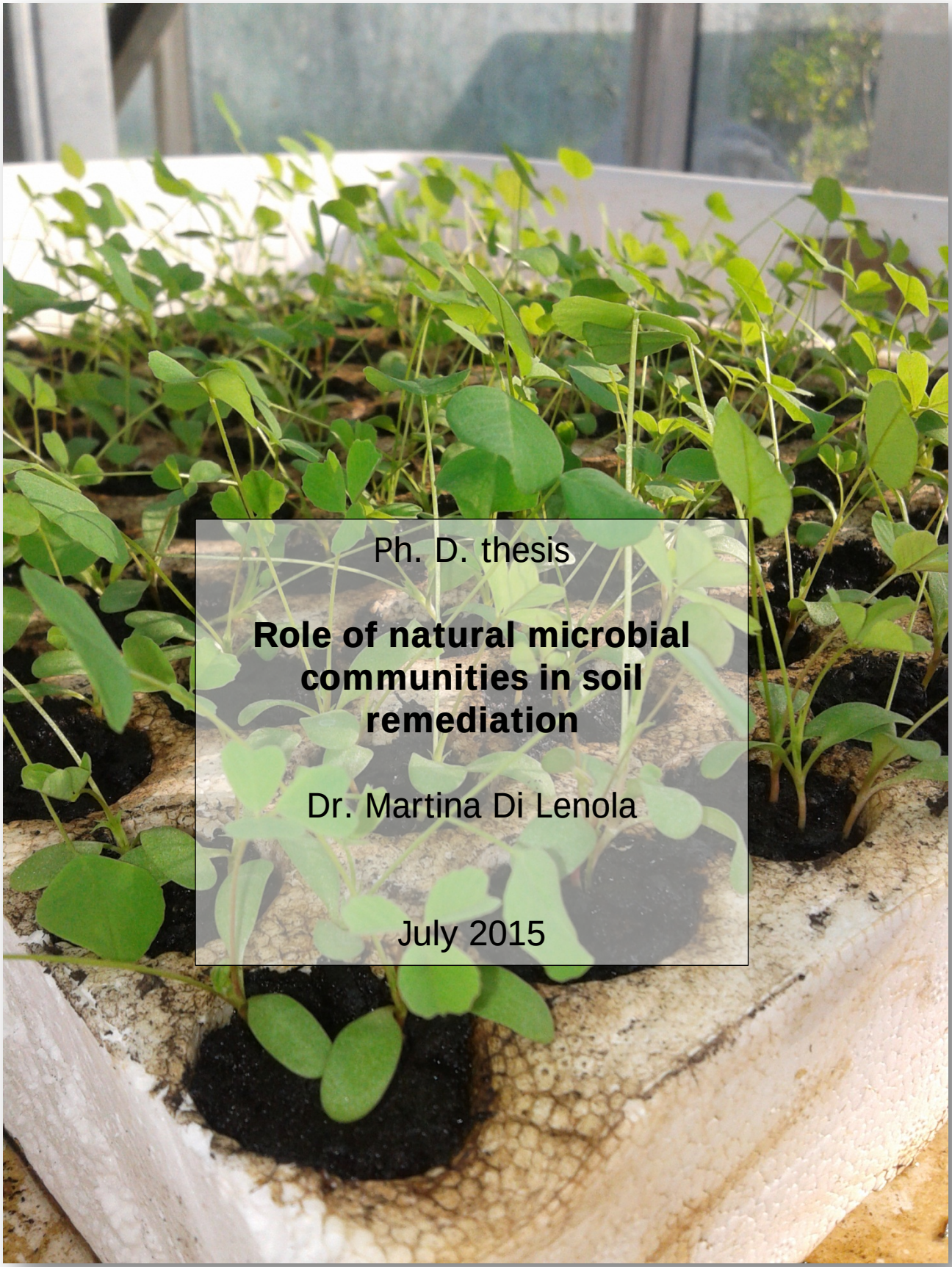

Ph. D. thesis

Role of natural microbial communities in soil remediation

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Role of natural microbial communities in soil remediation

