTU table, composed by 3587 OTUs, and the phylogenetic tree were calculated with FastTree2 (Price et al., 2010) using default parameters and the PyNast-aligned (Caporaso et al., 2010b) representative sequences as an input. The OTU table and the phylogenetic tree were used as inputs for the subsequent analyses of alpha- and beta-diversity. Bray-Curtis distance matrix on the log transformed OTU table was used to perform a Principal Coordinates Analysis, Canonical Analysis of Principal coordinates (CAP) and to conduct a permutational multivariate analyses of variance (PERMANOVA). Statistical analyses were conducted in PRIMER v. 6.1, PERMANOVA+ for PRIMER routines (Anderson et al., 2008) to test differences in bacterial community composition among the

three soil fractions and among the three plant species. A first analysis was conducted in a one-way anova to explore difference among the fraction considering as a factor “Fraction” as fixed and orthogonal (3 levels: rhizosphere/root surrounding soil/non-vegetated bulk). A second analysis was conducted exploring difference of the microbial assemblage among plants and fractions using the factor “Plant” (3 levels: *Medicago sativa, Centaurea nigrescens* and *Dactylis glomerata*) and the factor “Fraction” (2 levels: rhizosphere and root surrounding soil) both as a fixed and orthogonal, and their interaction (Fraction x Plant). Prior to perform the statistical analysis we verify that the data were not over-dispersed using PERMDISP for the factor “Fraction” ($F_{1,18}$=3.61 p=0.21) and the factor “Plant” ($F_{2,15}$=9.36 p=0.06). The shared OTUs among different fractions and plant species have been defined by Venn-diagram analysis using the software available at http://bioinformatics.psb.ugent.be. Diversity indexes were calculated using PAST (Hammer et al., 2001) and their statistical difference was evaluated with the analysis of variance considering the index as response variable and “Fraction” and “Plant” as explanatory categorical variable (see above for details). Raw sequences have been deposited at the ENA European Read Archive under accession number from SAMN07167786 to SAMN07167806, BioProject PRJNA388028, SubmissionID SUB2728610.

16S rRNA gene quantitative PCR (qPCR) was performed in a reaction volume of 15 µl with 1 µl of template using universal primers 27F and 1492R and the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). Thermal protocol was set up as follow: 98 °C (3 min), then 40 cycles at 98 °C (15 sec), 58°C (30 sec) and 72°C (1 min). Starting DNA concentration (ng/µl) was measured using a PowerWave HT Microplate Spectrophotometer (BioTek) and the number of 16S rRNA copies obtained with qPCR was normalized with the DNA concentration.

Bacteria isolation and identification

For bacterial isolation, the R samples obtained from the triplicate plants of the same species were pooled and homogenized. One gram of the resulting soil was suspended in 9 ml of physiological solution (0.9% NaCl), diluted in ten-fold series and plated onto mineral medium (Uhlik et al., 2011), adding biphenyl crystals on the plate lid as unique carbon source. Colonies were randomly picked after one week of incubation at 30°C, after the appearance of a stable number of colonies, and were spread three times on the same medium to obtain pure bacterial cultures. A collection of 165 biphenyl-utilizing rhizobacterial strains was established (70 isolates from MS and DG, 84 isolates from CN) and cryopreserved in 25% glycerol at -80°C. Strain code includes a different number according to the plant of origin (1: MS, 2: CN, 3: DG).

The genomic DNA of each isolate was extracted through boiling cell lysis (Ferjani et al. 2015). Bacterial strains were identified through 16S rRNA gene amplification and partial sequencing (Macrogen, Rep. of South Korea) as described by Mapelli et al. (2013). 16S rRNA nucleotide sequences were subjected to BLAST search (using the blastn suite) and were deposited in the ENA database under accession numbers LT838007–LT838169.

***bphA* gene detection, quantification and sequencing in soil metagenomes and strain genomes**

The presence of the genes encoding for biphenyl dioxygenase α subunit (*bphA*) was assessed in the metagenome of soil samples (B, S and R) through PCR as described by Iwai et al. (2010), using the primers BPHD-F3/R1, and further confirmed using 512F and 674R primer set (Leewis et al. 2016a). The latter primer set was also used for a *bphA* gene qPCR assay as described by Leewis et al. (2016a) and the relative abundance of *bphA* gene copies was expressed as a ratio over the total community 16S rRNA gene copy number. Bacterial isolates were grown overnight in Tryptic Soy Broth medium and then subjected to CTAB – phenol chloroform DNA extraction (Chouaia et al. 2010). *BphA* gene amplification was performed with 2 µl of DNA template in a final volume of 30 µl with primers 463F/674R (Petric et al. 2011), at the following conditions: buffer 1X, $MgCl_2$ 1.8 mM, dNTPs 0.2 mM, primers 1 µM, Taq 1.5 U per reaction. PCR thermal protocol was set up as follows: 10 min at 95°C, then 40 cycles of 95°C (15 s), 65°C (1 min), 72°C (2 min) and a final elongation step of 10 min at 72°C. All PCR reactions were performed utilizing FastStart™ High Fidelity PCR System (Roche). Genomic DNA of the model PCB-degrading strain *Paraburkholderia xenovorans* LB400 (DSMZ, Germany), was used as positive control for all *bphA* PCR reactions. PCR results were visualized on 1.2% agarose gel and PCR products that did not show the presence of aspecific bands were sequenced at Eurofins Genomics (Italy). Sequences were then identified using the BLASTn suite of the NCBI website (www.ncbi.nlm.nih.gov) and a Neighbor-Joining phylogenetic tree was then built using MEGA 5.1 (Tamura et al. 2011), computing the evolutionary distances using the Jukes-Cantor method. Nucleotide sequences were deposited in the ENA database under accession numbers LT840193–LT840239.

***In vitro* characterization of bacterial isolates for PGP traits and bioremediation potential**

In vitro screening for the presence of activities related to plant growth promotion was performed for the entire bacteria collection. Inorganic phosphate solubilization and the production of indole-3-acetic acid (IAA), ammonia, protease and exopolysaccharides (EPS) were assessed as described by Cherif et al. (2015); 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity was evaluated according to Belimov et al. (2005). Strains were also tested for abiotic stress resistance, namely the

ability to grow at 4°C and 42°C, and in presence of 5% NaCl and 20% polyethylene glycol (PEG) supplemented to the medium (Cherif et al. 2015). Catechol 2,3-dioxygenase activity was tested following the protocol described by Margesin et al. (2003).

***In vivo* assessment of plant growth promotion of tomato**

Tomato seeds were sown in commercial non sterile soil placed in trays and, after one week, uniform-sized seedlings were selected, each transplanted in separated 0.3 kg commercial soil pots and maintained under greenhouse conditions (≈ 110 photons m^{-2} s of light for 12h during the day and average temperature of 25°C). A subset of 11 rhizobacteria strains, selected based on a cluster analysis performed using MVSP (Kovach et al., 1999) combining the PGP activities and the abiotic stress tolerance traits, were inoculated separately on tomato plants. One week after transplantation, the tomato plants (n=5 for each treatment) were fertilized once with a bacterial suspension of the selected strains at a final concentration of 10^8 cells g^{-1} of soil (fresh weight). The inocula have been prepared according to Rolli and coauthors (2015) resuspending the bacterial cells in sterile tap water. Non-inoculated plants (n=5) were irrigated with the same amounts of sterile tap water and used as control. After 30 days, plants were harvested for the measurement of shoot/root length and weight. Statistical analysis was performed by a pairwise comparison between each bacterial strain inoculated with the negative control, using Student t-test.

RESULTS

Diversity and degradation potential of bacterial communities associated to non-vegetated and root-associated soil fractions in the SIN Caffaro

The □-diversity of bacterial communities colonizing non-vegetated bulk soil (B) and root-associated soil fractions (rhizosphere, R, and root surrounding soil, S) in the SIN Caffaro was significantly different (Figure 1A, PERMANOVA, $F_{2,18}$:3.48, p=0.0007, Supplementary Table 2A). Considering only the R and S soil fractions associated to the three plant species *M. sativa* (MS)*, C. nigrescens* (CN) and *D. glomerata* (DG), the bacterial population assemblage was significantly driven by both plant species and soil fraction (Figure 1B, PERMANOVA, $F_{1,12}$=4.72, p<0.001 and PERMANOVA, $F_{2,12}$=3.21, p<0.05 respectively, Supplementary Table 2B) but not by their interaction (PERMANOVA, $F_{2,17}$=1.67, p>0.05, Supplementary Table 2B). Quantification of the individual factors' contributions to the observed bacterial community variations, determined by PERMANOVA of Bray-Curtis, showed that the two factors "Fraction" and "Plant species" equally contributed to determine the observed

bacterial microbiome variation, explaining 21% and 18% of the variation, respectively (Supplementary Table 3), followed by the interaction of these two factors (11%). For the three fractions, not significant differences have been retrieved considering the OTU richness, but among the rhizosphere the MS and CN plants presented a lower number of OTUs (Figure 1C). OTU evenness was instead significantly different among fractions (ANOVA, $F_{2,20}$=21.63, p<0.001) but not between plants (Figure 1D). OTU diversity, calculated as inverse Simpson diversity index, was significantly lower in R compared to B and S (ANOVA, $F_{2,20}$=4.7, p<0.05). While not significant differences have been observed among the different plants in S fraction, plant species determined a strong effect in R fraction where DG hosted the significantly highest diversity, followed by CN and MS (Figure 1E).

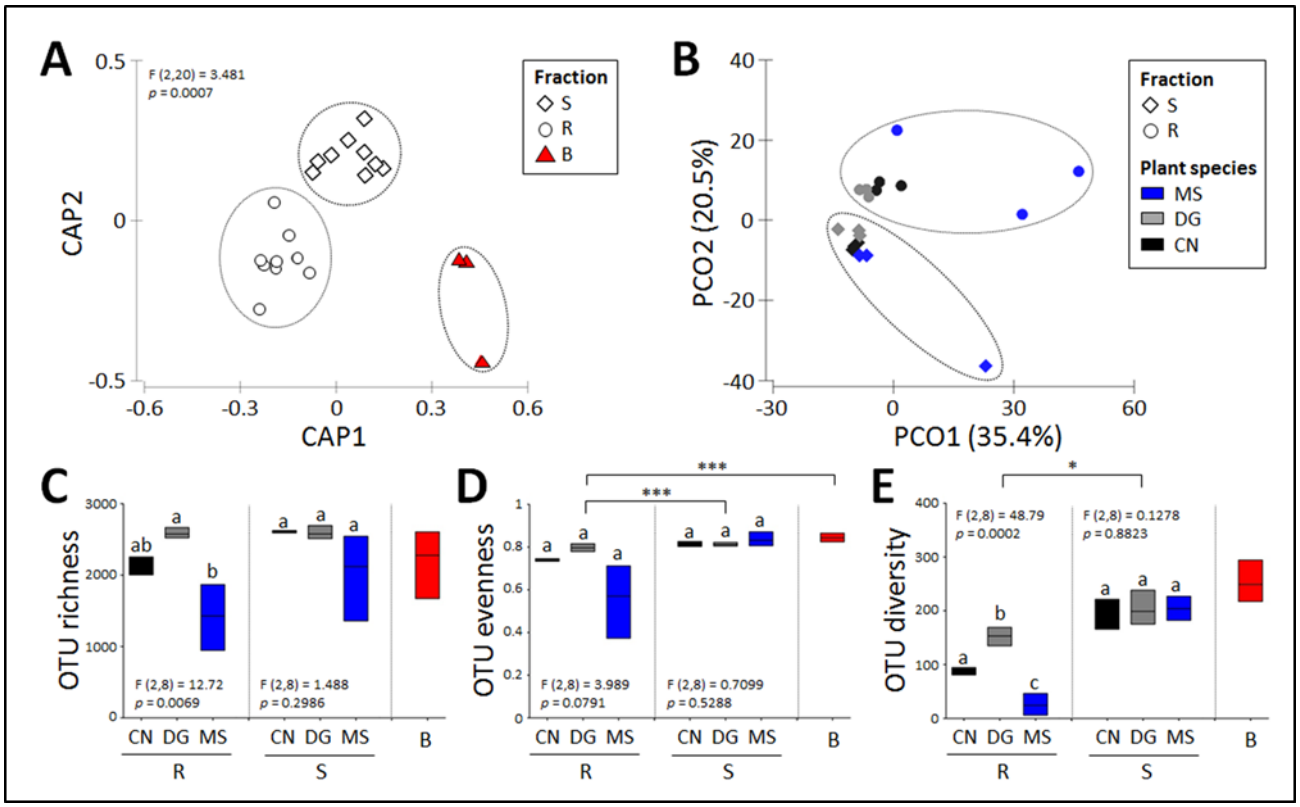

Figure 1. Analysis of bacterial community's structure and diversity of soil samples associated to spontaneous plants at SIN Caffaro. (A) Constraint (CAP) and (B) Unconstraint analysis of principal coordinates of the OTU_{97} obtained from 16S rRNA gene sequencing of the bacterial communities considering the different soil fractions (A) and the root-associated soil fractions according to the plant species (B). (C) OTU_{97} Richness, (D) OTU_{97} Evenness and (E) OTU_{97} diversity. Statistical analysis are indicated in within the plant species comparing each fraction while asterisks indicate the statistical significance among fractions (for details see the text). The letters R, S and B indicate respectively the rhizosphere, root surrounding soil and non-vegetated bulk soil.

The three soil fractions collected in the SIN Caffaro showed (i) low number of specific OTUs typical of each fraction, and (ii) a consistent group of shared OTUs (2831/3587) accounting the 97% of the 16S rRNA sequences (Figure 2A, Supplementary Table 4A). The rhizosphere and root surrounding soil associated to the three plant species shared a total of 1922/3436 and 2558/3436 OTUs, respectively, accounting up to 98% of relative abundance in S (Figure 2B and C, Supplementary Table 4B and C).

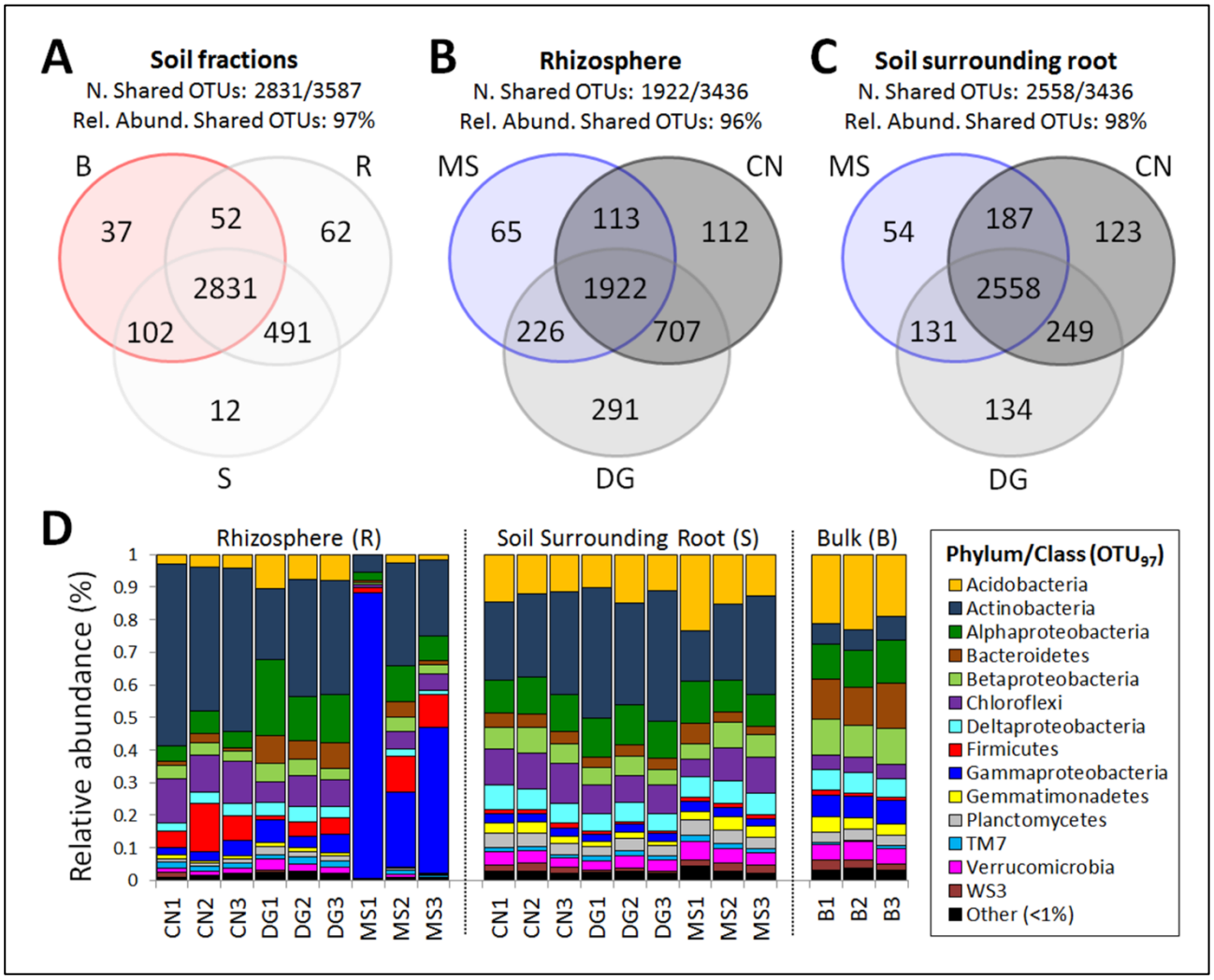

Figure 2. Shared microbiome and taxonomic diversity of bacterial communities associated to non-vegetated bulk soil and root-associated soil fractions of plants grown in the SIN Caffaro. Venn diagrams showing the shared and exclusive bacterial OTUs of **(A)** the three soil fractions (R, S and B). Specific and shared OTUs among the three plants in rhizosphere soil **(B)** and soil surrounding root **(C)** have been reported. Number of the shared OTUs and their relative abundance in all soil fractions, rhizosphere and soil surrounding root have been listed on the top of each Venn diagram. **(D)** Relative abundance of different bacterial phylum/class in the non-vegetated bulk soil (B), rhizosphere (R) and soil surrounding root (S) of the three plants (MS, CN and DG) representing OTUs showing more than 0.001% relative abundance of all reads. Phyla/classes representing less than 1% out of total reads were grouped as 'Other'.

The high number of shared OTUs in non-vegetated and plant-associated soils indicated that all the fractions hosted bacterial communities with similar phylogenetic composition and different in the structure, as demonstrated by the analysis of Beta and Alpha-diversity (Figure 1).

The taxonomic affiliation of OTUs (Figure 2D, Supplementary Table 5 and Supplementary Table 6) revealed that the soil fractions hosted 37 bacterial phyla, 105 classes (99.3% sequences classified), 153 orders (89% classified), 167 families (67% sequences classified) and 162 genera (23% sequences classified). The most represented phyla were present in all the soil fractions (R, S and B): *Proteobacteria* 34% (among these *Gammaproteobacteria* 16% and *Alphaproteobacteria* 10%), *Actinobacteria* 30%, *Chloroflexi* (8%) and *Acidobacteria* (8%). The three soil fractions were nevertheless dominated by different phyla/classes. In the non-vegetated soils *Proteobacteria* (on average 35%, mainly represented by *Alphaproteobacteria* and *Betaproteobacteria*) was the prevalent phylum followed by *Acidobacteria* (22%) and *Bacteroidetes* (12%) (Figure 2D). *Proteobacteria* dominated also the rhizospheres with *Gammaproteobacteria* (26%) and *Alphaproteobacteria* (9%) as the main *Proteobacteria*-classes, while in S fraction the *Actinobacteria* was the dominant phylum (30%).

Moving from the root-associated fractions (R and S) to the non-vegetated bulk soil, a strong reduction of the *Actinobacteria* phylum was observed in the bacterial communities, from an average of 33% in R to an average of 6% in B (Supplementary Table 5). An opposite trend has been observed for *Acidobacteria*, *Bacteroidetes, Verrucomicrobia* and *Gemmatimonadetes* phyla that were enriched in the non-vegetated bulk soil and strongly limited in R and S fractions (Supplementary Table 5). Finally, while *Firmicutes* bacteria were enriched in R (7%) respect to the other two fractions (1%), *Chloroflexi* were more prevalent in S (10%) respect to R (7%) and B (4%). Difference in taxa enrichment and selection were observed also according to the plant species in both R and S fractions (Supplementary Table 7). The MS and CN bacterial communities in R fractions were dominated by *Gammaproteobacteria* (52%) and *Actinobacteria* (50%) respectively, determining a higher dominance index value (0.32 and 0.28) in these communities respect to the DG one (0.15), while DG hosted a more equally distributed bacterial community with Evenness index of 0.62. An opposite trend has been observed in the S fraction, where DG was dominated by *Actinobacteria* (37%) while MS and CN presented several taxa equally distributed within the bacterial communities (Evenness 0.72 and 0.69, respectively, Supplementary Table 7).

The *bphA* gene was detected, by means of qualitative PCR using different primer sets, in the metagenomes of all B, S and R samples. Despite that, the quantification of *bphA* gene with primers

512F and 674R was possible only for two replica of the CN rhizosphere (*bph*A/16S rRNA ratio, CN R2: 0.56 ± 0.03; CN R3: 0.50 ± 0.08) and one replica of the DG S (*bph*A/16S rRNA ratio, DG S2: 0.08 ± 0.004), showing the highest *bphA*/16S rRNA gene ratio in the CN rhizosphere.

Identification of rhizosphere biphenyl-utilizing bacterial isolates

One-hundred sixty-five bacterial strains have been isolated from the rhizosphere of the three plant species MS, CN, DG in mineral medium supplemented by biphenyl as unique carbon source, hence considered as potential biphenyl degraders. The strains belonged only to 3 phyla, *Actinobacteria* (75%, 51%, 52% in MS, CN and DG collections, respectively), *Proteobacteria* (17%, 30%, 32% in MS, CN and DG collections, respectively) and *Bacilli* (8%, 11%, 13%, in MS, CN and DG collections, respectively). All the isolates belonged to phyla that were present in all the metagenome 16S rRNA high-throughput sequencing libraries, even though with a different relative abundance. All the isolates exhibited sequence identity with the closest described species in NCBI database higher than 97% (Supplementary Table 8) and their phylogenetic affiliation at the genus level is represented in Supplementary Figure 1. Among *Gammaproteobacteria, Pseudomonas* species were widespread in the three sub-collections, and constituted 15%, 23% and 13% of MS, CN, DG rhizosphere, respectively. *Acinetobacter* was isolated only from CN and DG rhizospheres (5% and 16%, respectively). Among the phylum *Bacilli*, the genus *Bacillus* was the most represented, isolated from the rhizosphere of all plant species (7%, 13%, 6% in CN, DG, MS, respectively). Seventy-three percent of all the isolates from the rhizosphere of the three plant species belonged to three genera: *Arthrobacter* (18, 14, 10 strains from DG, MS, CN rhizospheres, respectively), *Microbacterium* (7, 24, 11 strains from DG, MS, CN rhizospheres, respectively), *Pseudomonas* (7, 8, 13 strains from DG, MS, CN rhizospheres, respectively). MS rhizosphere showed the lowest number (6) of cultivable genera within the biphenyl-utilizing bacteria, widely dominated by *Microbacterium* (44% of the isolates) and *Arthrobacter* (29% of the isolates), compared with CN and DG rhizosphere that included 11 genera.

Functional characterization of the cultivable rhizosphere biphenyl-utilizing bacteria

The isolate collection has been screened *in vitro* for PGP activities, bioremediation potential and abiotic stress resistance (results are detailed for each isolate in Supplementary Table 8). Production of IAA and proteases were the PGP related activities most represented among the collection, present in more than 50% of the strains, in similar percentages among sub-collections isolated from each plant species (Figure 3).

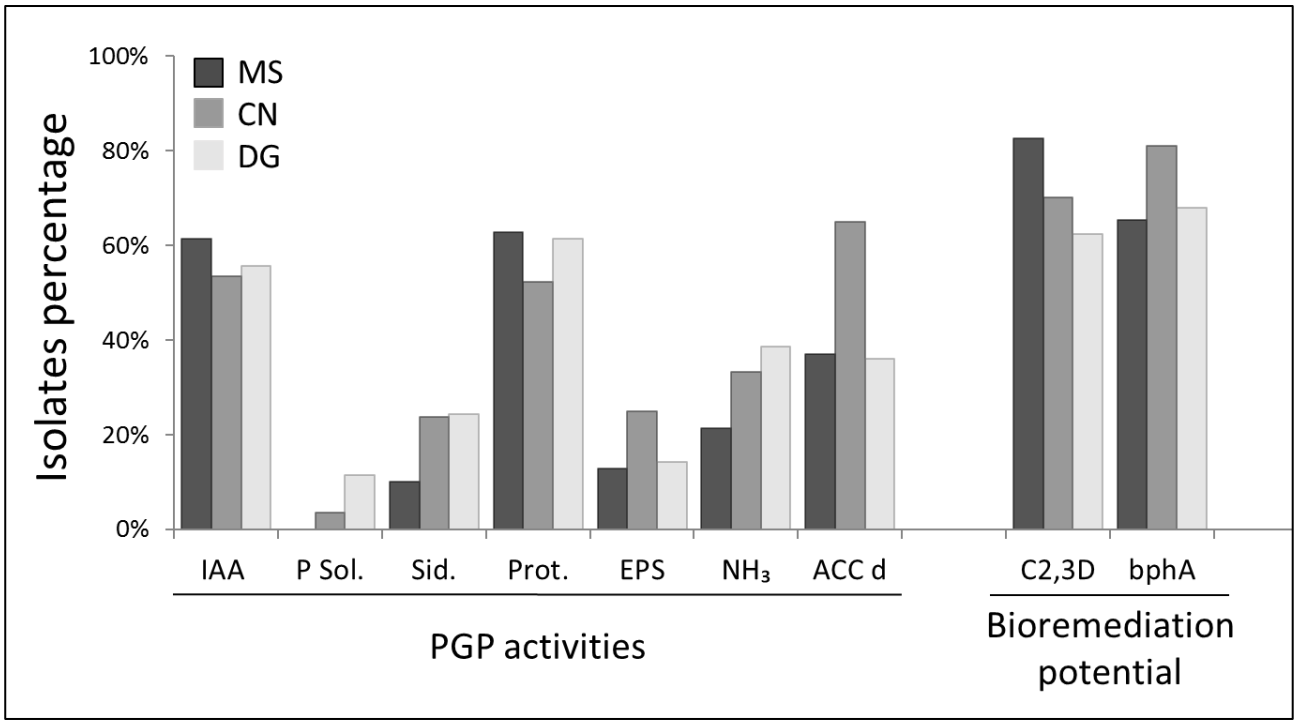

Figure 3 Characterization of the bacteria collection for plant growth promotion (PGP) traits and degradation capacity. Percentage of strains isolated from MS, CN, DG rhizosphere that resulted positive to PGP and degradation screenings. Among PGP activities: IAA = indolacetic acid production, P. Sol. = inorganic phosphate solubilisation, Sid. = siderophore production, Prot. = protease production; EPS = exopolysaccharides release, NH_3=ammonia production, ACCd = ACC-deaminase activity. Activities related with bioremediation potential: C 2,3 D = 2,3-catechol dioxygenase activity and bphA = positive PCR amplification of the *bphA* gene.

A large fraction of the collection produced ammonia (21%, 33% and 39% of the MS, CN and DG strains, respectively), EPS (13%, 25% and 14%, respectively) and siderophores (10%, 24% and 24%, respectively). ACC deaminase activity was detected in higher percentage among CN (65%) than MS (37%) and DG (36%) sub-collections. Phosphate solubilization activity was present in a small proportion among CN (4%) and DG (11%) isolates, while it was absent in MS sub-collection. Concerning the biodegradation potential, cathecol 2,3-dioxygenase activity was widespread in the entire collection with 83%, 70% and 63% of the MS, CN and DG active isolates, respectively. Biphenyl dioxygenase α subunit gene (*bphA*) was detected in the genomic DNA of 65%, 81%, and 68% of the MS, CN and DG isolates, respectively. PCR products presenting one single band of the expected size (211 bp) in agarose gel electrophoresis were sequenced, obtaining 45 partial *bphA* gene sequences (9, 19 and 17 from MS, CN and DG isolates, respectively). Eighty percent of the sequences showed high nucleotide identity (>99%) with *Pseudomonas pseudoalcaligenes* KF707 *bphA*, and the remaining ones showed >99% nucleotide identity with *Rhodococcus wratislaviensis* strain P13

bphA1 and *Rhodococcus opacus bphA1* genes (Supplementary Table 9). The *bphA* sequences were clustered in a phylogenetic tree with *bphA* sequences of reference strains having demonstrated PCB degradation ability. The sequences were clustered in two groups, both including known PCB-degraders (Supplementary Figure 2): *Pseudomonas* KF707-like sequences clustered together with *Paraburkholderia xenovorans* LB400, while *Rhodococcus*-like sequences grouped separately.

All strains were able to cope with the osmotic stress induced by the addition of 20% of PEG in the growth medium (Supplementary Table 2). The majority of the strains (71% on average) from each plant species proved to grow in presence of salt in the growth medium, while a percentage comprised between 58% and 71% tolerated high (42°C) and low (4°C) temperatures for growth (Figure 3 and Supplementary Table 8).

A total of eleven strains has been selected basing on PGP activities and abiotic stress tolerance traits (Supplementary Figure 3) and have been tested *in vivo* on tomato as a model plant. The strains were affiliated to different species belonging to the genera *Pseudomonas*, *Acinetobacter Arthrobacter* and *Curtobacterium,* and presented an array of different *in vitro* PGP traits, abiotic stress tolerance and bioremediation potential (Supplementary Table 10).

Despite showing only 1 and 2 *in vitro* PGP traits, respectively, strains 2-30 and 2-50, both isolated from CN plant and affiliated to different species of the genus *Arthrobacter* (Supplementary Table 10), significantly promoted plant growth compared to the non-inoculated control (Figure 4).

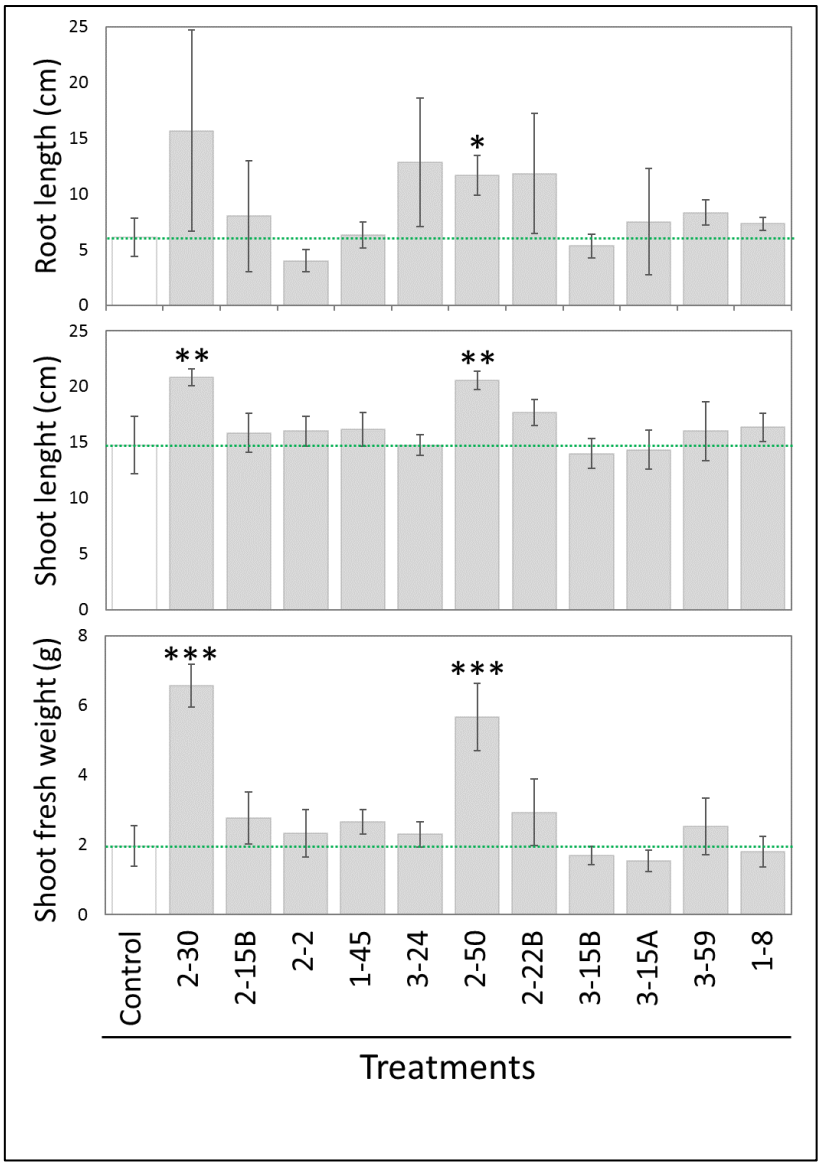

Figure 4. Results of *In vivo* plant growth promoting test on tomato under greenhouse conditions, according to (A) shoots length, (B) root length and (C) fresh weight measurement. Screened strains and plant measurement results are reported on the X and Y-axes respectively. The data were calculated as average of five plants per treatment and Student t-test was adopted to statistically analyze the data. The star indicates statistically significant differences (* = $p<0.05$, ** = $0.001<p<0.05$, *** = $p<0.001$).

Both strains mainly affected shoot development, significantly increasing shoot length ($p<0.01$) and fresh weight ($p<0.001$). While 2-30 did not showed effect on the root development, 2-50 significantly promote also the root length ($p<0.05$). Strain 2-50 possesses, moreover, both the tested bioremediation potential traits (Supplementary Table 10).

DISCUSSION

Plant influence on soil bacterial community structure and degradation potential

In vegetated soils, physico-chemical properties together with plant root exudation and turnover shape the structure of the microbial community. Resulting in the so-called "rhizosphere effect", every plant species presents a specific pattern of root exudation and interacts with the soil community selecting a specific microbiota recruited from the surrounding soil (Berg and Smalla, 2009; Haichar et al., 2008). Here we compare the bacterial communities inhabiting the rhizosphere and root surrounding soils of three spontaneous plant species with a non-vegetated bulk soil from a site heavily contaminated with chlorinated persistent organic pollutants and heavy metals, demonstrating that both the soil fraction and the plant species cooperated in the selection of a specific bacterial assemblage in the root-associated soils. The bacterial communities inhabiting all soil fractions hosted the typical phyla that dominate soil ecosystems, i.e. *Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi* and *Proteobacteria* (Bulgarelli et al., 2013). R and S fractions, that are directly under plant root influence, showed an increase of *Actinobacteria* and *Proteobacteria.* Within the *Actinobacteria*, the order *Gaiellales* was retrieved at higher percentage in all the plant-associated fractions and in particular in the CN rhizosphere. This bacterial order was previously detected in the root system of rice (Hernandez et al., 2015) and maize planted on heavy metals polluted soil (Touceda-Gonzalez et al., 2015), however it is still poorly studied and includes only one described species (Albuquerque et al., 2011). Plant exudates enriched also the orders *Sphingomonadales* and *Pseudomonadales* in the DG and MS rhizospheres, respectively. Members of these orders have already been retrieved in PCB polluted soils, involved in biphenyl degradation and for some genera also in PCB degradation (Leewis et al., 2016a; Leigh et al., 2007; Hu et al. 2015; Jayanna and Gayathri 2015). The phylum *Chloroflexi*, comprising a well known anaerobic PCB degraders group (Jugder et al., 2015), was retrieved at relative abundance higher than 1% exclusively in the S fractions, being particularly enriched in MS1. It is possible that an anoxic microniche occurred in this sample, allowing the selection of *Chloroflexi* members, in agreement with previous finding of *Chloroflexi* enrichment in the rhizosphere of *Medicago sativa* during a PCB polluted soil phytoremediation trial (Tu et al. 2011) and of *Sparganium* sp. during biostimulation of the autochthonous microbiota in historically PCB polluted sediments (Di Gregorio et al., 2013).

Studies on a hydrocarbon-contaminated soil proved that the pollutant concentration rather than rhizosphere effect of planted willows had a major role in shaping the bacterial community (Yergeau

et al., 2014; Bell et al., 2014). In a previous work (Di Guardo et al., 2017) we suggested that soil pollutant profiles of different former agricultural fields in the SIN Caffaro acted as drivers of the bacterial community composition. Here we demonstrated that in one of the previously analyzed fields, the rhizosphere effect of spontaneous plants adapted to the occurring soil contamination significantly influenced bacterial assemblages in the root-associated soil fraction, R and S. We speculate that in the rhizosphere an enhanced pollutant degradation, according to the higher abundance of taxa previously associated to PCB metabolism, contributed to shape bacterial diversity (Ridl et al. 2016; Leewis et al. 2016b). Our results indicated that autochthonous vegetation, not specifically selected for rhizoremediation efficiency, but rather naturally adapted to counteract phytotoxicity and the specific polluted soil conditions, can establish a strong relationship with the soil bacterial community, potentially sustaining PCB detoxification and in turn the natural attenuation process. The diversity of R bacterial communities was significantly influenced by the three plant species, which selected a root-associated microbiome having a peculiar structure. Despite this, the occurrence of a high percentage of OTUs shared among the different soil fractions, may suggest a strong selection force toward specific taxa constituted by the high pollution level, and possibly related to contaminant detoxification.

Different plant species, including MS, were previously demonstrated to enrich and stimulate the PCB-degrading bacterial communities, inducing an increase of *bphA* gene copy number and its expression levels in the rhizosphere compared to the non-vegetated bulk soil (Li et al., 2013; Tu et al., 2011; Pagé et al., 2015). In this work, the *bphA* gene was detected in the metagenome of all the soil fractions and could be quantified in CN rhizosphere and DG soil surrounding roots. The copy number ratio between this gene and the 16S rRNA bacterial gene was higher in R than in S samples, leading to speculate that bacterial populations harboring degrading potential were enriched in the rhizosphere fraction. However, the spread of *bphA* gene in the bacterial microbiota of the SIN Caffaro soil indicated an intrinsic PCB attenuation potential in this site, regardless of soil fraction or plant species, suggesting a stronger role of edaphic conditions rather than vegetation.

Isolates potential for rhizoremediation

The root system of plant growing on other PCB polluted sites, was shown to host PCB metabolizing bacteria (Ionescu et al., 2009; Leigh et al., 2006). Hence, we applied a cultivation approach to identify indigenous bacteria in the rhizosphere of plants naturally able to cope with the high level of pollution in the SIN Caffaro, having PGP and PCB degrading potentials. The synergistic effect of these traits has indeed the potential to sustain rhizoremediation approaches, in which both plants

and bacteria are involved in remediation (Vergani et al. 2017). The cultivation approach applied to MS, CN, DG rhizospheres, due to the selective conditions determined by biphenyl as unique carbon source, led to isolate only bacteria species belonging to three of the thirty-seven phyla identified in the soil metagenome by 16S rRNA high-throughput sequencing (*Actinobacteria, Proteobacteria* and *Firmicutes*). The high taxonomic similarity shown by the three sub-collections, in which four species over the twenty-one detected accounted for the 78% of the whole collection, suggests that the cultivable biphenyl-utilizing bacteria likely were mainly selected by the soil characteristics occurring at the SIN Caffaro site rather than the plant species. The detection of *bphA* gene in 72% of the isolates indicates that these strains harbor the genetic information to initiate the upper pathway of PCB degradation. Moreover, the large majority of the isolates belonged to the genera *Arthrobacter, Microbacterium* and *Pseudomonas*, taxa previously isolated from the rhizosphere of plants growing in PCB contaminated soils and known for their capacity to grow on biphenyl and, for some strains, to degrade PCB (Kurzawova et al., 2012; Uhlik et al., 2011; Leigh et al., 2007; Gilbert and Crowley 1997). Most of the *bphA* sequences amplified from the rhizobacteria collection showed higher similarity with the biphenyl dioxygenase α subunit of *Pseudomonas pseudoalcaligenes* KF707, a model strain studied for its ability to metabolize PCB through 2,3-dioxidation (Furukawa, 1994). This large group of sequences clusters together with the *bphA* sequence of the reference strain *Paraburkholderia xenovorans* LB400. The functional diversity between *Paraburkholderia xenovorans* LB400 and *Pseudomonas pseudoalcaligenes* KF707 is determined by minor differences in the gene sequence (Furukawa and Fujihara, 2008), whose detection was not possible in our analysis due to the short sequence coverage of the used primer set. Functional redundancy for PCB degradation in the SIN Caffaro soil is supported by *bph*A sequences displaying sequence divergence. A second cluster of sequences was affiliated with a gene previously sequenced in *Rhodococcus* spp., including the reference strain *R. jostii* RHA1, which shows a different range of substrates and a low degree of homology compared with the *bphA* proteins produced by *Pseudomonas* KF707 and *Paraburkholderia* LB400 (Masai et al., 1995). The *bphA* sequences of the SIN Caffaro isolates were not related to the phylogenetic identification of the isolates, reflecting that *bph* genes are frequently associated to mobile elements and can be spread through horizontal gene transfer (Pieper, 2005). Catechol 2,3-dioxygenase activity was widespread throughout the collection and all the isolated strains harboring *bphA* gene also presented this activity. Biphenyl dioxygenase and catechol 2,3-dioxygenase are enzymes involved in the metabolism of several root-derived and xenobiotic aromatic compounds, so these results support the hypothesis that plants foster organic

contaminant degradation by their root-associated bacteria (Fuchs et al., 2011). In particular, since catechol is a toxic metabolite produced by the degradation of biphenyl in the benzoate lower pathway, its degradation capability is essential for a complete mineralization of PCB that can occur through co-metabolism by bacteria harboring the upper and/or lower degradation pathways (Leewis et al. 2016a).