Ruolo delle comunità microbiche del suolo nel recupero di suoli degradati

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A Francesco
che ho scelto per la vita

e a mamma e papà
che mi hanno dato e insegnato la vita

I am among those who think that science has great beauty. *A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. We should not allow it to be believed that all scientific progress can be reduced to mechanisms, machines, gearings, even though such machinery also has its beauty. Neither do I believe that the spirit of adventure runs any risk of disappearing in our world. If I see anything vital around me, it is precisely that spirit of adventure, which seems indestructible and is akin to curiosity.*

cit. Marie Curie

SUMMARY

Soil degradation is defined as a change in the soil health status resulting in a diminished capacity of the ecosystem to provide goods and services for its beneficiaries. Soil degradation is responsible for soil biodiversity loss and in turn for many ecosystem functions disappearance. Many of the functions performed by soil organisms and in particular by microorganisms can provide essential ecosystem services to human society. Most of the species in soil are microorganisms (mainly bacteria and fungi) which are considered the chemical engineers of the soil, responsible for the decomposition of plant organic matter into nutrients readily available for plants, promoting soil fertility and plant productivity. Moreover, they have a key role in soil remediation both in the bulk soil (bioremediation) and in the rhizosphere (Plant-assisted bioremediation). Different threats can act directly on soil biodiversity (e.g. chemical pollution) or indirectly, through one of the soil degradation processes (e.g. land-use change can affect soil biodiversity by favoring erosion). Soil degradation can alter plant productivity, soil resilience and resistance capacity.

Mitigation is intervention intended to reduce ongoing degradation. This comes in at a stage when degradation has already begun and the main aim is to halt further degradation and to start improving resources and their functions. The addition of organic matter, through animal or biosolid waste composted, and the restoration of vegetation are suitable strategies to recover and to improve soil condition by erosion and/or contamination. Compost and plant presence can act directly and indirectly to increase microbial diversity and to promote microbial activity and contaminant degradation.

In this study we analyzed the role of autochthonous microbial communities in recovering two kinds of degraded soils after applying compost from different sources and/or planting two different species for different purposes (e.g. *R. officinalis* for improving soil carbon content and *M. sativa* for PCB degradation). The experimental studies were performed in laboratory microcosms using samples from an agricultural soil degraded with a low organic matter content, and samples from an area chronically contaminated by persistent organic compounds (polychlorinated biphenyls, PCB).

The results of the two recovery strategies applied in this work show how the simultaneous application of compost and plant can improve the overall soil quality, promoting the structure and functioning of soil microbial communities and their capability to improve soil quality in terms both of increasing in nutrients and in contaminant decreasing.

RIASSUNTO

I fenomeni di degrado del suolo possono essere definiti come cambiamenti nello stato di salute del suolo risultanti in una riduzione della capacità dell'ecosistema di fornire beni e servizi. I fenomeni di degrado sono i principali responsabile della perdita di biodiversità del suolo e di conseguenza della scomparsa delle funzioni ecosistemiche da esso fornite. Diverse sono le minacce che possono ridurre la biodiversità del suolo sia direttamente (ad esempio attraverso la contaminazione) che indirettamente, attraverso uno dei processi di degrado del suolo (quali ad esempio cambiamenti nell' utilizzo del suolo che portano all'erosione). Alla base di tutti i servizi ecosistemi ci sono i microorganismi del suolo, in grado di fornire servizi di supporto e essenziali per la vita.

Le strategie di recupero sono interventi destinati a ridurre i fenomeni di degrado. Questo può avvenire quando fenomeni di degrado sono già in atto e l'obiettivo principale è quello di arrestare l'ulteriore degrado e di cominciare a migliorarne le qualità. L'aggiunta di materia organica, proveniente da escrementi animali o da rifiuti solidi urbani, e il ripristino o l'impianto di vegetazione, sono delle potenziali strategie per il ripristino ed il miglioramento delle condizioni del suolo. Ciò avviene non solo in modo diretto attraverso i benefici immediati forniti sia dalla pianta che dal compost ma soprattutto grazie al miglioramento della struttura e funzione delle comunità microbiche del suolo, nonché all'incremento della loro biodiversità.