PGP activities have been frequently reported in bacteria from polluted soils (Franchi et al., 2016; Thijs et al., 2014; Croes et al., 2013), according to the ability of plants to select beneficial bacteria when growing under phytotoxic and stress conditions. Likewise, IAA production and ACC deaminase activity are PGP traits well represented in the cultivable biphenyl-utilizing rhizobacteria hosted by all the three plant species. Bacterial IAA production can influence root proliferation and elongation, thereby affecting nutrient and water uptake by plants (Lambrecht et al., 2000) besides phytoextraction and phytostabilization in soils contaminated by heavy metals (Ma et al., 2011). Moreover, indole and its derivatives are considered inter-kingdom signal molecules and play a role as biofilm regulators, a further feature in PGP bacteria-plant interactions (Lee et al. 2015). ACC deaminase activity is known to lower ACC level in plant cells interfering with ethylene biosynthesis and thereby decreasing plant stress response potentially deriving from chemical phytotoxicity (Glick, 2010). Therefore, the isolated strains showed a notable potential in supporting plant adaptation and growth in the highly polluted soil of the SIN Caffaro. Moreover, a significant fraction of isolates tolerated moderate saline, osmotic and temperature stresses. These phenotypes are not directly related to PGP or biodegradation activity, but could confer to the strains a higher fitness in soils with complex and uneven pollutant fingerprints and subjected to seasonal changes (Di Guardo et al. 2017).

Two strains belonging to the genus *Arthrobacter,* one of the most abundant in our collection, also proved to promote the growth of a model plant (tomato) under greenhouse conditions. Since *in vitro* screening alone is not always sufficient to evaluate the actual PGP potential of bacterial strains (Cardinale et al., 2015), this result is encouraging for further *in vivo* tests with plant species selected for rhizoremediation trials, considering that these bacteria are also well adapted to the heavy pollution of the SIN Caffaro soil.

CONCLUSION

In this work we reported that three spontaneous plant species selected in the strongly PCB-polluted soil of a historical contaminated site, differentially affected the composition of the bacterial community in the root-associated soil fractions. The selective pressure imposed by the persistent chlorinated organic pollutants, heavy metals and metalloids in the soil, putatively affected the soil shared microbiome and determined an intrinsic functional potential for natural attenuation in the root-associated soil fractions. Rhizosphere bacterial strains harboring *bphA* gene and displaying catechol dioxygenase and PGP abilities, such as IAA production and ACC deaminase activity, are a potential resource for the improvement of plant growth and the detoxification in the heavily contaminated soils of SIN Caffaro, possibly exploitable for future rhizoremediation interventions.

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Chapter 3.

Set up of phyto-rhizoremediation processes of a PCB polluted soil in greenhouse conditions: evaluation of microbiota biostimulation and characterization of potential bacterial degraders

INTRODUCTION

As mentioned in the previous chapters, soil excavation and *ex situ* physical and chemical treatments are not applicable to large areas like the former agricultural fields inside the SIN Caffaro, because of the excessive economic and environmental impact. Rhizoremediation represents a low-cost strategy that could restore the functionality of the polluted soil in an area already heavily impacted by soil consumption (Segura et al 2009; Vergani et al. 2017; ISPRA 2017), since the plant rhizosphere is considered a favourable niche for microbial proliferation, activity and metabolism of organic pollutants (Philippot et al. 2013; Musilova et al. 2016). The main challenge of rhizoremediation is that it requires a site-specific design that considers the pollution profile together with the soil chemical and physical properties and the climate conditions. In fact, these factors are critical for plant growth and for the bioavailability of the target contaminant to microbial degradation. Moreover, plant species and developmental stage modify the pattern of root exudates and differentially affect the soil microbial communities' assembly and degradation abilities (Segura et al. 2009; Costa et al. 2008; Chaparro et al. 2014). In order to evaluate the biostimulation performance of different plant species and soil treatments for the development of a suitable rhizoremediation strategy, an experimental trial was set up in greenhouse conditions with the soil collected from the SIN Caffaro. The plant species and the different soil treatments were chosen after an extensive literature research on phytoremediation considering the main contaminants of the SIN Caffaro (Polychlorinated biphenyls [PCB], arsenic [As] and mercury [Hg]) and preferring plant species that were phylogenetically related to the spontaneous ones still growing in the former agricultural fields of the SIN Caffaro. The commercial availability of the seeds and the plants cultivability in greenhouse conditions were also considered. A complete list of the different treatments with the relative controls is reported in Table 1. *Medicago sativa, Phalaris arundinacea, Festuca arundinacea* and *Brassica juncea* were selected due to their ability to increase PCB depletion during phytoremediation trials in comparison with non-planted controls (Checkol et al. 2004; Li et al. 2013; Dzantor et al., 2000; Tu et al. 2011;). In one treatment *F. arundinacea* was associated to *Cucurbita pepo pepo,* in the attempt to improve the overall phytoextraction performance, since plants belonging to the family of *Cucurbitaceae* are known to uptake PCB from the soil (Whitfield Aslund et al. 2008).

Table 1. List of the ten planted treatments and the respective non-planted controls. C1 and C6 represent additional controls accounting for soil fertilization and pollution respectively.

Treatment	Control
P1 *Phalaris arundinacea*	**C2** non planted fertilized soil
P2 *Phalaris arundinacea* + redox cycle	**C3** non planted fertilized soil + redox cycle
P3 *Festuca arundinacea*	**C2** non planted fertilized soil
P4 *Festuca arundinacea* + *Cucurbita pepo spp pepo*	**C2** non planted fertilized soil
P5 *Festuca arundinacea +* redox *cycle*	**C3** non planted fertilized soil + redox cycle
P6 *Festuca arundinacea +* compost	**C5** non planted fertilized soil + compost
P7 *Medicago sativa* + AMF + rizobia	**C2** non planted fertilized soil
P8 S*alix caprea* + *Athyrium filix-femina*	**C7** non planted fertilized soil + soil from plantlets
P9 *Brassica juncea*	**C2** non planted fertilized soil
P10 *Brassica juncea +* thiosulphate	**C4** non planted fertilized soil + thiosulphate
C1 non planted non fertilized soil	
C6 non polluted soil planted with *Medicago sativa* + *Festuca arundinacea*	

In two separate treatments *P. arundinacea* and *F. arundinacea* were associated to repeated flooding conditions, to create a redox cycle with the aim to couple a reductive dechlorination process with aerobic PCB degradation, thereby favouring high chlorinated congeners removal (Meggo et al. 2013; Meggo and Schnoor 2013; Chen et al. 2014). Some treatments were set up to address the phytoextraction of As and Hg from the soil. In one treatment, we associated *Brassica juncea* with the addition of ammonium thiosulfate, a compound that was reported to improve Hg uptake using this plant species (Wang et al. 2014; Franchi et al. 2016). Likewise, the fern *Pteris vittata* was adopted for its ability to accumulate and detoxify As, and because its rapid growth and high biomass make it appropriate for phytoremediation (Lessl et al. 2014; Alagic et al. 2013).

3.1 Effect of time and treatment on microbial communities' structure and enzymatic activity

Even though it is known that rhizosphere effect and different soil conditions shape the microbial communities structure in soils (Philippot et al. 2013), it is still poorly understood how bacteria and fungi constituting the soil microbiome interact with different plant species and respond to combined abiotic factors, such as mineral fertilization and pollutant concentration. Besides microbial community structure, microbial enzymatic activity can change and increase in response to biostimulation, ultimately turning into enhanced biodegradation capacity of contaminants in polluted soils (Mukherjee et al. 2014). In the first part of this work, we aimed to assess the influence of the selected plant species and soil treatment on the bacterial and fungal communities' structure and their effect in stimulating the overall soil microbial activity at different times of sampling.

MATERIALS AND METHODS

Collection site description and chemical-physical characterization of the soil

The agricultural soils within the SIN Caffaro present a mixed and uneven contamination of chlorinated persistent organic pollutants, heavy metals and metalloids (see Chapter 1 for further details). The sampling site for the collection of the soil used for the experiment set up was chosen after a geostatistical analysis of the former agricultural fields described in Chapter 1. The geostatistical analysis was conducted basing on the pollutant concentration measurements performed on 120 samples collected along a vertical soil profile in different stations distributed in the site to map its contamination, aiming to select a soil displaying a contaminants concentration representing an average of the levels measured in the entire area. The collected soil was sieved, homogenized using the Japanese slab cake method (Pitard 1993) and divided in five portions that were chemically characterized before being transferred into pots for the greenhouse experiment (T0, Supplementary table 1).

Greenhouse rhizoremediation experiment set up

The homogenized soil collected inside the SIN Caffaro was transferred into polypropylene 6-liters pots to set up the rhizoremediation trial under greenhouse conditions. Each treatment was set up in triplicate and fertilized with ENNEKAPPA (Ilsagroup) and NutriLeaf® 20-20-20 (Biograd). Controls pot for each treatment were also prepared in triplicate, as well as the non-fertilized control. Redox cycle was obtained in pots by alternating seven days of flooding using

tap water with seven days of drying conditions for all the duration of the experiment. Ammonium thiosulfate (SIGMA ALDRICH) was spiked in the soil at a final concentration of 8 g/Kg.

Soil sampling for microbiological analyses

Soil samples were collected destructively from triplicate pots at different sampling times: at the beginning of the experiment (only non-planted controls, T0) and after three months (T1), six months (T2) and eighteen months (T4). The soil contained in each pot was sieved and homogenized using the Japanese slab cake method, and then triplicate samples were collected for each treatment and stored in sterile plastic bags at 4°C or -20°C until analysis.

Evaluation of the soil microbial hydrolytic activity and Automated Ribosomal Internal Spacer Analysis (ARISA) community fingerprinting

Microbial hydrolytic activity was evaluated following the FDA hydrolysis test set up by Green et al. (2006). The test was performed on T1, T2 and T4 soil samples and statistical analysis was carried out by a pairwise comparison between each treatment and the relative control, using the Student t-test. ARISA fingerprinting of the bacterial communities was carried out according to Mapelli et al. (2013a) on T0, T2 and T4 soil samples. ARISA fingerprinting of fungi (F-ARISA) was performed as described by Banning et al (2011) and analysed in the same way of bacterial ARISA (B-ARISA) with modified binning of the peaks (±1 bp from 100 to 300 bp, ±3 bp from 300 to 600 bp and ±5 bp from 600 to 900 and ±10 bp from 900 to 1200 bp). For detailed description of the FDA hydrolysis method and ARISA dataset analysis refer to Chapter 1.

RESULTS

ARISA fingerprinting of soil microbial communities

The ARISA fingerprinting was applied both on the bacterial (B-ARISA) and fungal (F-ARISA) communities. B-ARISA fingerprinting performed on all the treatments at the three sampling times (T0, T2 and T4) showed that bacterial communities clustered separately according to the time as graphically represented by the Constrained Analysis of Principal Coordinates (Figure 1A). Statistical analysis proved that soil samples host a significantly different bacterial community according to the time (PERMANOVA: $F(2,119)=6.494$; $p=0.0001$) and treatment (PERMANOVA: $F(16,119)=4.158$; $p=0.0001$).

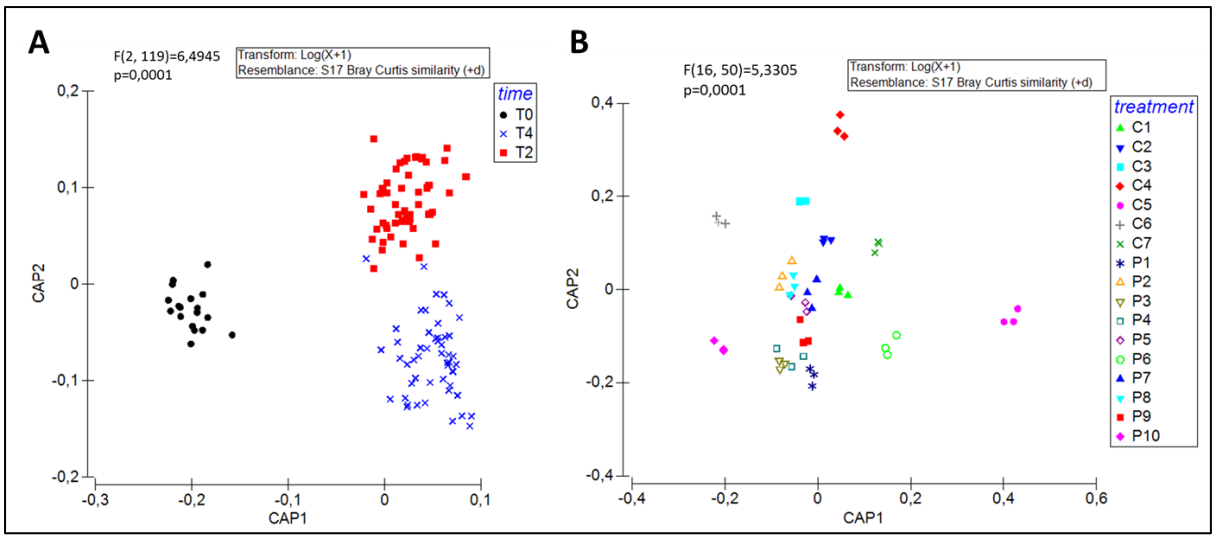

Figure 1. Constrained analysis of principal coordinates (CAP) of bacterial communities ARISA fingerprinting .**A.** Distribution of all the samples collected at T0, T2 (six months) and T4 (eighteen months) according to the factor "time". **B.** Distribution of samples collected at T4 according to the factor "treatment".

Pairwise comparisons of bacterial communities after six months of biostimulation (T2, Table 2A) showed that the majority of the planted treatments already harboured a different bacterial community compared to their non-planted controls, with the exception of treatments P2 (*Phalaris arundinacea* with redox cycle), P3 (*Festuca arundinacea*), P5 (*Festuca arundinacea* with redox cycle) and P10 (*Brassica juncea* with ammonium thiosulphate). At the end of the experiment (T4) bacterial communities of different soil treatments grouped separately according to CAP analysis (Figure 1B), as confirmed by statistics (PERMANOVA: $F(2,50)=5.330$; $p=0.0001$). Pairwise PERMANOVA analysis (Table 2B) revealed that only the planted treatments P2 (*Phalaris arundinacea* with redox cycle) and P8 (*Salix caprea* and *Athyrium filix-femina*) did not significantly affect the bacterial community structure compared to their controls, while all the other treatments had a significant effect. Pairwise test of non-planted controls C1 (non-fertilized soil) and C2 (fertilized soil) revealed that mineral fertilization had a significant role in altering the bacterial communities at the end of the experiment, after eighteen months of biostimulation (Table 2B) while this effect was not yet detectable at T2 (Table 2A).

Table 2. Pairwise comparisons (PERMANOVA) of the bacteria ARISA fingerprinting results of the planted treatments with the respective control at sampling time T2 and T4. P values of statistically different comparisons (P<0.05) are represented in bold.

A. ARISA Groups (bacteria - T2)		**t**	**P (MC)**
C1 non planted non fertilized soil	**C2** non planted fertilized soil	1,1403	0,297
C2 non planted fertilized soil	**C3** non planted fertilized soil + redox cycle	1,7952	0,0619
C2 non planted fertilized soil	**C4** non planted fertilized soil + thiosulphate	1,972	**0,0305**
C2 non planted fertilized soil	**C5** non planted fertilized soil + compost	3,4508	**0,0051**
C2 non planted fertilized soil	**C7** non planted fertilized soil + soil from plantlets	2,8672	**0,0079**
P1 *Phalaris arundinacea*	**C2** non planted fertilized soil	1,9075	**0,0359**
P2 *Phalaris arundinacea* + redox cycle	**C3** non planted fertilized soil + redox cycle	0,9635	0,4509
P3 *Festuca arundinacea*	**C2** non planted fertilized soil	1,5879	0,1083
P4 *Festuca arundinacea* + *Cucurbita pepo spp pepo*	**C2** non planted fertilized soil	2,1986	**0,0217**
P5 *Festuca arundinacea +* redox *cycle*	**C3** non planted fertilized soil + redox cycle	1,1484	0,3053
P6 *Festuca arundinacea +* compost	**C5** non planted fertilized soil + compost	2,0267	**0,0318**
P7 *Medicago sativa* + AMF + rizobia	**C2** non planted fertilized soil	1,8522	**0,0416**
P8 S*alix caprea* + *Athyrium filix-femina*	**C7** non planted fertilized soil + soil from plantlets	2,0577	**0,0279**
P9 *Brassica juncea*	**C2** non planted fertilized soil	2,4658	**0,0116**
P10 *Brassica juncea +* thiosulphate	**C4** non planted fertilized soil + thiosulphate	1,4734	0,1125
B. ARISA Groups (bacteria - T4)		**t**	**P (MC)**
C1 non planted non fertilized soil	**C2** non planted fertilized soil	1,9175	**0,0343**
C2 non planted fertilized soil	**C3** non planted fertilized soil + redox cycle	2,1292	**0,0254**
C2 non planted fertilized soil	**C4** non planted fertilized soil + thiosulphate	2,4925	**0,0129**
C2 non planted fertilized soil	**C5** non planted fertilized soil + compost	3,0684	**0,0065**
C2 non planted fertilized soil	**C7** non planted fertilized soil + soil from plantlets	2,066	**0,0282**
P1 *Phalaris arundinacea*	**C2** non planted fertilized soil	2,0579	**0,0271**
P2 *Phalaris arundinacea* + redox cycle	**C3** non planted fertilized soil + redox cycle	1,7754	0,0509
P3 *Festuca arundinacea*	**C2** non planted fertilized soil	2,3118	**0,0177**
P4 *Festuca arundinacea* + *Cucurbita pepo spp pepo*	**C2** non planted fertilized soil	2,6525	**0,0096**
P5 *Festuca arundinacea +* redox *cycle*	**C3** non planted fertilized soil + redox cycle	2,1923	**0,0197**
P6 *Festuca arundinacea +* compost	**C5** non planted fertilized soil + compost	2,119	**0,0246**
P7 *Medicago sativa* + AMF + rizobia	**C2** non planted fertilized soil	2,5476	**0,0124**
P8 S*alix caprea* + *Athyrium filix-femina*	**C7** non planted fertilized soil + soil from plantlets	1,3727	0,1529
P9 *Brassica juncea*	**C2** non planted fertilized soil	2,2005	**0,0207**
P10 *Brassica juncea +* thiosulphate	**C4** non planted fertilized soil + thiosulphate	2,2355	**0,017**

The comparison of F-ARISA fingerprints revealed that fungal communities of the treatment can be clustered according to the experimental times, as shown by the Principal Coordinate Analysis (PCoA) reported in Figure 2 and demonstrated statistically (PERMANOVA: F(2,68)=8.0673; p=0.0001).

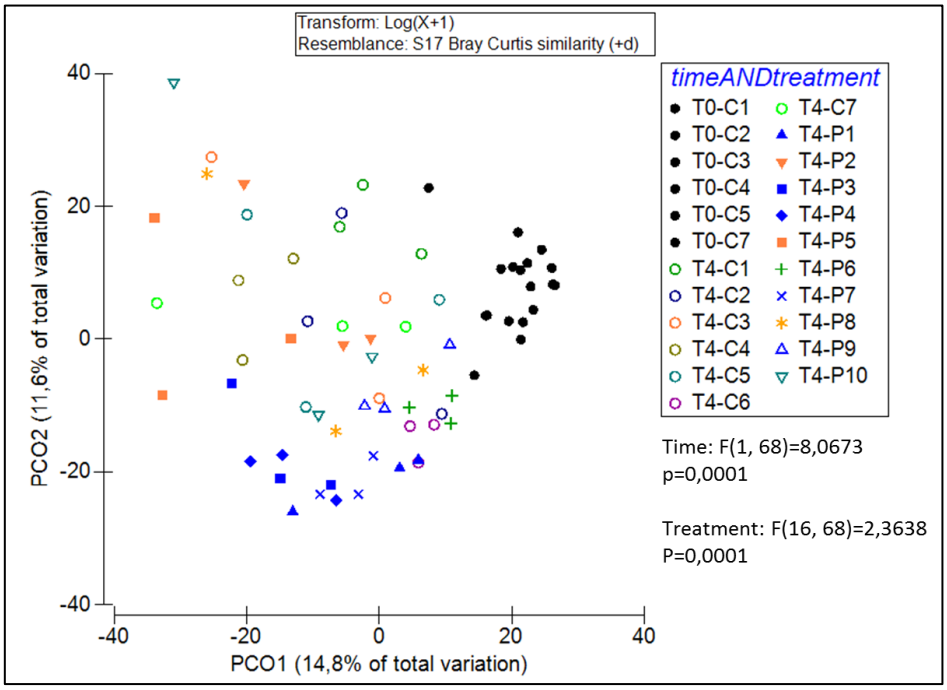

Figure 2. Principal coordinate analysis (PCoA) of fungal communities ARISA fingerprinting at sampling time T0 and T4. The distribution of all the samples is shown according to both factors "time" and "treatment".

The fungal communities were also observed at the end of the experiment (T4). In this case, no significant differences were detected when applying a pairwise test to compare each planted treatment with its control (Table 3) and when comparing different soil conditions within the non-planted controls. No significant change was detected either between fertilized and non-fertilized non-planted soils (Table 3). Differently from the case of bacterial communities, a significant difference between the fungal community of the non-polluted control and the other control and treatments was observed only in six cases (Supplementary table 3).

Table 3. Pairwise comparisons (PERMANOVA) of the fungi ARISA fingerprinting results of the planted treatments with the respective control at sampling time T4. No statistically different comparisons (P<0.05) are present.

ARISA Groups (fungi - T4)		t	P (MC)
C1 non planted non fertilized soil	**C2** non planted fertilized soil	1,0065	0,4279
C2 non planted fertilized soil	**C3** non planted fertilized soil + redox cycle	1,5592	0,095
C2 non planted fertilized soil	**C4** non planted fertilized soil + thiosulphate	1,3495	0,1661
C2 non planted fertilized soil	**C5** non planted fertilized soil + compost	1,1392	0,3124
C2 non planted fertilized soil	**C7** non planted fertilized soil + soil from plantlets	1,0494	0,381
P1 *Phalaris arundinacea*	**C2** non planted fertilized soil	1,4445	0,136
P2 *Phalaris arundinacea* + redox cycle	**C3** non planted fertilized soil + redox cycle	1,5971	0,0965
P3 *Festuca arundinacea*	**C2** non planted fertilized soil	1,5299	0,1053
P4 *Festuca arundinacea* + *Cucurbita pepo spp pepo*	**C2** non planted fertilized soil	1,5189	0,0994
P5 *Festuca arundinacea +* redox *cycle*	**C3** non planted fertilized soil + redox cycle	1,2722	0,204
P6 *Festuca arundinacea +* compost	**C5** non planted fertilized soil + compost	1,6321	0,0851
P7 *Medicago sativa* + AMF + rizobia	**C2** non planted fertilized soil	1,4995	0,125
P8 *Salix caprea* + *Athyrium filix-femina*	**C7** non planted fertilized soil + soil from plantlets	1,3669	0,1665
P9 *Brassica juncea*	**C2** non planted fertilized soil	1,6921	0,0687
P10 *Brassica juncea* + thiosulphate	**C4** non planted fertilized soil + thiosulphate	1,4274	0,1431

Soil hydrolytic activity

FDA test performed on soils sampled after three months of greenhouse experiment (T1) revealed that *Phalaris arundinacea* exposed to a redox cycle (treatment P2) significantly increased the soil hydrolytic activity compared to the relative non-planted control (Student T test: p=0.019, Figure 3A). After six months of experiment (T2) two different soil treatment recorded significantly increased activity compared to their control, *Festuca arundinacea* with redox cycle and *Brassica juncea* (Figure 3B). FDA test was then repeated at the end of the rhizoremediation trial after eighteen months of treatment (T4) and results showed that all the planted treatments increased the soil hydrolytic activity compared to the non-planted controls (Figure 3C). At sampling time T1, the fertilized, non-planted control (C2) reported enahnced activity (Student T test: p=0.026) compared to the non-fertilized, non-planted control (C1), while no significant differences were recorded between these two treatments at times T2 and T4.

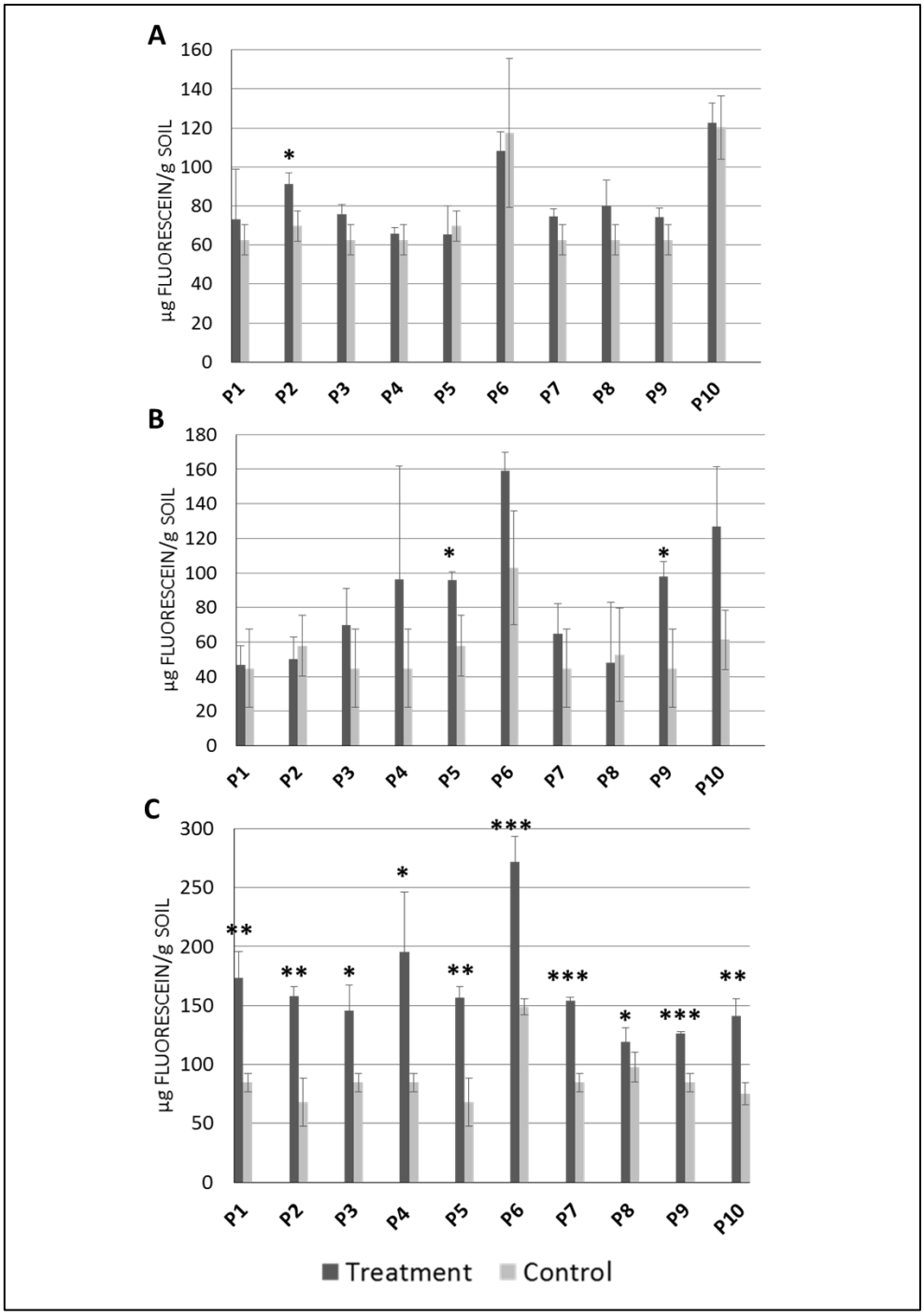

Figure 3. Results of the fluorescein diacetate (FDA) hydrolytic activity test. The histograms report the quantity of fluorescein (µg) produced after the incubation of 1 g of soil from each planted treatment and the relative control at different sampling times: **A.** Three months (T1). **B.** Six months (T2). **C.** Fifteen months (T4).

DISCUSSION

Different plant species exert the so-called "rhizosphere effect" selecting specific communities both of bacteria and fungi (Costa et al. 2008; Dassen et al. 2017). Studies on contaminated soils reported that fertilization treatment have a role in altering the soil bacterial and fungal communities, even though the effect may change depending on plant species and soil pollution profile (Leewis et al. 2016; Pérez de Mora et al. 2006; Ridl et al. 2016). In this study, comparing the ability of different soil treatments to shape the microbiota structure, we observed changes in the microbial communities over time during the rhizoremediation pot experiment for both bacteria and fungi, whereas the bacterial community was also significantly influenced according to the applied treatment. Bacteria and fungi react differentially to rhizostimulation and their response seems to be strongly related to the soil contamination levels and the plant compartment (Bell et al. 2014; Tardif et al. 2016). Our results showed that while the presence of plants and the different soil conditions (i.e. redox cycle, mineral fertilization, addition of compost or ammonium thiosulphate) shaped the bacterial communities at the end of the rhizoremediation experiment, after eighteen months of biostimulation, they did not exert effect on the fungal ones. Mineral fertilization seemed to have an effect in non-planted soils only on bacteria and after prolonged treatment (eighteen months). In addition, when comparing the microbial communities' structure of a planted, non-polluted soil (C6) with those of polluted soils, our results confirmed that pollution was a driver of the whole soil microbiome structure (see chapter 1) and it seemed to be the only factor influencing the fungal communities.

Evaluating the biostimulation effect on soil enzymatic activity through FDA hydrolysis test, we observed that after three months (T1) *Phalaris arundinacea* subjected to a redox cycle was the only treatment showing a statistically significant positive effect compared to its control. After six months (T2), the same results was obtained by *Festuca arundinacea* subjected to a redox cycle, suggesting that the application of alternate flooding conditions, independently from the used plant species, were more rapid in stimulating the soil microbial activity compared to the other treatments. These results are particularly interesting for PCB-polluted soils rhizoremediation, implying that aerobic microorganisms can more easily degrade low-chlorinated congeners that are produced by the dechlorination process during the anoxic periods (Meggo and Schnoor, 2013), leading to enhanced PCB depletion in the soil. The only other plant species promoting the soil enzymatic activity after six months (T2) was *Brassica*

juncea, in agreement with previous reports that indicate plants belonging to the genus *Brassica* as able to enhance PCB degradation in the rhizosphere soil (Singer et al. 2003; Javorska et al. 2009). All the planted treatments were successful in increasing the overall soil hydrolytic activity after eighteen months of biostimulation, indicating that the establishment of a "rhizosphere effect" was independent on the plant species but was effective only with full plant development. Notably, the effect of the mineral fertilization of the soils had a role in increasing the soil hydrolytic activity only in the short time, at the beginning of the greenhouse experiment, as resulted from the comparison of the unplanted non-fertilized (C1) and fertilized (C2) controls at all the sampling times. For this reason, biostimulation at the end of the experiment could be attributed only to the plant rhizodeposition effect.

CONCLUSION

Overall, the study of microbial community structure highlighted that planted species played a role in shaping the bacterial but not the fungal communities' structure of the polluted soil. This suggests the need of further research for the development of rhizoremediation strategies for the SIN Caffaro, especially to better understand the drivers of diversity on the fungal fraction of the potentially degrading microbial community. In addition, we proved the positive role of long-term rhizostimulation rather than mineral fertilization in enhancing the microbial enzymatic activity in the soil, which may increase contaminants degradation rates during rhizoremediation. Given the currently available data on the soil microbial communities' structure and activity, it is possible to state that all the tested treatments revealed a rhizoremediation potential. Further analyses, including chemical analyses aimed to measure the pollutants' concentration in the soils, are required to identify the most promising treatments and assess their ability to enhance soil detoxification.

3.2 Isolation and characterization of PCB-degrading bacteria with PGP activity for application in assisted rhizoremediation

The efficiency of the rhizoremediation process may be increased by bioaugmentation with bacterial strains combining biodegradation potential with plant growth promoting (PGP) traits, that help the plant to counteract the toxic effects of the polluted soil (Abilash et al. 2016; Thijs et al. 2014). Autochthonous bioaugmentation is of major interest because it relies on strains already adapted to the site ecological conditions and prevents the introduction of potentially dangerous species (El Fantroussi and Agathos 2005). For this reason, the soil planted with *Phalaris arundinacea* and exposed to a redox cycle (P2), which showed the highest stimulation effect on soil hydrolityc activity in the short time (T1), was selected for the isolation of bacterial strains able to grow on biphenyl and plant secondary metabolites (PSMs) as unique carbon source. These compounds are known to foster PCB degradation through the activation of the *bph* patway (Vergani et al. 2017a). The bacterial isolates were identified and characterized *in vitro* to evaluate their potential for PCB biodegradation and plant growth promotion, aiming to select strains that could be utilized for autochthonous bioaugmentation in assisted PCB -rhizoremediation strategy at the SIN Caffaro.

MATERIALS AND METHODS

Bacteria Isolation

Isolation of bacteria was performed from the three replicates of treatment P2 at sampling time T1. One gram of soil was suspended in 9 ml of physiological solution (0.9% NaCl), diluted in ten-fold series and plated onto Petri dishes containing agar mineral medium (Uhlik et al. 2011) supplemented with biphenyl, limonene or naringin (SIGMA Aldrich) as unique carbon source. Biphenyl crystals were placed on the dishes lid, limonene was let evaporate from a vial inside a jar where Petri dishes were incubated and naringin was dissolved in ethanol and added to the autoclaved mineral medium to a final concentration of 2 g/L (Donnelly et al. 1994). After one week of incubation at 30°C, 30 colonies for each replicate and each medium (n=270 colonies) were randomly picked and spread three times on mineral medium supplemented with biphenyl. Isolates were labelled with codes including numbers 1, 2, 3 and letters B, L, and N according to the sample replicate and the isolation medium (B=biphenyl, L=limonene, N=naringin) followed by progressive numbers.

Bacteria identification and *bphA* gene amplification

The DNA of each isolate was extracted through CTAB – phenol chloroform DNA extraction (Chouaia et al. 2010) and the collection was de-replicated using the ribosomal internal transcribed spacers (ITS)-PCR protocol (Mapelli et al., 2013b). One representative strain from each ITS profile group was identified through 16S rRNA gene amplification and partial sequencing (Macrogen, Rep. of South Korea) as described by Mapelli et al. (2013b) and the sub-collection was then stored at -80°C in R2A liquid medium with 25% glycerol for further analysis. 16S rRNA nucleotide sequences were subjected to BLAST search (using the blastn suite) for taxonomic identification. The presence of the genes encoding for biphenyl dioxygenase α subunit (*bphA*) was assessed through PCR with primers 512F and 674R according to Leewis et al. (2016) and further confirmed as described by Iwai et al. (2010) using BPHD-F3/R1 primer set. PCR reactions for the amplification of *bphA* gene were performed utilizing FastStart™ High Fidelity PCR System (Roche) and the DNA of the PCB-degrading bacteria *Paraburkholderia xenovorans* LB400 (DSMZ, Germany) was used as positive control. PCR results were visualized on 1.2% agarose gel and PCR products that showed a single band of the predicted size were sequenced at Eurofins Genomics Srl (Italy). Nucleotide sequences were then analysed using the blastn suite to assess nucleotide percentage identity with known dioxygenases deposited at the NCBI database.

Screening of bacterial strains activities for assisted rhizoremediation

Catechol 2,3-dioxygenase activity was tested according to Margesin et. al (2003): bacteria were grown in tryptic soy broth (TSB) medium for 48 hours at 30°C in mixing conditions, then 50 µl of the bacterial suspension were added to 150 µl of a catechol solution (catechol 90 mM, Tris-acetate buffer pH 7.5 50 mM) and incubated in the dark for two hours at room temperature. The result was considered positive if the samples developed a green-brownish colour compared to the negative control.

Biosurfactant production was evaluated with an emulsification test. Bacterial strains were grown in TSB medium for 48 hours in agitation, and then mineral oil was added tothe culture at a 1:1 ratio. The two phases were mixed by vortexing thoroughly for two minutes. After 30 minutes the emulsification between the two phases was evaluated and measured comparing it to a positive control containing 10% SDS instead of the bacterial culture. The stability of the emulsification was evaluated by incubating the samples for 48 hours at room temperature and comparing the height of the emulsification after 30 minutes and after 48 hours. 1-

aminocyclopropane-1-carboxylate (ACC) deaminase activity was tested following the protocol described by Belimov et al. (2005).

***In vivo* PGP test on *Arabidospis thaliana* seedlings**

Plant growth promotion on *Arabidopsis thaliana* was assessed as follows: *A*. *thaliana* seeds were surface-sterilized by soaking them in 70 % ethanol for 2 minutes and in 1 % sodium hypochlorite for 20 minutes. After five washing steps with sterile distilled water the seeds were disposed on plates containing MS medium supplemented with sucrose (Murashige and Skoog basal salt mixture 2.15 g/L, sucrose 15 g/L, agar 8 g/L) and vernalized at 4°C in the dark for 72 hours. The plates were then transferred for seeds germination into a growth chamber at 22°C, 50% humidity and 16 hours of light per day. After three days, *Arabidopsis* seedlings were moved on the same medium into new plates prepared by severing 1/3 of the solid medium. Seven plantlets for each plate were disposed vertically on the cut's edge. The bacterial strains to be tested for plant growth promotion activity were inoculated separately with a toothpick at the opposite side of the seedlings, in parallel to the cut (see Figure 5A). Five replicate plates for each strain and for a non-inoculated control were set up, wrapped with Parafilm and placed vertically in the growth chamber for two weeks. Plant fresh weight was then measured for each plate and a Student T-test statistical analysis was applied to perform a pairwise comparison between the plants inoculated with each bacterial strain and the negative control.

Resting cell assay for PCB degradation assessment

Bacterial strains were grown at 28°C on a rotatory shaker in mineral medium supplemented with sodium pyruvate 30 mM and biphenyl crystals to induce the *bph* genes expression. A 1% inoculum was then transferred into flasks containing 250 ml of the same medium and incubated at the same conditions. The culture was filtered in sterile conditions using a funnel filled with glass wool to remove biphenyl crystals, washed two times with sterile physiological solution (0.9% NaCl) and suspended in 200 ml of mineral medium in order to obtain an optical density=1 at 600 nm. 100 ml of the suspension were collected and autoclaved to be used for the setup of negative controls. Four replicates for each strain and the relative negative control were set up as follows: 20 ml of the bacterial suspension were transferred into 100 ml serum bottles and spiked with 20 µl of 1% PCB mixture (Delor 103). An additional negative control was set up with *Pseudomonas sp.* JAB1 strain with disrupted *bph* operon (provided by Prof. Ondrej Uhlik, unpublished data). The bottles were sealed and incubated for 48 hours on a

rotatory shaker at 28°C, and then the reaction was stopped by freezing the bottles at -20°C. The content of individual PCB congeners present in the microcosms were determined using gas chromatography-mass spectrometry (GC-MS; 450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA). PCBs were analysed in ethyl acetate extracts according to the method described by Čvančarová and co-authors (2012). Total PCB content was expressed for each microcosm as a sum of the individual congeners measured (Supplementary table 6) and PCB degradation ability was assessed by a statistical comparison (Student T-test) between each strain and its inactivated control or the JAB1 control.

RESULTS

Bacteria isolation and identification

Starting from 270 pure isolates obtained from the soil associated to *Phalaris arundinacea* and subjected to a redox cycle, we obtained a collection of 128 bacterial strains able to grow on mineral medium supplemented with biphenyl as unique carbon source. 66% of these strains were isolated on biphenyl, 29% on limonene and 5% on naringin. Basing on the ITS clustering of the 128 bacteria, ITS-PCR revealed 56 distinct ITS profiles. The great majority of the collection belonged to the phylum *Actinobacteria*, with only one strain affiliated to *Proteobacteria.* At the genus level, twenty-eight strains (50% of the whole collection) were identified as *Arthrobacter sp.* and seven as *Pseudoarthrobacter sp.* (13%). *Gordonia sp., Rhodococcus sp.,* and *Streptomyces sp.* represented together the 33% of the collection with six strains each. Two isolates belonged to the genus *Micromonospora* and the only *Proteobacteria* isolated belonged to the genus *Pseudomonas* (Table 4).

Table 4. Taxonomic affiliation at the genus level of the bacterial strains isolated from the soil of treatment P2 (*Phalaris arundinacea* with redox cycle) at sampling time T1. The number of isolates and the relative percentage in the collection is reported for each bacterial genus.

Genus	N° of isolates	%
Arthrobacter	28	50%
Pseudarthrobacter	7	13%
Gordonia	6	11%
Micromonospora	2	4%
Rhodococcus	6	11%
Streptomyces	6	11%
Pseudomonas	1	2%

Characterization of the isolates for enhanced rhyzoremediation potential

The screening for activities significant for rhizoremediation has been performed on 56 isolates representative of each ITS profile and the results are summarized in Figure 4, while Supplementary table 4 indicates the results detailed for each bacterial strain. *BphA* gene was detected in 35 out of 56 isolates using primers 512F/674R and all the obtained partial gene sequences showed high nucleotide sequence identity (99%) with *Rhodococcus* sp. partial *bphA* gene sequences (Supplementary table 5A). Strains 2B7, 2B8 (identified as *Arthrobacter* sp.), 2B23, 2B27, 3B12 (*Rhodococcus* spp.) 2N21, 2N22 and 2N24 (*Streptomyces* spp.) also displayed PCR products of the predicted size (542 base pairs) with primers F3/R1. In this case, partial gene sequences of *Rhodococcus* strains were identified as *bphA* genes (100% of identity), while sequences of *Arthrobacter* 2B7 and 2B8 strains and of *Streptomyces* 2N21 strains showed higher identity to the α-subunit of a generic rieske non-heme iron oxygenase and to 3-phenylpropionate dioxygenase respectively (Supplementary table 5B). The majority of the isolates (49 out of 56, 88%) was able to produce biosurfactants/bioemulsifiers, producing an emulsification with mineral oil that remained stable after 48 h for the 80% of the isolates. Catechol dioxygenase activity through meta-cleavage and ACC deaminase activity were detected in the 32% and 39% of the isolates respectively.