Il principale obiettivo di questa attività di ricerca è stato quello di studiare il ruolo delle comunità microbiche autoctone nel recuperare suoli degradati tramite l'applicazione di compost di diversa origine e l'introduzione di specie vegetali selezionate ad hoc (*R. officinalis* per il suolo a basso contenuto di sostanza organica e *M. sativa* per il suolo contaminato da PCB).

Gli studi sperimentali sono stati effettuati in microcosmi di laboratorio utilizzando campioni provenienti da un suolo agrario degradato per basso contenuto di sostanza organica e da un'area contaminata da composti organici persistenti (Policlorobifenili, PCB).

I risultati delle due strategie di recupero proposte in questo lavoro mostrano come l'applicazione simultanea di compost e di specifiche specie vegetali sia in grado di migliorare la qualità del suolo, promuovendo le migliori condizioni per le comunità microbiche in esso presenti e quindi favorendone sia l'attività che le loro capacità di fornire funzioni ecosistemiche chiave quali il ciclo dei nutrienti e la decontaminazione.

LIST OF FIGURES AND TABLES

pp.:

FIGURES

TABLES

PART I - INTRODUCTION

1 Terrestrial ecosystem

Terrestrial ecosystem are generally classified according to main characteristics of plant communities (e.g. more or less abundance of representative species and specific life cycles depending on seasonal changes) which are affected both by abiotic conditions and presence and survival of animal communities of a specific area (Smith and Smith, 2007). Soil is a complex biological system and an extremely heterogeneous habitat, which is not uniformly occupied by soil organisms. It consists of a mosaic of inorganic minerals resulting from rock weathering, and organic material that is partly decomposed product of plants and other organisms. Soil is fundamental and irreplaceable; it governs plant productivity of terrestrial ecosystems and it maintains biogeochemical cycles because microorganisms in the soil degrade, sooner or later, virtually all organic compounds including persistent xenobiotics and naturally occurring polyphenolic compounds (Nannipieri *et al*., 2003).

It is well established that apart from plant (being the primary producers), a range of other organisms play key roles in terrestrial ecosystems. The functional importance of their diversity, however, is poorly known. Roughly, terrestrial ecosystems can be divided into an aboveground and a belowground compartment, which are tightly linked by plants. Plants acquire water and nutrients from the soil, where soil biota influence the availability and acquisition of nutrients. Living roots directly interact with mutualistic (e.g. mycorrhizal fungi, N-fixing bacteria), pathogenic (e.g. disease causing bacteria and fungi) and herbivorous soil organisms (e.g. plant-feeding insects and nematodes). Mycorrhizal fungi enhance plants' nutrient and water uptake capacity by extending the root system. N-fixing bacteria increase nutrient availability by capturing atmospheric nitrogen, while root herbivores and root pathogens reduce the root system and plant growth. Indirectly, soil organisms (e.g. saprophytic bacteria, fungi, nematodes, micro-arthropods) are crucial for plant nutrient provision by decomposing plant derived organic matter and releasing mineralized nutrients. Aboveground herbivores and pathogens may reduce plant photosynthetic capacity and biomass (De Deyn and Van der Putten 2005) (Figure 1.1).

In the present thesis, the belowground soil and its associated communities will be considered.