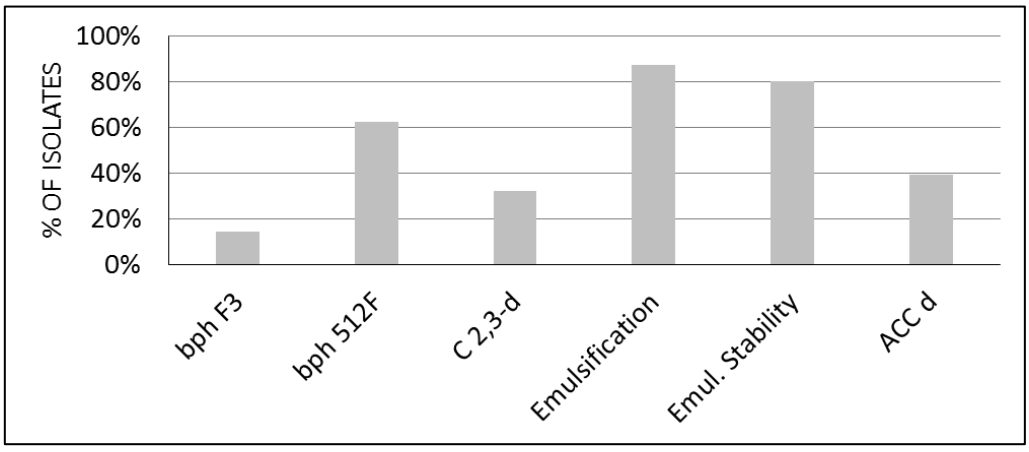

Figure 4: Results of the screening of the bacterial collection for rhizoremediation potential. The histograms represent the percentage of isolates displaying positive results in each test. BphA F3 and 512F=PCR amplification of the *bphA* gene with primers F3/R1 and 512F/674R; C 2,3 D = 2,3-catechol dioxygenase activity; Emul.=emulsification activity; Emul. St.=emulsification stability; ACCd=ACC-deaminase activity.

***In vivo* PGP activity and PCB degradation ability of selected bacterial strains**

Strains 2B7, 2B8, 2B23, 2B27 and 3B12 were selected for a PGP test on the model plant *Arabidopsis thaliana.* All the tested strains had an effect on the plant root system by promoting lateral root development, as exemplified in Figure 5A. This resulted in a significant increase of plant fresh weight compared to the non-inoculated control for strains 2B23, 2B27 and 3B12, all belonging to the genus *Rhodococcus,* while no significant differences were observed for *Arthrobacter* strains 2B7 and 2B8(Figure 5B).

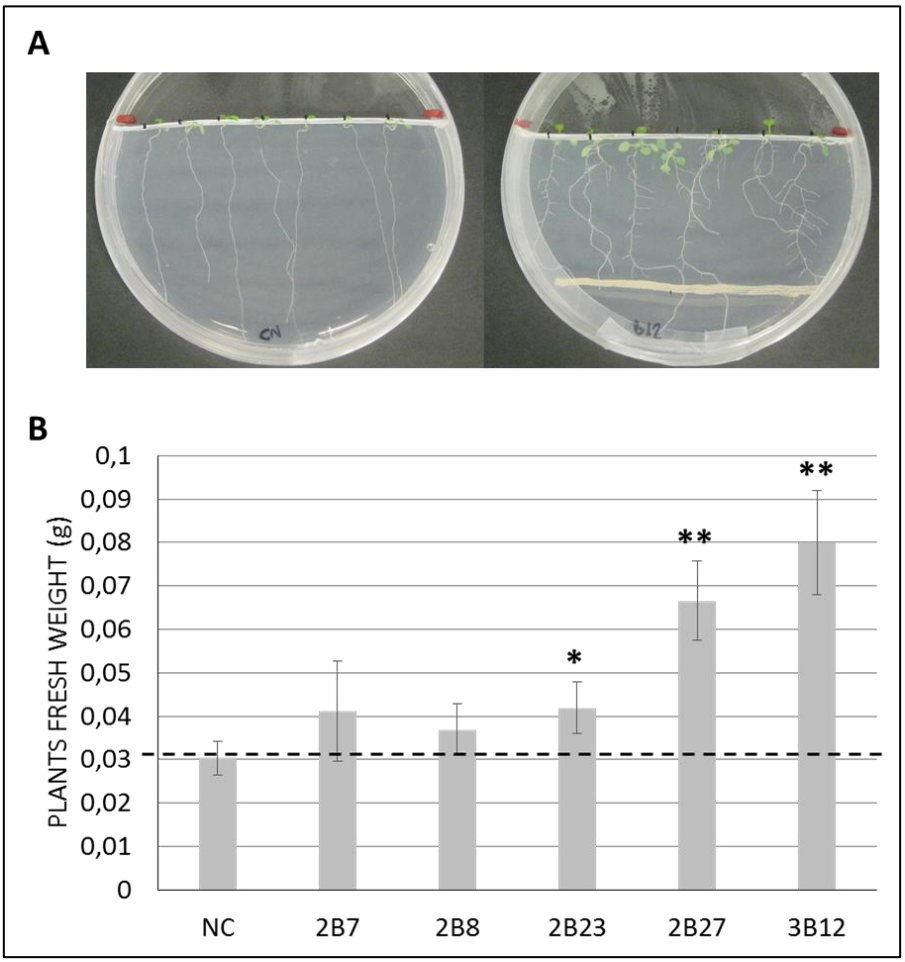

Figure 5: Results of plant growth promoting assay on *Arabidopsis thaliana.* **(A)** The pictures exemplify the promotion of lateral root development by *Rhodococcus jostii* strain B12 compared to the non-inoculated negative control (NC). **(B)** Screened strains and plant fresh weight measurement results are reported on the X-and Y-axes, respectively. Student T-test was adopted to statistically analyse the data comparing the inoculated strains with the negative control. The stars indicate statistically significant differences (*=$p<0.05$, **=$0.001<p<0.05$). NC=negative control.

Strains 2B7, 3B12, 2B23 and 2B27 were also subjected to a resting cell assay to assess their ability to degrade PCB using the commercial mixture Delor 103. A complete list of all the PCB

congeners measured after 48 h of incubation is reported in Supplementary table 6. Bottles inoculated with *Rhodococcus* strains 3B12, 2B23 and 2B27, showed a significant decrease in the total PCB content after 48 h of incubation compared to the ones with autoclaved controls (Table 5), and strain 3B12 also displayed a decrease compared to the control strain *Pseudomonas* sp. JAB1 (Student T-test, p=0.02654).

Table 5. Average total PCB content calculated as a sum of the different congeners measured in each replicate serum bottle. P values of the statistical analysis comparing each strain with its inactivated control are reported and significant values (p<0.05) are highlighted in bold. Tot.=total, X=inactivated control.

Sample	**2B7**	**2B7X**	**3B12**	**3B12X**	**2B23**	**2B23X**	**2B27**	**2B27X**
Average tot. PCB content (µg)	154,05	164,10	119,03	170,16	139,66	171,72	128,01	157,30
Standard deviation	10,73	2,30	5,84	12,89	5,97	8,60	14,16	13,79
p (Student T test)	0,18515		**0,00077**		**0,00544**		**0,04247**	

Rhodococcus strains 3B12 and 2B23 showed a significant decrease of all the five most abundant PCB congeners measured, mainly dichloro- (PCB 5+8) and trichlorobiphenyls (PCB 17, PCB 18, PCB 28+31, PCB 33), while strain 2B27 was related only to the depletion of PCB 28+31 and PCB 5+8. No total PCB depletion was instead observed in bottles inoculated with *Arthrobacter* sp. strain B7 even though a significant decrease in the content of PCB 33+53 was measured (Figure 6).

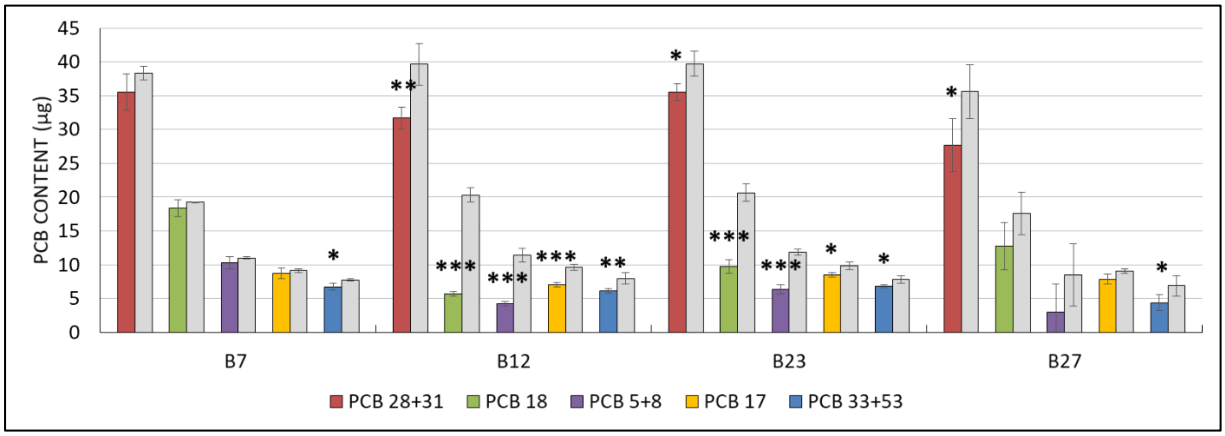

Figure 6: Results of the resting cell assay for the evaluation of the PCB degradation ability of bacterial strains. The average content of the most abundant PCB congeners measured in the flasks is reported for each strain tested. The stars indicate statistically significant depletion when comparing the bacterial strains with their inactivated control (values represented in grey) according to Student T-test (*=p<0.05, **=0.001<p<0.05, ***=p<0.001).

DISCUSSION

In contrast with a previous bacterial collection obtained from rhizosphere soil sampled within the same area of the SIN Caffaro (see chapter 2; Vergani et al. 2017b), here we could isolate exclusively strains affiliated to the phylum *Actinobacteria*. This different result is possibly due to the combination of the restrictive isolation conditions and especially to the different origin of soil that was sampled in association to a different plant species, *Phalaris arundinacea*, subjected to repeated flooding. Bacteria belonging to the *Actinobacteria* class are known for their metabolic versatility and the potential for bioremediation of persistent organic pollutants, including PCB (Arenskotter et al. 2004; Fu et al. 2014; Vergani et al. 2017a), as shown by the results of our *in vitro* screening. Most of the strains harboured the *bphA* gene that is essential to begin the aerobic pathway responsible for PCB degradation, and some were also able to cleave catechol, a toxic metabolite produced during the degradation pathways of aromatic compounds. The majority of the isolates produced a stable emulsification with mineral oil, so they are potentially able to increase hydrophobic contaminant bioavailability for degradation (Kugler et al. 2015). This is a common trait among the class *Actinobacteria*, including the genus *Arthrobacter, Gordonia* and *Rhodococcus*, known to produce molecules able to disperse organic pollutants contaminations (Kugler et al. 2015). This is pivotal also for the remediation of PCB polluted soils, since one of the main limits to the biodegradation of these molecules is that they are highly hydrophobic and tightly bound to the soil organic matter (Passatore et al. 2014). As observed for 16S rRNA-based identification of the isolates, also the affiliation of *bphA* partial gene sequences resulted different from our previous findings (Vergani et al. 2017b), with all of the sequences clustering as *Rhodococcus* type. However, the amplification of a longer *bphA* gene fragment with primers F3/R1 revealed a broader sequence diversity, as we obtained three different types of Rieske non-heme dioxygenase. This finding implies the necessity of further investigation to clarify the functionality of these genes, given the great diversity of enzymes included in this family, not always related to PCB degradation (Iwai et al. 2010). ACC deaminase activity was detected in all the isolates affiliated to the genus *Streptomyces* and in twelve *Arthrobacter* and five *Pseudoarthrobacter* strains. These bacteria may represent a useful resource to sustain the growth of plants selected for rhizoremediation, since ACC deaminase activity is a well known direct mechanisms of PGP bacteria to help plants in counteracting the environmental stress (e.g. soil phytotoxicity) mediated by the level of the phytohormone

ethylene. Three *Rhodococcus* sp. strains also showed to promote *Arabidopsis thaliana* growth by inducing lateral root development, a relevant ability for rhizoremediation as a wider extension of the plant root system is a highly desirable trait that would result in an increase of the soil volume subjected to the influence of rhizodeposition. Interestingly, the same *Rhodococcus* strains also displayed a significant effect on PCB depletion, according to the resting cell assay, a result that finds confirmation in the previous literature (Masai et al. 1995; Toussaint et al. 2012). Overall these findings reveal the potential of *Rhodococcus* strains to sustain plant growth and PCB biodegradation. The effectiveness of these strains in terms of root development promotion and complete PCB mineralization should be however further assessed to evaluate their actual possibilities of application in the field.

CONCLUSION

We established from the soil planted with *Phalaris arundinacea* and treated with a redox cycle a collection of *Actinobacteria* displaying different activities that suggest their potential to enhance PCB rhizoremediation. In particular, three strains belonging to the genus *Rhodococcus* showed both the abilities to promote plant root development in the model plant *A. thaliana* and to degrade PCB, making them ideal candidate to sustain PCB rhizoremediation through a site-tailored bioaugmentation approach. Further investigation should be directed to test these strains *in vivo* on plants of interest for rhizoremediation and to better characterize their PCB degradation activity.

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General conclusions and future perspectives

Rhizoremediation, which is based on the beneficial interactions between plants and microbial populations inhabiting the rhizosphere, is recognized as a sustainable tool for the detoxification of PCB polluted soils. The efficiency of this biotechnology *in situ* has been, nevertheless, poorly assessed, since the upscaling from laboratory to greenhouse conditions to the field was rarely implemented. A major challenge for the setup of an efficient *in situ* rhizoremediation strategy is to understand how site-specific environmental conditions, especially the pollution fingerprint, affect the autochthonous degrading bacterial populations and whether these are able to establish positive interactions with the introduced plant species, in order to enhance the naturally occurring biodegradation process. In this PhD project I focused on the heavily polluted Site of National Priority (SIN) Caffaro, which can be considered a case study for the rhizoremediation of extended areas presenting strong and uneven PCB contamination. In the first part of the work the soil microbiome of three former agricultural fields within the SIN was considered, revealing that the bacterial communities' structure, their phylogenetic diversity and the soil microbial activity were related with the soil physical and chemical parameters, both along a soil depth profile and across the surface of the area of collection. These findings suggest the adaptation of the microbial communities to the high xenobiotics concentrations in the soil, possibly resulting in PCB biodegradation abilities that should be further examined by studying the degrading genes (*e.g.* the *bph* operon) diversity and functionality in the soil metagenome. The natural attenuation potential of the bacterial microbiome was also unraveled in a second study focused on three spontaneous plant species which were naturally selected in the most polluted field (Vergani et al. 2017b). A molecular ecology approach pointed out that the phylogenetic composition of the bacterial communities was conserved along a soil gradient from the non-vegetated to the root-associated soils, while the distribution of the bacterial taxa in the root-associated soil fractions was related to the rhizosphere effect driven by the three plant species. A wide collection of bacterial isolates able to metabolize biphenyl was established from the rhizosphere soil of the three plants, and *in vitro* screening showed that biodegradation and plant growth promoting (PGP) potential were widespread in the rhizosphere cultivable microbiome. Two *Arthrobacter* sp. strains, moreover, were demonstrated to display PGP activity *in vivo* on model tomato plants, suggesting their possible application as bioenhancers on plant species of interest for rhizoremediation.

During the second part of this PhD project a rhizoremediation microcosm-scale experiment was set up on the SIN Caffaro soil in greenhouse conditions. The biostimulation effect, conveyed by different plant species and soil treatments, was evaluated during a 24 months assay. Plant species and

treatments were identified basing upon an extensive literature screening, aimed to select the species/treatments that in previous studies showed to be effective in PCB rhizoremediation (Vergani et al. 2017a, Terzaghi et al. 2018). The results of bacterial molecular fingerprinting and biochemical analysis of soil surrounding the plant roots revealed that the plants, when compared with unplanted control microcosms, had an effect on the soil microbiota in i) driving a change in the community structure and ii) stimulating the activity of organic matter (OM) hydrolysis. The latter finding is especially encouraging for the success of the rhizoremediation trial, since the stimulation of the soil microbiota leading to an increase in the OM hydrolytic activity may contribute to enhance PCB bioavailability and in turn their degradation in the polluted soil (Terzaghi et al. 2018). Notably, the effect on the phylogenetic diversity was significant in particular for the bacterial community rather than the fungal one, indicating that the bacterial fraction of the soil microbiota is the most prone to biostimulation and, according to the literature data, harbors the most described degrading genes.

Furthermore, Stable Isotope Probing (SIP) experiments set up with soil biostimulated for 24 months spiked with ^{13}C-labelled 4-chlorobyphenyl proved that this PCB congener was mineralized by the soil microbial community (unpublished data). The degrading populations will be possibly identified with further analysis of the ^{13}C-containing soil metagenome (Uhlik et al. 2013). Chemical analysis of soil and plant tissues are ongoing to assess whether and to which extent PCB depletion and extraction occurred in the differentially treated soils. From biostimulated soil a collection of *Actinobacterial* strains displaying *in vitro* biodegradation and PGP activities was also obtained. Three strains belonging to the genus *Rhodococcus* were in particular characterized for their PCB degradation capacity and for the ability to promote *Arabidopsis thaliana* growth and root development under laboratory conditions. Since *A. thaliana* root exudates previously showed to promote PCB degradation by a *Rhodoccoccus* bacterial strain (Pham et al. 2015; Toussaint et al. 2012), our results open future research perspectives on the investigation of this plant-bacteria interaction for PCB rhizoremediation purposes.

The results obtained during this PhD thesis disclosed the existence of a PCB natural attenuation potential within the autochthonous microbial communities of the SIN Caffaro polluted soil, and pointed out that rhizoremediation could be an effective strategy to enhance the soil detoxification. Further research should be focused to better characterise the degrading microbiome inhabiting the soil and to identify the best plant-treatment combination with the support of chemical investigations assessing the rate of PCB removal from soil. Also, *in vivo* test with PCB-degrading and

PGP bacterial strains are required to assess their potential as bioaugmentation tools to sustain a rhizoremediation intervention.

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Supplementary Material

SUPPLEMENTARY MATERIAL to CHAPTER 1

Supplementary table 1: Average values of the chemical parameters (pH, total organic carbon, As, Hg and PCBs concentrations) measured in the soil samples collected along the depth gradient (0-100 cm) from the areas A, R and T. TOC=total organic carbon; Tot.=total.

Sample	pH	TOC (g/Kg)	As (mg/Kg)	Hg (mg/Kg)	PCB ≤ 5 Cl (µg/Kg)	PCB > 5 Cl (µg/Kg)	Tot. PCB (µg/Kg)
A1 0-10	7,870	3,810	70,000	22,000	5661,864	27484,000	33145,864
A1 10-20	8,120	2,830	77,900	21,800	6298,222	28386,300	34684,522
A1 20-30	8,200	2,670	81,900	23,700	6585,148	26026,100	32611,248
A1 30-40	8,380	1,770	45,700	10,400	3302,470	16847,500	20149,970
A1 40-60	7,980	1,200	29,100	2,240	707,809	4105,020	4812,829
A2 0-10	8,030	3,140	70,200	11,100	836,824	4406,733	5243,557
A2 10-20	8,140	2,600	93,400	5,760	1051,267	5070,393	6121,660
A2 20-30	7,910	1,790	82,000	3,380	554,222	2229,304	2783,526
A2 30-40	7,990	1,720	40,900	0,781	60,922	246,965	307,887
A2 40-60	8,160	1,580	25,000	0,480	31,157	151,995	183,152
A2 60-80	8,010	1,070	25,100	0,791	44,222	152,484	196,706
A2 80-100	7,970	0,380	22,200	0,275	19,824	51,455	71,279
A3 0-10	7,860	0,900	65,400	2,240	159,794	1059,408	1219,202
A3 10-20	7,920	1,060	65,000	2,300	220,771	1301,403	1522,174
A3 20-30	7,950	0,350	71,000	3,260	364,479	2013,437	2377,917
A3 30-40	8,040	0,670	60,900	2,090	129,640	883,228	1012,868
A3 40-60	8,010	0,910	55,600	1,680	66,428	500,894	567,322
A3 60-80	8,230	0,025	19,400	0,303	38,761	250,035	288,795
A3 80-100	8,580	0,620	7,600	0,159	11,021	35,128	46,149
R1 0-10	7,980	2,170	39,600	3,510	836,377	7135,426	7971,802
R1 10-20	7,510	0,340	40,700	3,350	780,460	7674,733	8455,193
R1 20-30	8,060	0,580	40,300	3,090	540,923	4632,387	5173,310
R1 30-40	7,840	0,880	29,200	1,380	317,463	3147,377	3464,839
R1 40-60	7,850	0,490	21,500	0,288	12,599	90,046	102,645
R1 60-80	7,860	0,320	19,300	0,081	3,487	18,858	22,345
R1 80-100	8,110	0,025	17,300	0,065	2,700	15,132	17,832
R2 0-10	7,650	1,370	29,000	0,930	42,499	431,842	474,342
R2 10-20	7,670	1,880	29,500	0,901	50,332	658,788	709,119
R2 20-30	7,540	1,890	31,400	0,962	42,816	439,414	482,230
R2 30-40	7,780	1,540	30,200	0,603	28,835	311,017	339,851
R2 40-60	7,790	1,200	22,000	0,137	8,631	31,735	40,366
R2 60-80	7,830	1,040	22,200	0,069	1,676	6,775	8,451
R2 80-100	7,970	1,210	20,900	0,053	2,485	5,546	8,032
R3 0-10	7,650	2,540	53,500	1,940	69,270	739,460	808,730
R3 10-20	7,580	1,900	43,900	1,650	79,002	831,560	910,562

Sample	pH	TOC (g/Kg)	As (mg/Kg)	Hg (mg/Kg)	PCB < 5 Cl (µg/Kg)	PCB > 5 Cl (µg/Kg)	Tot. PCB (µg/Kg)
R3 20-30	7,790	1,930	55,000	2,120	100,561	885,121	985,682
R3 30-40	7,670	2,080	56,900	1,950	55,709	887,357	943,067
R3 40-60	7,720	1,500	41,800	0,319	2,585	22,653	25,238
R3 60-80	7,880	1,860	23,900	0,140	1,025	5,453	6,479
R3 80-100	7,810	0,888	19,000	0,056	0,511	1,539	2,050
T1 0-10	8,070	1,960	58,700	10,200	785,073	1872,048	2657,121
T1 10-20	7,020	1,600	58,800	10,600	1051,641	2514,886	3566,527
T1 20-30	8,250	0,550	53,100	8,120	704,614	1661,486	2366,100
T1 30-40	8,090	0,850	71,700	12,800	1314,168	2373,754	3687,922
T1 40-60	8,160	0,440	17,000	0,526	34,748	115,476	150,224
T1 60-80	8,020	0,280	19,300	0,143	5,497	13,645	19,143
T1 80-100	8,240	0,850	14,800	0,112	2,285	9,669	11,954
T2 0-10	7,720	1,880	43,900	3,960	149,586	799,939	949,526
T2 10-20	7,830	1,460	49,900	4,430	126,261	1020,979	1147,240
T2 20-30	8,050	1,650	48,000	4,290	190,152	979,365	1169,517
T2 30-40	7,980	0,460	30,200	1,810	41,198	272,627	313,826
T2 40-60	8,220	0,230	18,100	0,444	7,137	21,461	28,598
T3 0-10	8,040	2,330	47,400	9,750	278,625	1240,796	1519,422
T3 10-20	7,920	1,220	49,400	10,800	333,166	1411,480	1744,647
T3 20-30	8,000	1,440	59,000	12,100	375,058	1514,566	1889,624
T3 30-40	8,020	1,570	63,900	11,700	351,711	1955,705	2307,416
T3 40-60	7,980	1,010	47,800	3,880	175,911	786,370	962,281
T3 60-80	8,010	0,590	26,900	1,300	46,428	83,021	129,449
T3 80-100	8,110	1,080	22,600	0,801	35,658	60,382	96,040

Supplementary table 2: Results of FDA hydrolysis test on soil collected along the depth gradient (0-100 cm) and across the surface (0-40 cm) of sampling areas A (**A**), R (**B**) and T (**C**). The table reports the total concentration of fluorescein calculated for 1 gram of soil. Tot=total; F=fluorescein.

A. Sampling area A

Depth gradient			**Surface (0-40 cm)**	
Sample		**Tot. F. (µg/g soil)**	**Sample**	**Tot. F. (µg/g soil)**
A1	0-10	435,4	A1	242,0
A1	10-20	190,4	A2	212,0
A1	20-30	147,3	A3	240,9
A1	30-40	113,6	A4	217,7
A1	40-60	110,1	A5	189,2
A1	60-80	4,3	A6	214,8
A1	80-100	1,4	A7	207,8
A2	0-10	262,0	A8	222,4
A2	10-20	227,9	A9	232,2
A2	20-30	84,8	A10	91,1
A2	30-40	141,4	A11	232,9
A2	40-60	78,5	A12	65,5
A2	60-80	135,0	A13	102,5
A2	80-100	54,1	A14	90,0
A3	0-10	148,8	A15	144,6
A3	10-20	268,0	A16	99,1
A3	20-30	190,5	A17	129,8
A3	30-40	169,9		
A3	40-60	101,1		
A3	60-80	37,9		
A3	80-100	16,8		

B. Sampling area R

Depth gradient			Surface (0-40 cm)	
Sample		**Tot. F. (µg/g soil)**	**Sample**	**Tot. F. (µg/g soil)**
R1	0-10	235,6	R1	91,9
R1	10-20	211,4	R2	94,4
R1	20-30	176,9	R3	74,2
R1	30-40	100,0	R4	73,5
R1	40-60	62,9	R5	40,3
R1	60-80	40,7	R6	66,5
R1	80-100	23,5	R7	51,9
R2	0-10	86,6	R8	95,7
R2	10-20	149,1	R9	37,4
R2	20-30	138,7	R10	68,1
R2	30-40	80,5	R11	61,5
R2	40-60	51,4	R12	77,1
R2	60-80	42,7	R13	116,1
R2	80-100	30,7	R14	135,2
R3	0-10	197,1	R15	130,7
R3	10-20	182,7	R16	153,0
R3	20-30	178,1	R17	59,1
R3	30-40	184,6	R18	71,1
R3	40-60	130,1	R19	80,2
R3	60-80	48,8		
R3	80-100	44,7		

C. Sampling area T

Depth gradient			Surface (0-40 cm)	
Sample		**Tot. F. (µg/g soil)**	**Sample**	**Tot. F. (µg/g soil)**
T1	0-10	286,8	T1	89,8
T1	10-20	182,4	T2	74,9
T1	20-30	128,9	T3	119,7
T1	30-40	173,5	T4	84,5
T1	40-60	75,7	T5	91,5
T1	60-80	50,1	T6	107,9
T1	80-100	16,6	T7	101,4
T2	0-10	168,5	T8	101,3
T2	10-20	209,8	T9	220,6
T2	20-30	177,4	T10	87,7
T2	30-40	90,0	T11	83,8
T2	40-60	69,1	T12	86,2
T2	60-80	40,8	T13	102,4
T2	80-100	20,6	T14	152,6
T3	0-10	207,6	T15	124,4
T3	10-20	130,6	T16	98,9
T3	20-30	145,1	T17	109,0
T3	30-40	119,1	T18	142,4
T3	40-60	79,1	T19	142,1
T3	60-80	88,1	T20	94,4
T3	80-100	59,8	T21	193,5
			T22	183,6
			T23	220,0
			T24	158,0
			T25	145,5
			T26	206,9
			T27	239,8
			T28	148,8

Supplementary table 3: Statistical analysis (distLM) of the correlations between the bacterial communities' structure of the soil samples collected along the depth gradient and the soil physical-chemical parameters. Significant values ($p<0.05$) are marked in bold. **A**. Marginal test and **B**. Sequential test.

A. Marginal test

Variable	SS(trace)	Pseudo-F	P	Prop.
clay	3512,5	3,8256	**0,001**	6,39E-02
fine loam	1715,5	1,8053	**0,025**	3,12E-02
large loam	2984,4	3,2173	**0,001**	5,43E-02
fine sand	2509,9	2,6813	**0,001**	4,57E-02
large sand	1805,9	1,9037	**0,023**	3,29E-02
pH	2046,6	2,1673	**0,016**	3,73E-02
TOC	3339	3,6244	**0,001**	6,08E-02
As	5550	6,2941	**0,001**	0,10104
Hg	3708	4,054	**0,001**	6,75E-02
PCB ≤ 5 Cl	3044,6	3,2861	**0,003**	5,54E-02
PCB > 5 Cl	3075,3	3,3212	**0,001**	5,60E-02
Tot. PCB	3079,9	3,3264	**0,001**	5,61E-02

B. Sequential test

Variable	R^2	Pseudo-F	P	Prop.	Cumul.	res.df
clay	6,39E-02	3,8256	**0,001**	6,39E-02	6,39E-02	56
fine loam	9,52E-02	1,9022	**0,023**	3,13E-02	9,52E-02	55
large loam	0,13716	2,6237	**0,003**	4,19E-02	0,13716	54
fine sand	0,16105	1,5094	0,063	2,39E-02	0,16105	53
large sand	0,19247	2,0232	**0,016**	3,14E-02	0,19247	52
pH	0,21545	1,4937	0,063	2,30E-02	0,21545	51
TOC	0,26678	3,5003	**0,001**	5,13E-02	0,26678	50
As	0,31616	3,538	**0,001**	4,94E-02	0,31616	49
Hg	0,34791	2,3372	**0,005**	3,18E-02	0,34791	48
PCB ≤ 5 Cl	0,39646	1,881	**0,014**	2,47E-02	0,39646	46
PCB > 5 Cl	0,39646	No test		-1,64E-15	0,39646	46
Tot. PCB	0,37178	1,7862	**0,023**	2,39E-02	0,37178	47

Supplementary table 4: Statistical analysis (distLM) of the correlations between the ARISA community fingerprinting of the soil samples collected across the surface of the three areas and the soil physical-chemical parameters. Significant values ($p<0.05$) are marked in bold. **A**. Marginal test and **B**. Sequential test.

A. Marginal test

Variable	SS(trace)	Pseudo-F	P	Prop.
clay	5964	9,2587	**0,001**	0,13564
fine loam	1712,4	2,3909	**0,003**	3,89E-02
large loam	1535,4	2,1349	**0,012**	3,49E-02
fine sand	1274,2	1,7609	**0,043**	2,90E-02
large sand	1796,8	2,5139	**0,003**	4,09E-02
pH	5027,2	7,6167	**0,001**	0,11434
TOC	1249,5	1,7257	**0,041**	2,84E-02
As	4640,4	6,9615	**0,001**	0,10554
Hg	1908	2,6765	**0,007**	4,34E-02
PCB < = 5 Cl	1528,9	2,1254	**0,019**	3,48E-02
PCB > 5 Cl	1658	2,312	**0,013**	3,77E-02
Tot. PCB	1655,5	2,3084	**0,009**	3,77E-02

B. Sequential test

Variable	R^2	Pseudo-F	P	Prop.	Cumul.	res.df
clay	0,13564	9,2587	**0,001**	0,13564	0,13564	59
fine loam	0,16064	1,7277	**0,019**	2,50E-02	0,16064	58
large loam	0,20069	2,856	**0,001**	4,01E-02	0,20069	57
fine sand	0,22626	1,8504	**0,007**	2,56E-02	0,22626	56
large sand	0,24074	1,0491	0,416	1,45E-02	0,24074	55
pH	0,2693	2,1103	**0,002**	2,86E-02	0,2693	54
TOC	0,29196	1,6967	**0,018**	2,27E-02	0,29196	53
As	0,33588	3,4387	**0,001**	4,39E-02	0,33588	52
Hg	0,35575	1,573	**0,023**	1,99E-02	0,35575	51
PCB ≤ 5 Cl	0,37285	1,3634	0,078	1,71E-02	0,37285	50
PCB > 5 Cl	0,38683	1,1172	0,289	1,40E-02	0,38683	49
Tot. PCB	0,38683	No test	No test	-2,30E-16	0,38683	49

Supplementary table 5. Relative abundance of the bacterial taxonomic groups in the soil samples collected along the depth gradient at 0-10 and 20-30 cm. Each class of the phylum *Proteobacteria* is reported in the table.

Phyla	A1 0-10	A1 20-30	A2 0-10	A2 20-30	A3 0-10	A3 20-30	R1 0-10	R1 20-30	R2 0-10	R2 20-30	R3 0-10	R3 20-30	T1 0-10	T1 20-30	T2 0-10	T2 20-30	T3 0-10	T3 20-30
Other	1,7	1,7	1,1	2,1	1,3	1,5	0,9	0,9	1	0,9	0,9	0,8	0,6	1,1	0,8	0,9	0,7	0,6
Acidobacteria	22	21,5	15,1	20,4	17,6	16,7	16,3	18,1	13	18,1	13,9	16,9	13,9	17,4	13,6	17	14,8	16,3
Actinobacteria	6,7	8,5	11,1	7,5	14,5	13,5	15,4	13	17,1	13,6	17,2	17	17,7	15	19,1	14,9	16,1	16,6
Bacteroidetes	11,2	5	16,9	3,4	2,4	9,1	11,5	6,4	12,2	7,3	10,7	7,2	9,7	5,7	9,3	8,1	11,2	4,9
Chloroflexi	4,9	7	4,8	9,3	8,7	6,5	4,7	5,6	5,9	6,4	6	7,7	6,5	9,3	7,1	7,3	6,3	8,1
Firmicutes	0,4	2	0,6	1,4	2,5	1,2	0,8	2	0,7	2	1	3,2	1,4	4	1,2	1,8	0,8	2,8
Gemmatimonadetes	4,5	6,6	2,1	6,1	5,8	4	1,7	3,4	1,7	3,8	1,7	3,5	1,1	3,4	2	3,3	1,9	3,1
Nitrospirae	1,7	2,4	1,4	3,7	2,7	1,4	1,4	2,5	1,5	2,4	1,7	2,1	1,4	3,1	1,6	2,5	0,9	2
Planctomycetes	7	5,2	6,4	5,6	5,7	6,9	8,4	6,6	7,4	7	7,7	6	6,3	4,8	6,3	6,3	7,8	6,3
Alphaproteobacteria	12,1	11,9	10,7	9,6	9,8	12,2	10,2	10,4	10,1	9,4	10,6	9,8	14,9	10,1	11,2	9,5	12,8	12,9
Betaproteobacteria	7,4	6,1	7,3	9,7	7,3	6,8	7,5	9,7	8,7	8,9	8,3	7,8	6,1	7	7,4	7,9	6	5
Deltaproteobacteria	4,1	4,1	4,7	6,4	6,9	5,3	5,8	7,2	5,6	6,9	6,2	6,6	5,9	6,8	6,7	6,8	4,9	4,7
Gammaproteobacteria	5,8	8,6	9,2	5,2	5,1	6,2	5,8	5,5	5,3	4,7	5,4	3,6	5	5,6	5	4,9	7,1	9,4
Verrucomicrobia	4	3,4	4	2,3	2,7	3,7	5,7	4,9	5,9	4,2	5,2	4,2	5,4	3,5	5,1	5	5,8	4,5
WS3	0,4	1,3	1,1	3	3,5	1,7	1,6	1,9	1,9	2,3	1,5	1,8	1	1,4	1,2	1,7	0,9	1,2

SUPPLEMENTARY MATERIAL to CHAPTER 2

Supplementary table 1. The Good's coverage value has been calculated for each sample in order to evaluate the quality of the sequencing process.

Bulk soil (B)	Good's coverage	Rhizosphere (R)	Good's coverage	Soil Surrounding root (S)	Good's coverage
B1	0.98544	CN1	0.99482	CN1	0.987667
B2	0.982849	CN2	0.995275	CN2	0.987637
B3	0.929602	CN3	0.997801	CN3	0.986612
		DG1	0.990533	DG1	0.985785
		DG2	0.991123	DG2	0.990792
		DG3	0.989995	DG3	0.986448
		MS1	0.998011	MS1	0.904878
		MS2	0.999444	MS2	0.986783
		MS3	0.984487	MS3	0.986987

Supplementary table 2. PERMANOVA for the 16S rRNA gene-based Illumina dataset. **(A)** Main test comparison of the distance matrix generated according to OTUs distribution of bacterial communities associated to the three fractions (Rhizosphere, Soil surrounding root and Bulk) using one-way PERMANOVA. **(B)** Main test comparison of the distance matrix generated according to OTUs distribution of bacterial communities in the two fractions (Rhizosphere and Soil surrounding root) and three plant species (MS, NC and DG) using two-way PERMANOVA. Df= degree of freedom, MS = mean of square, F= F-statistic and *p* is the statistical *p* value. In bold the statistical significant terms.

A) Factor	Df	MS	F	*p*
Fraction	**2**	**2294.7**	**3.481**	**0.0007**
Residual	18	659.21		
Total	20			

B) Factor	Df	MS	F	p
Fraction	**1**	**2320.4**	**4.7151**	**0.0024**
Plant	**2**	**1579.2**	**3.2089**	**0.0078**
FractionXPlant	2	817.77	1.6617	0.0967
Residual	12	492.12		

Supplementary Table 3. Estimates of components of variation of the two-way PERMANOVA between plant species and soil fractions with their interaction. In bold the statistical significant terms (see Supplementary Table 2).

Source	Estimate	Sq.root	%
Fraction	**203.14**	**14.253**	**21**
Plant	**181.18**	**13.46**	**18**
FractionxPlant	108.55	10.419	11
Residual	492.12	22.184	50

Supplementary Table 4. Shared OTUs of **(A)** soil fractions (R, S and B) and plant species (MS, NC and DG) in **(B)** rhizosphere and **(C)** soil surrounding root fractions.

A) Soil fractions	Number of elements
B	3022
R	3436
S	3436
Overall number of unique elements	3587
Shared OTUs	2831

B) Rhizosphere	Number of elements
CN	2854
DG	3146
MS	2326
Overall number of unique elements	3436
Shared OTUs	1922

C) Root surrounding soil	Number of elements
CN	3117
DG	3072
MS	2930
Overall number of unique elements	3436
Shared OTUs	2558

Supplementary Table 5. Relative abundance of the bacterial taxonomic groups in the soil samples associated to the plant species at the SIN Caffaro site. CN = *Centaurea nigrescens* DG = *Dactylis glomerata,* MS = *Medicago sativa*. OTU_{97} are reported in percentage.

(A) Phylum/Class	Rhizosphere (R)								
	CN1	CN2	CN3	DG1	DG2	DG3	MS1	MS2	MS3
Acidobacteria	2.9	4.0	4.2	10.6	7.7	8.0	0.17	2.7	1.6
Actinobacteria	55.7	44.0	50.2	21.8	36.0	35.0	5.42	31.6	23.4
Alphaproteobacteria	4.8	7.0	4.9	23.4	13.5	14.9	2.46	10.9	7.6
Bacteroidetes	1.2	2.7	0.9	8.5	5.8	7.8	0.41	4.9	1.3
Betaproteobacteria	4.2	3.6	3.4	5.5	5.1	3.4	0.73	4.3	2.8
Chloroflexi	13.6	11.3	12.8	6.4	9.5	8.2	0.85	5.3	4.9
Deltaproteobacteria	2.7	3.7	3.7	4.1	4.7	3.5	0.24	2.2	1.3
Firmicutes	5.0	14.8	7.7	1.1	4.4	5.0	1.33	11.2	10.3
Gammaproteobacteria	2.1	2.8	4.6	7.2	3.5	5.7	87.80	22.9	44.6
Gemmatimonadetes	1.4	0.8	1.1	1.1	1.2	0.9	0.01	0.4	0.2
Other (<1%)	1.0	1.3	1.7	2.5	2.3	1.8	0.04	0.7	0.3
Planctomycetes	0.8	0.8	1.1	2.7	1.7	1.8	0.03	0.5	0.3
TM7	1.9	1.6	1.6	1.2	2.1	1.8	0.43	1.4	0.8
Verrucomicrobia	1.3	1.2	1.5	3.5	2.2	2.0	0.07	0.9	0.4
WS3	1.5	0.4	0.6	0.5	0.4	0.4	0.01	0.1	0.2

(B) Phylum/Class	Soil Surrounding root (S)								
	CN1	CN2	CN3	DG1	DG2	DG3	MS1	MS2	MS3
Acidobacteria	14.6	12.0	11.3	10.3	14.9	11.0	23.5	15.2	12.8
Actinobacteria	23.8	25.7	31.6	39.8	31.3	40.2	15.2	23.5	30.2
Alphaproteobacteria	10.2	11.2	11.5	12.2	12.1	11.3	12.9	9.7	9.7
Bacteroidetes	4.4	4.1	3.8	3.2	3.6	3.5	6.4	3.0	2.5
Betaproteobacteria	6.6	7.8	5.9	5.2	6.1	4.8	4.6	7.9	7.0
Chloroflexi	11.1	11.3	12.2	8.8	8.1	8.8	5.5	10.3	11.1
Deltaproteobacteria	7.4	6.1	6.0	5.4	5.9	5.2	6.4	6.9	6.4
Firmicutes	1.4	1.3	1.5	0.9	0.7	0.8	1.1	1.2	1.4
Gammaproteobacteria	2.9	2.7	2.5	2.1	2.5	2.6	3.1	2.8	2.3
Gemmatimonadetes	3.0	3.4	2.5	1.6	1.8	1.3	2.7	4.0	3.5
Other (<1%)	2.7	2.7	2.1	2.3	2.7	2.2	4.4	2.7	2.3
Planctomycetes	4.4	4.1	3.2	3.0	3.9	3.0	4.6	4.1	3.4
TM7	1.2	1.2	1.0	1.5	1.5	1.3	2.0	1.6	1.2
Verrucomicrobia	4.1	3.9	3.1	2.9	4.0	3.4	5.6	4.5	3.7
WS3	2.1	2.6	1.9	0.8	0.9	0.6	1.9	2.6	2.4

(C) Phylum/Class	Bulk		
	B1	B2	B3
Other (<1%)	3.0	3.7	3.0
WS3	3.2	2.7	2.0
Verrucomicrobia	4.9	5.5	4.8
TM7	0.6	0.6	0.8
Planctomycetes	3.2	3.2	3.3
Gemmatimonadetes	4.5	3.7	3.6
Gammaproteobacteria	6.7	6.7	7.2
Firmicutes	1.7	0.8	0.8
Deltaproteobacteria	6.1	6.5	5.7
Chloroflexi	4.5	4.4	4.3
Betaproteobacteria	11.1	9.9	11.0
Bacteroidetes	12.1	11.6	14.0
Alphaproteobacteria	11.0	11.4	13.4
Actinobacteria	6.2	6.4	7.0
Acidobacteria	21.2	23.1	19.2

Supplementary Table 6. Relative abundance of the bacterial taxonomic groups in the soil samples associated to the plant species at the SIN Caffaro site. Relative abundance is indicated at the Order levels for each detected Phylum/Class. CN = *Centaurea nigrescens* DG = *Dactylis glomerata,* MS = *Medicago sativa*. OTU_{97} are reported in percentage. The table is provided as Excel file.

Supplementary Table 7. Taxa distribution among the three plant species in **(A)** rhizosphere and **(B)** root surrounding soil fraction (left tables) and respective diversity indices (right tables).

(A) Phylum/Class	Rhizosphere		
	CN	DG	MS
Acidobacteria	3.7	8.7	1.5
Actinobacteria	50.0	30.9	20.1
Alphaproteobacteria	5.6	17.2	7.0
Bacteroidetes	1.6	7.4	2.2
Betaproteobacteria	3.7	4.7	2.6
Chloroflexi	12.6	8.0	3.7
Deltaproteobacteria	3.3	4.1	1.2
Firmicutes	9.2	3.5	7.6
Gammaproteobacteria	3.2	5.5	51.8
Gemmatimonadetes	1.1	1.1	0.2
Other (<1%)	1.3	2.2	0.3
Planctomycetes	0.9	2.1	0.3
TM7	1.7	1.7	0.9
Verrucomicrobia	1.3	2.6	0.5
WS3	0.8	0.4	0.1

Diversity index	Rhizosphere		
	CN	DG	MS
Dominance_D	0.2835	0.1544	0.3224
Simpson_1-D	0.7165	0.8456	0.6776
Shannon_H	1.834	2.224	1.585
Evenness_e^H/S	0.4173	0.6163	0.3254
Brillouin	1.639	2.01	1.424
Menhinick	1.5	1.499	1.5
Margalef	3.04	3.039	3.04
Equitability_J	0.6773	0.8213	0.5854
Fisher_alpha	4.894	4.892	4.894
Berger-Parker	0.5	0.3087	0.518

(B) Phylum/Class	Root surrounding Soil		
	CN	DG	MS
Acidobacteria	12.7	12.1	17.2
Actinobacteria	27.0	37.1	23.0
Alphaproteobacteria	11.0	11.9	10.7
Bacteroidetes	4.1	3.4	4.0
Betaproteobacteria	6.8	5.4	6.5
Chloroflexi	11.5	8.6	9.0
Deltaproteobacteria	6.5	5.5	6.6
Firmicutes	1.4	0.8	1.2
Gammaproteobacteria	2.7	2.4	2.7
Gemmatimonadetes	2.9	1.6	3.4
Other (<1%)	2.5	2.4	3.1
Planctomycetes	3.9	3.3	4.0
TM7	1.1	1.4	1.6
Verrucomicrobia	3.7	3.4	4.6
WS3	2.2	0.7	2.3

Diversity index	Root surrounding Soil		
	CN	DG	MS
Dominance_D	0.1308	0.1849	0.1199
Simpson_1-D	0.8692	0.8151	0.8801
Shannon_H	2.333	2.126	2.38
Evenness_e^H/S	0.6871	0.5586	0.7206
Brillouin	2.11	1.916	2.154
Menhinick	1.5	1.5	1.501
Margalef	3.04	3.04	3.041
Equitability_J	0.8614	0.7849	0.879
Fisher_alpha	4.894	4.894	4.896
Berger-Parker	0.27	0.371	0.2302

Supplementary Table 8. Identification, PGP traits, abiotic stress tolerance and degradation potential of the rhizobacteria associated to *Medicago sativa*, *Centaurea nigrescens* and *Dactylis glomerata*. The list includes the taxonomic classification of all strains and the results of the physiological tests and *bphA* PCR assay performed. ACCd = ACC-deaminase activity; IAA = auxin production; P. Sol. = inorganic phosphate solubilization; Sid. = siderophore production; Prot. = protease production; EPS = exopolysaccharides release; NH_3=ammonia production; PEG = polyethylene glycol; C 2,3 D = 2,3-catechol dioxygenase activity; bphA = positive PCR amplification of the *bphA* gene. The table is provided as Excel file.

Supplementary Table 9. Classification of the *bphA* gene sequences. Information on the *bphA* gene. ID % = % of identity; Acc n = accession number of the closest relative sequence in NCBI. Strain code indicate the plant of origin (1: MS, 2: CN, 3: DG).

Strain	Species	Closest *bphA* sequence	ID %	Acc. N.
1_12B	*Pseudomonas moraviensis*	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	99	KP972446
1_19	*Arthrobacter scleromae*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
1_25	*Arthrobacter scleromae*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
1_28	*Streptomyces erythrochromogenes*	Rhodococcus opacus partial bphA1 gene for biphenyl 2,3-dioxygenase alpha subunit	100	AJ544524
1_37	*Arthrobacter phenanthrenivorans*	Rhodococcus opacus partial bphA1 gene for biphenyl 2,3-dioxygenase alpha subunit	100	AJ544524
1_41	*Microbacterium natoriense*	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	99	KP972446
1_45	*Pseudomonas baetica*	Rhodococcus opacus partial bphA1 gene for biphenyl 2,3-dioxygenase alpha subunit	100	AJ544524
1_51	*Microbacterium natoriense*	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	99	KP972446
1_58	*Microbacterium natoriense*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_10	*Bacillus aryabhattai*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_13	*Acinetobacter calcoaceticus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_14B	*Agromyces terreus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_15B	*Acinetobacter calcoaceticus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_16	*Rahnella aquatilis*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_17	*Bacillus cereus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_28	*Microbacterium takaoensis*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_34	*Microbacterium yannicii*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_36	*Microbacterium yannicii*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_38A	*Agromyces cerinus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_42	*Microbacterium phyllosphaerae*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_43	*Agromyces cerinus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_46	*Pseudomonas salomonii*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_47	*Microbacterium arthrosphaerae*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_49	*Pseudomonas koreensis*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_5	*Bacillus megaterium*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_55	*Promicromonospora sukumoe*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
2_57	*Pseudomonas putida*	Rhodococcus opacus partial bphA1 gene for biphenyl 2,3-dioxygenase alpha subunit	100	AJ544524
2_9	*Paenibacillus lautus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862

Strain	Species	Closest *bphA* sequence	ID %	Acc. N.
3_1	*Microbacterium yannicii*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_12	*Microbacterium yannicii*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_13	*Microbacterium yannicii*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_15A	*Acinetobacter calcoaceticus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_19	*Arthrobacter nitroguajacolicus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_2	*Acinetobacter calcoaceticus*	Rhodococcus opacus partial bphA1 gene for biphenyl 2,3-dioxygenase alpha subunit	100	AJ544524
3_21	*Bacillus toyonensis*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_22	*Arthrobacter oxydans*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_32A	*Pseudomonas putida*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_33	*Arthrobacter oryzae*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_36	*Microbacterium natoriense*	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit	99	KP972446
3_38A	*Bacillus cereus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_48	*Arthrobacter scleromae*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_55	*Pseudomonas putida*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_57	*Arthrobacter humicola*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_6	*Buttiauxella agrestis*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862
3_9	*Acinetobacter calcoaceticus*	Pseudomonas pseudoalcaligenes KF707 = NBRC biphenyl dioxygenase alpha subunit	99	AP014862

Supplementary Table 10. Identification, PGP activity, abiotic stress traits and bioremediation potential of the eleven strains selected for the *in vivo* PGP test

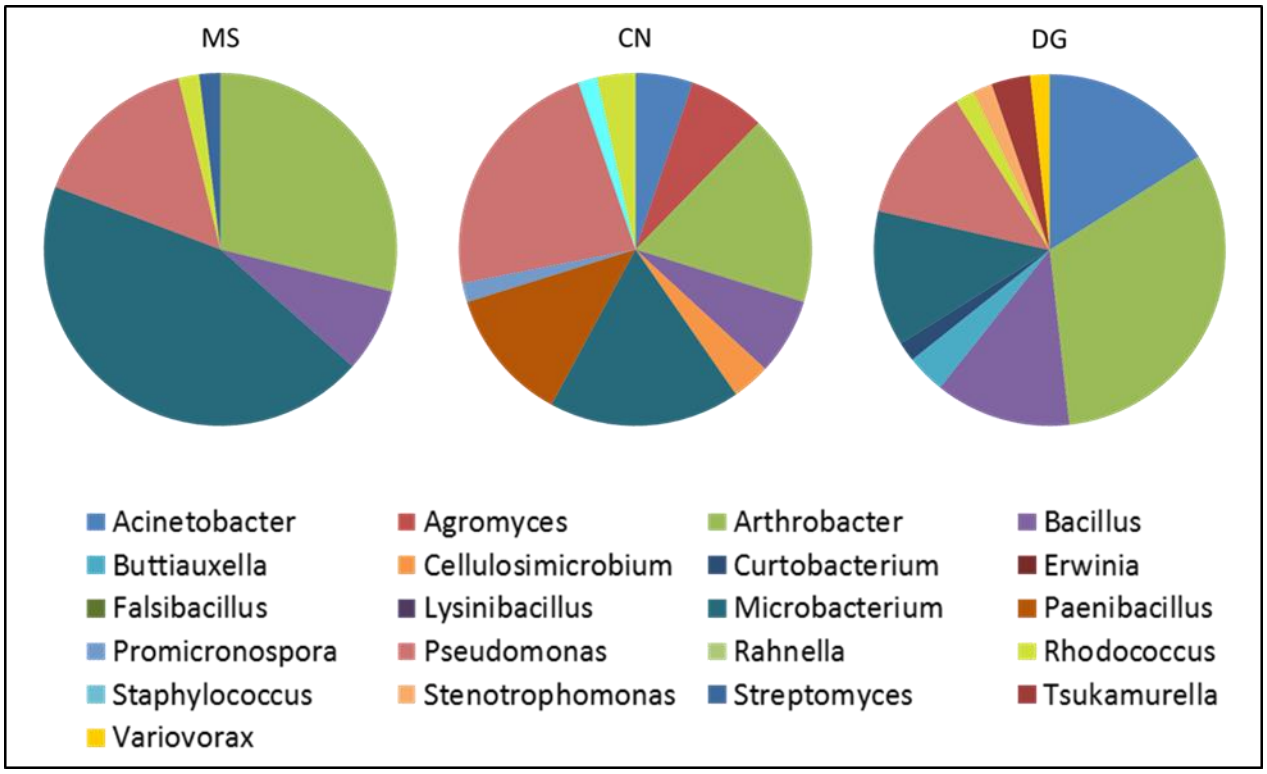
Plant	Strain	Identification[#] (% identity)	PGP activity							Abiotic stress tolerance				Bioremediation potential	
			ACCd	IAA	P sol	Sid	Prot	EPS	NH_3	4°C	42°C	20%PEG	5% NaCl	C 2,3 D	*bphA*
MS	1-45	*Pseudomonas baetica* (98)	0	1	0	0	1	0	0	1	0	1	0	1	1
	1-8	*Pseudomonas salomonii (99)*	0	1	0	0	0	0	0	1	1	1	1	1	0
CN	2-15B	*Acinetobacter calcoaceticus* (99)	0	0	1	0	0	0	1	1	1	1	1	1	1
	2-2	*Pseudomonas reinekei (99)*	0	0	0	1	0	0	1	1	1	1	1	1	0
	2-22B	*Pseudomonads baetica (99)*	0	1	0	1	1	0	1	1	0	1	1	1	0
	2-30	*Arthrobacter aurescens (99)*	0	1	0	0	0	0	0	1	1	1	1	1	0
	2-50	*Arthrobacter nitroguajacolicus* (99)	1	0	0	0	1	0	0	1	0	1	1	1	1
DG	3-15A	*Acinetobacter calcoaceticus (99)*	0	1	0	0	0	0	0	1	1	1	1	1	1
	3-15B	*Arthrobacter aurescens (99)*	1	1	0	1	1	0	1	1	0	1	1	0	0
	3-24	*Pseudomonas baetica (99)*	0	1	0	0	1	0	1	1	0	1	0	1	1
	3-59	*Curtobacterium flaccumfaciens (99)*	1	1	0	0	1	1	0	0	0	1	1	1	0

Identification was determined according to the partial sequence of the 16S rRNA gene

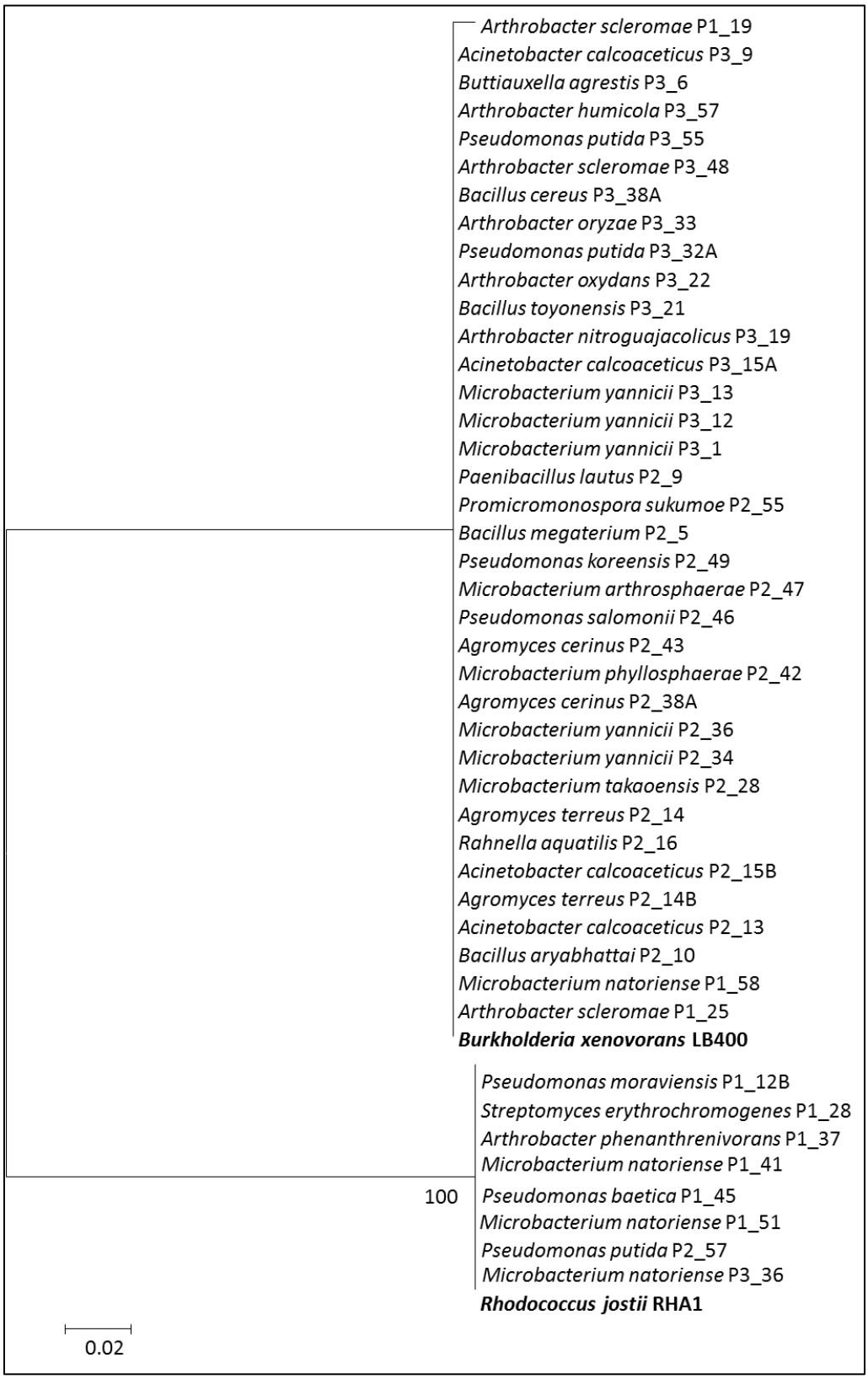
ACCd: ACC-deaminase activity; IAA: auxin production; Sol P: inorganic phosphate solubilization; Sid: siderophore production; Prot. = protease production; ESP: exopolysaccharide production; NH_3=ammonia production; PEG = polyethylene glycol; C 2,3 D = 2,3-catechol dioxygenase activity; *bphA* = positive PCR amplification of the *bphA* gene.

0/1: absence/presence of the activity.

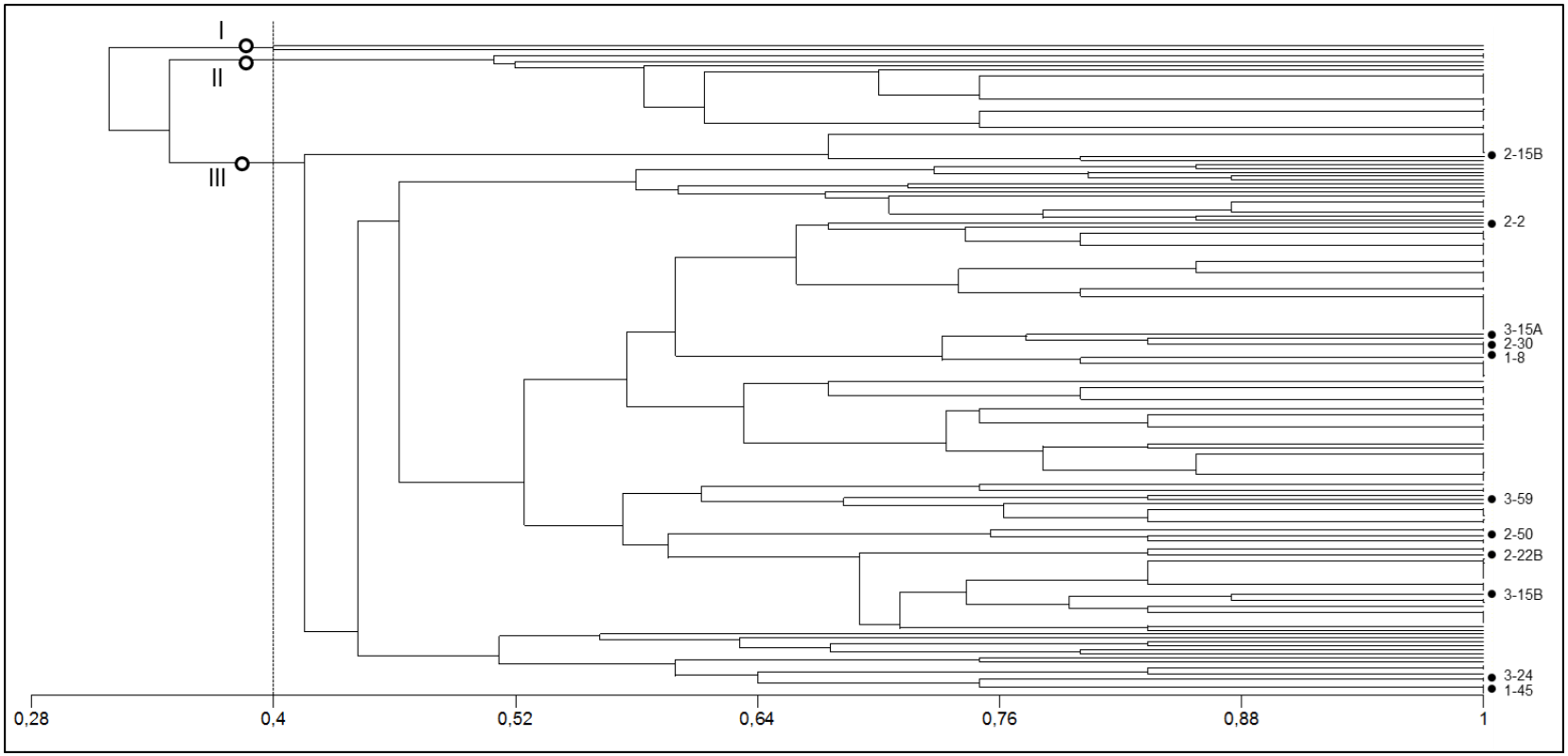
Supplementary Figure 1. Identification of biphenyl-utilizing rhizobacteria from spontaneous plants. Phylogenetic classification is reported at the genus level for the bacteria isolated from the rhizosphere of MS, CN and DG.



Supplementary Figure 2. Phylogenetic tree of the *bphA* gene sequences of the isolated strains. Reference strains *P. xenovorans* LB400 and *R. jostii* RHA1 are indicated in bold.



Supplementary Figure 3. Selection of PGP bacteria for the *in vivo* assay. Cluster analysis combining the PGP activities and the abiotic stress tolerance traits of the strains. Black dots and codes are indicated for the selected PGP strains.



SUPPLEMENTARY MATERIAL TO CHAPTER 3

Supplementary table 1. Physical-chemical parameters of the soil from the SIN Caffaro after homogenisation and partitioning in five portions for pot setup (T0). Total PCB is reported as a sum of the PCB congeners measured in the samples (n= 20).

Parameters		Soil portions				
		1	**2**	**3**	**4**	**5**
Soil texture (> 2 mm e < 20 mm)	% p/p	15	14	16	17	13
Dry fraction (< 2 mm)	% p/p	85	86	84	83	87
Organic carbon	g/kg s.s.	12,3	13,6	12,1	12	11,8
Arsenic (As)	mg/kg s.s.	44,3	44,8	43,8	45,2	48,4
Mercury (Hg)	mg/kg s.s.	6,4	6,2	6,4	6,2	6,6
PCB (Total)	mg/kg s.s.	6,4340	6,1734	5,7678	6,6824	7,1000
PCB 28 (TriCB)	mg/kg s.s.	0,2754	0,2699	0,2617	0,2928	0,3082
PCB 52 (TetraCB)	mg/kg s.s.	0,2238	0,2155	0,2051	0,2308	0,2428
PCB 101 (PentaCB)	mg/kg s.s.	0,3989	0,3806	0,3524	0,4099	0,4349
PCB 138 (EsaCB)	mg/kg s.s.	0,5961	0,5821	0,5506	0,6240	0,6734
PCB 153 (EsaCB)	mg/kg s.s.	0,6413	0,6050	0,5693	0,6725	0,6981
PCB 180 (EptaCB)	mg/kg s.s.	0,5224	0,4997	0,4778	0,5455	0,5778
PCB 95 (PentaCB)	mg/kg s.s.	0,2001	0,1911	0,1778	0,2040	0,2154
PCB 99 (PentaCB)	mg/kg s.s.	0,1421	0,1360	0,1250	0,1464	0,1540
PCB 110 (PentaCB)	mg/kg s.s.	0,3091	0,2924	0,2743	0,3194	0,3392
PCB 128 (EsaCB)	mg/kg s.s.	0,0965	0,0916	0,0866	0,1006	0,1069
PCB 146 (EsaCB)	mg/kg s.s.	0,0647	0,0625	0,0582	0,0675	0,0713
PCB 149 (EsaCB)	mg/kg s.s.	0,3908	0,3757	0,3500	0,4067	0,4345
PCB 151 (EsaCB)	mg/kg s.s.	0,1579	0,1517	0,1417	0,1630	0,1744
PCB 170 (EptaCB)	mg/kg s.s.	0,2617	0,2502	0,2357	0,2729	0,2902
PCB 177 (EptaCB)	mg/kg s.s.	0,1153	0,1103	0,1039	0,1204	0,1287
PCB 183 (EptaCB)	mg/kg s.s.	0,0821	0,0784	0,0722	0,0843	0,0899
PCB 187 (EptaCB)	mg/kg s.s.	0,3061	0,2936	0,2742	0,3190	0,3374
PCB 194 (OctaCB)	mg/kg s.s.	0,1999	0,1897	0,1782	0,2139	0,2214
PCB 196+203 (OctaCB)	mg/kg s.s.	0,1667	0,1608	0,1505	0,1756	0,1841
PCB 209 (DecaCB)	mg/kg s.s.	1,2831	1,2366	1,1226	1,3132	1,4174

Supplementary table 2. Pairwise comparison (PERMANOVA) of bacterial ARISA fingerprints between the non-polluted planted control (C6) and all the polluted soil treatments and controls at sampling time T2 and T4. Significant P values (P<0.05) are marked in bold.

ARISA (bacteria)	T2		T4	
Groups	**t**	**P(MC)**	**t**	**P(MC)**
C1, C6	2,3983	**0,0139**	3,0216	**0,0071**
C2, C6	2,5454	**0,0133**	2,9384	**0,0068**
C3, C6	1,8528	**0,0513**	2,7404	**0,01**
C4, C6	2,3982	**0,0182**	2,8368	**0,0092**
C5, C6	3,0839	**0,0072**	3,4083	**0,0036**
C7, C6	2,674	**0,0105**	2,3134	**0,0204**
C6, P1	2,2056	**0,0193**	2,4507	**0,0135**
C6, P2	2,3772	**0,0144**	2,4302	**0,0127**
C6, P3	1,8252	**0,048**	3,0208	**0,0068**
C6, P4	2,5379	**0,0107**	3,0528	**0,0064**
C6, P5	2,6531	**0,0101**	3,3343	**0,0046**
C6, P6	2,7321	**0,0088**	2,8036	**0,009**
C6, P7	2,4528	**0,0136**	2,739	**0,0087**
C6, P8	2,5759	**0,0118**	2,4108	**0,0148**
C6, P9	2,5775	**0,0107**	2,8366	**0,0074**
C6, P10	2,0998	**0,0257**	2,9126	**0,0077**

Supplementary table 3. Pairwise comparison (PERMANOVA) of fungi ARISA fingerprinting between treatments planted with different species in the same soil conditions at sampling time T4. Significant P values (P<0.05) are marked in bold.

ARISA (fungi)	T4	
Groups	**t**	**P(MC)**
C1, C6	1,7156	0,0721
C2, C6	1,6202	0,0911
C3, C6	1,8939	0,0511
C4, C6	2,2937	**0,0173**
C5, C6	1,8469	0,0528
C7, C6	1,7758	0,067
C6, P1	1,6763	0,0647
C6, P2	2,0325	**0,0353**
C6, P3	1,7627	0,0587
C6, P4	1,9911	**0,0324**
C6, P5	1,8183	0,0556
C6, P6	1,9693	**0,0318**
C6, P7	2,117	**0,0246**
C6, P8	1,7408	0,0723
C6, P9	2,561	**0,0136**
C6, P10	1,605	0,1047

Supplementary table 4. Taxonomic identification and *in vitro* screening of activities linked to the rhizoremediation potential of all the strains of the PCR-ITS de-replicated collection (n=56). Gray squares indicate positive results. Nd=not determined. Bph F3 and 512F = PCR amplification of the *bphA* gene with primers F3/R1 and 512F/674R; C 2,3 D = 2,3-catechol dioxygenase activity; Emul. = emulsification activity; Emul. St. = emulsification stability; ACCd = ACC-deaminase activity.

	Isolate identification			Rhizoremediation potential					
Strain	Identification	Id. %	Accession N°	bph F3	bph 512F	C 2,3-d	Emul.	Emul. St.	ACC d
1B1	*Gordonia amicalis*	99	KM113029		■	■	■	■	
1B10	*Arthrobacter pascens*	100	KF515608		■	■	■	■	■
1B16	*Arthrobacter oryzae*	99	KU877631		■		■	■	■
1B18	*Arthrobacter oryzae*	99	HF585031		■	■	■	■	■
1B21	*Micromonospora chalcea*	99	KX607093			nd	nd	nd	nd
1B23	*Arthrobacter oryzae*	99	KF424279		■		■		
1B3	*Gordonia amicalis*	100	KM113029		■	■	■	■	
1B4	*Gordonia terrae*	99	KJ995852			■	■	■	
1B9	*Arthrobacter oryzae*	99	AB648958		■		■	■	■
1L10	*Gordonia terrae*	99	KM113032			■	■	■	
1L11	*Gordonia terrae*	99	KM113032		■	■	■	■	
1L16	*Pseudarthrobacter siccitolerans*	99	KY753248				■		■
1L20	*Rhodococcus aetherivorans*	99	JN180179			■	■	■	
1L5	*Streptomyces mutabilis*	99	KY007178				■		■
1N10	*Streptomyces albogriseolus*	99	FJ486338				■	■	■
1N31	*Gordonia amicalis*	99	KU904410			■	■	■	
2B10	*Arthrobacter pascens*	100	KF515608				■	■	
2B13	*Rhodococcus aetherivorans*	99	JN180179		■	■	■	■	
2B15	*Arthrobacter sulfonivorans*	99	CP013747		■	■	■	■	
2B16	*Arthrobacter pascens*	99	JN903380				■	■	
2B17	*Pseudarthrobacter siccitolerans*	99	KY753220		■		■	■	■
2B19	*Arthrobacter oryzae*	100	KT443873		■		■	■	■
2B2	*Arthrobacter oryzae*	99	KU877631		■	■	■	■	■
2B23	*Rhodococcus jostii*	99	KF976882	■	■		■	■	
2B27	*Rhodococcus rhodochrous*	99	KM461687	■	■		■	■	
2B28	*Arthrobacter pascens*	100	KF515608		■		■	■	■
2B29	*Pseudarthrobacter scleromae*	100	KC788089				■	■	■

	Isolate identification			Rhizoremediation potential					
Strain	**Identification**	**Id. %**	**Accession N°**	**bph F3**	**bph 512F**	**C 2,3-d**	**Emul.**	**Emul. St.**	**ACC d**
2B3	*Arthrobacter oryzae*	99	KF424279						
2B30	*Arthrobacter oryzae*	99	HF585031						
2B5	*Pseudarthrobacter scleromae*	100	KC788089						
2B7	*Arthrobacter pascens*	100	KF515608						
2B8	*Arthrobacter pascens*	100	KC934751						
2L10	*Arthrobacter pascens*	99	KF515608						
2L21	*Streptomyces griseorubens*	99	EU841555						
2L22	*Arthrobacter pascens*	100	KF515608						
2L27	*Arthrobacter pascens*	99	KC934751						
2L29	*Pseudarthrobacter oxydans*	99	KY646071						
2L30	*Arthrobacter pascens*	100	KF515608						
2N20	*Pseudarthrobacter oxydans*	99	KY646071						
2N21	*Streptomyces torulosus*	99	GU479440						
2N22	*Streptomyces torulosus*	100	GU479440						
2N24	*Streptomyces neyagawaensis*	100	JN566030						
3B10	*Rhodococcus aetherivorans*	99	JN180179						
3B12	*Rhodococcus jostii*	99	KJ571086						
3B14	*Micromonospora sediminicola*	100	KU973997			nd	nd	nd	nd
3B15	*Arthrobacter pascens*	100	KF515608						
3B20	*Arthrobacter sulfonivorans*	99	CP013747						
3B21	*Arthrobacter oryzae*	100	HF585031						
3B23	*Pseudarthrobacter scleromae*	100	KC788089						
3B26	*Arthrobacter pascens*	100	KF515608						
3B3	*Arthrobacter oryzae*	99	KU877631						
3B4	*Arthrobacter pascens*	100	KF515608						
3B5	*Arthrobacter pascens*	100	KF515608						
3B7	*Arthrobacter oryzae*	99	KC778368						
3B8	*Arthrobacter pascens*	100	KF515608						
3L27	*Pseudomonas putida*	99	KT380614						

Supplementary table 5. Identification of the *bphA* partial nucleotide sequences according to the NCBI database. **A.** PCR products amplified with primers 512F and 674R. **B.** PCR products amplified with primers F3 and R1.

A. Primers 512F/674R

Strain	Closest relative	Acc. N°	Id. %
1B3	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	KP972446	98
1B10	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	KP972446	99
1L11	Rhodococcus wratislaviensis strain G10 putative Rieske non-heme iron oxygenase alpha subunit gene	KP972448	100
2B3	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	KP972446	99
2B7	Rhodococcus wratislaviensis strain G10 putative Rieske non-heme iron oxygenase alpha subunit gene	KP972448	100
2B8	Rhodococcus wratislaviensis strain G10 putative Rieske non-heme iron oxygenase alpha subunit gene	KP972448	100
2B13	Rhodococcus wratislaviensis strain G10 putative Rieske non-heme iron oxygenase alpha subunit gene	KP972448	100
2B23	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	KP972446	99
2B27	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	KP972446	99
2B28	Rhodococcus wratislaviensis strain G10 putative Rieske non-heme iron oxygenase alpha subunit gene	KP972448	99
2L29	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	KP972446	98
2N22	Rhodococcus wratislaviensis strain G10 putative Rieske non-heme iron oxygenase alpha subunit gene	KP972448	100
2N24	Rhodococcus wratislaviensis strain G10 putative Rieske non-heme iron oxygenase alpha subunit gene	KP972448	100
3B3	Rhodococcus wratislaviensis strain G10 putative Rieske non-heme iron oxygenase alpha subunit gene	KP972448	100
3B8	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	KP972446	98
3B10	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	KP972446	99
3B12	Rhodococcus wratislaviensis strain P13 biphenyl 2,3-dioxygenase alpha subunit (bphA1) gene	KP972446	99

B. Primers F3/R1

Strain	Closest relative	Acc. N°	Id. %
2B23	Uncultured Rhodococcus sp. clone NS7 biphenyl 2,3-dioxygenase alpha subunit (bphAa) gene	JN675900	100
2B27	Uncultured Rhodococcus sp. clone NS7 biphenyl 2,3-dioxygenase alpha subunit (bphAa) gene	JN675900	100
3B12	Uncultured Rhodococcus sp. clone NS7 biphenyl 2,3-dioxygenase alpha subunit (bphAa) gene	JN675900	100
2B7	Arthrobacter sp. 3YC3 putative Rieske non-heme iron oxygenase alpha subunit gene	DQ166965	97
2B8	Arthrobacter sp. 3YC3 putative Rieske non-heme iron oxygenase alpha subunit gene	DQ166965	99
2N21	Streptomyces scabiei 3-phenylpropionate dioxygenase alpha subunit	FN554889	90

Supplementary table 6. Resting cell assay results. The table reports the average PCB content measured for each bacterial strain. The PCB congeners were measured in each replicate serum bottle after 48 h of incubation with the PCB mixture Delor 103 and the bacterial suspension. X=inactivated control represented by the autoclaved strain culture. JAB1=*Pseudomonas* sp. strain JAB1.

PCB congener	B7	B7X	B12	B12X	B23	B23X	B27	B27X	JAB1
101+90	0,30	0,26	0,24	0,26	0,26	0,29	0,29	0,28	0,26
110	0,51	0,39	0,34	0,40	0,38	0,45	0,51	0,45	0,40
118	0,34	0,17	0,20	0,23	0,21	0,26	0,26	0,32	0,20
15	2,78	2,55	1,79	2,46	2,26	2,38	1,90	2,33	2,23
16	9,23	8,79	8,31	9,17	10,13	9,50	8,81	9,15	9,18
17	9,64	9,14	7,07	9,64	9,31	9,84	7,86	9,08	9,29
18	20,50	19,25	5,67	20,32	11,44	20,64	12,70	17,53	19,79
19	2,25	2,13	1,98	2,11	2,40	2,14	2,23	2,27	2,13
22	6,25	5,73	4,95	6,04	5,92	6,00	5,20	5,76	5,45
25+26	1,77	1,69	0,62	1,73	0,72	1,74	0,92	1,43	1,51
27+24	1,15	1,07	0,40	1,18	0,70	1,19	0,88	1,10	1,11
28+31	40,35	38,31	31,69	39,65	38,14	39,72	27,68	35,63	36,83
32	5,87	5,82	4,71	6,01	5,99	6,01	5,28	5,71	5,59
33+53	7,80	7,75	6,11	7,95	7,41	7,83	4,39	6,87	7,22
37	3,51	2,98	2,23	2,99	2,41	2,68	2,43	2,93	2,41
4	4,55	4,28	1,83	4,61	3,00	4,45	3,10	3,96	4,46
40	1,44	1,34	1,24	1,34	1,43	1,44	1,33	1,49	1,33
41+64+71+72	5,85	5,54	4,93	5,49	5,86	5,70	5,46	5,68	5,12
42	3,54	3,54	3,11	3,49	3,69	3,63	3,39	3,57	3,45
44	3,87	3,66	2,47	3,84	3,43	3,98	3,28	3,70	3,66
45	1,69	1,56	1,48	1,57	1,90	1,62	1,56	1,59	1,54
48+47	4,52	4,26	3,94	4,37	4,64	4,51	4,25	4,29	4,36
49	4,46	4,22	3,90	4,58	4,61	4,53	4,34	4,38	4,18
5+8	11,73	11,01	4,21	11,45	7,02	11,80	2,98	8,47	11,33
52	3,75	3,58	3,26	3,67	3,91	3,67	3,64	3,71	3,53
56+60	3,54	3,12	2,74	3,17	2,87	3,32	2,98	3,32	2,89
6	1,14	1,16	0,21	1,14	0,50	1,09	0,43	0,84	1,13
63	0,17	0,15	0,13	0,15	0,15	0,17	0,15	0,16	0,16
66+95	5,31	4,70	4,03	4,81	4,53	4,83	3,70	4,79	4,31
70+76	4,02	3,54	3,06	3,73	3,41	3,77	3,51	3,84	3,24
74	1,98	1,76	1,58	1,85	1,70	1,79	1,81	1,94	1,63
84+92+89	0,18	0,19	0,19	0,27	0,36	0,23	0,22	0,22	0,29
87	0,02	0,01	0,01	0,02	0,01	0,02	0,02	0,02	0,02
97	0,20	0,17	0,16	0,22	0,16	0,25	0,21	0,20	0,19
99+113	0,30	0,26	0,23	0,27	0,26	0,28	0,29	0,27	0,26
Tot. PCB (sum)	**174,50**	**164,10**	**119,03**	**170,16**	**151,14**	**171,72**	**128,01**	**157,30**	**160,68**

Activities performed during the PhD

Paper published in international journals

Vergani L, Mapelli F, Zanardini E, Terzaghi E, Di Guardo A, Morosini C, Raspa G, Borin S. 2017. Phyto-rhizoremediation of polychlorinated biphenyl contaminated soils: An outlook on plant-microbe beneficial interactions. *Science of the Total Environment* 575:1395–1406.

Vergani L, Mapelli F, Marasco R, Crotti E, Fusi M, Di Guardo A, Armiraglio S, Daffonchio D, Borin S. 2017. Bacteria associated to plants naturally selected in a historical PCB polluted soil show potential to sustain natural attenuation. *Frontiers in Microbiology* 8, 1385. doi:10.3389/fmicb.2017.01385.

Terzaghi E, Zanardini E, Morosini C, Raspa G, Borin S, Mapelli F, **Vergani L**, Di Guardo A. 2018. Rhizoremediation half-lives of PCBs: Role of congener composition, organic carbon forms, bioavailability, microbial activity, plant species and soil conditions, on the prediction of fate and persistence in soil. *Science of the Total Environment* 612:544-560.

Oral dissertations in national and international meetings

Vergani L, Mapelli F, Marasco R, Crotti E, Fusi M, Daffonchio D, Borin S. 2017. Bacteria associated to plants naturally selected in a historical PCB polluted soil show potential to sustain natural attenuation. Mirobial Diversity Conference 2017. Bari, Italy 25 November 2017

Vergani L, Mapelli F, Marasco R, Crotti E, Fusi M, Daffonchio D, Borin S. 2017. Bacteria associated to plants naturally selected in a historical PCB polluted soil show potential to sustain natural attenuation. BioBio Conference 2017. Prague, Czech Republic 26 June 2017.

Vergani L. "Pollutant profiles drive spatial pattern of soil bacterial communities" Cortona Procarioti 2016. Cortona, Italy 1 May 2016.

Coauthor of oral presentation in national and international meetings

Mapelli F, **Vergani** L, Marasco R, Fusi M, Daffonchio D, Borin S. "Spatial pattern of soil bacterial diversity in a mixed and uneven polluted site and assessment of rhizoremediation potential" Microbial Diversity Conference, Perugia 27-29 October 2015.

Marasco R, Rolli E, **Vergani** L, Fusi M, Booth J M, Soussi A, Fodelianakis S, Blilou I, Cardinale M, Cherif A, Borin S, Daffonchio D. "Bacterial diversity and functional services within the rhizosheat of a desert plant." Microbial Diversity Conference, Perugia 27-29 October 2015.

Marasco R, Rolli E, **Vergani** L, Soussi A, Fusi M, Fodelianakis S, Booth J M Blilou I, Cardinale M, Cherif A, Borin S, Daffonchio D. "Bacterial diversity and functional services within the rhizosheat of a desert plant". 3rd Florence Conference on Phenotype MicroArray Analysis of Cells, Florence 10-12 September 2015.

Poster presentations in national and international meetings

Vergani L, Mapelli F, Marasco R, Chouaia B, Di Guardo A, Zanardini E, Armiraglio S, Daffonchio D, Borin S. "Microbial facilitators for phytoremediation in PCB polluted soil". XXI Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Portici (NA) 14-16 September 2016.

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Marasco R, Rolli E, **Vergani** L, Soussi A, Fusi M, Fodelianakis S, Booth J M, Cardinale M, Borin S, Daffonchio D. “Bacterial assemblages associated with unique root morphology of a desert plant”. BAGECO13 Conference, Milan 14-18 June 2015.

Participation to workshops and summer schools

“Mothur Workshop 2016” held by Patrick D. Schloss for the analysis of output data from high-throughput DNA sequencing technologies. Detroit, Michigan, August 31 to September 2, 2016.

Internship abroad

Five months-internship at the laboratory of Professor Ondrej Uhlik at the Department of Biochemistry and Microbiology, University of Chemistry and Technology (UCT), Prague, Czech Republic.

Teaching activities

Collaboration to the laboratory courses of microbiology for students attending the bachelor and master programmes in "Plant, food and agri-environmental biotechnology" and "Science of crop production and protection" at University of Milan.

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