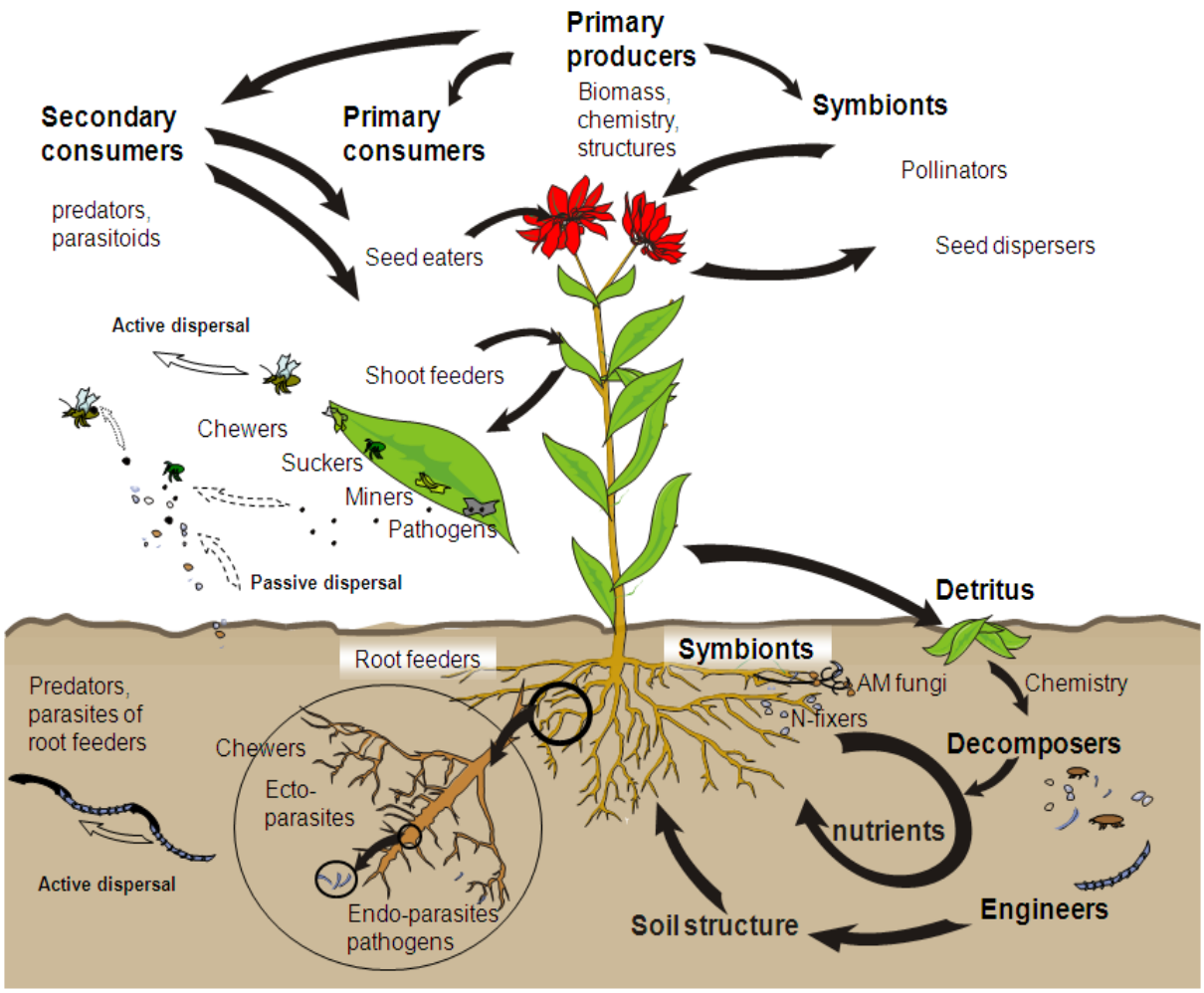

Figure 1.1- Interdependency of aboveground and belowground biodiversity. Modified from (De Deyn and Van der Putten 2005)

1.1 Biodiversity and functioning of belowground soil communities

The living population inhabiting soil includes macrofauna, mesofauna, microfauna and microflora. Soil microorganisms live within the pores left between soil particles, free or attached to surfaces, such as in water films surrounding soil particles. The pore space can be of various shapes and sizes, depending on the texture and structure of the soil. Texture characterizes the relative importance of clay (<5 μm), silt (5-50 μm) and sand particles (>50 μm). The smaller the particles is, the more space they leave between them that can be filled by water and/or soil organisms. Indeed, a high density of small pores can result in less water availability for plants and small animals due to the intrinsic physical properties of water. Soil texture also largely determines other soil characteristics, such as pH and organic matter content. Given the poor water retention capacity of sandy soils, nutrients and lime will be easily washed out, making these soils more acidic. Moreover, clay minerals can form aggregates with the humic compounds in the soil, thereby protecting organic material and affecting its availability in the soil. Soil organisms also directly modify soil architecture, creating further habitats within the pores, by building networks of solid structures.

Soils are the habitat and resource for a large part of global biodiversity: over one fourth of all living species on earth are strict soil or litter dwellers. They are home to a prodigious diversity of life, which can often be several orders of magnitude greater than that present aboveground or in the canopy of rainforests (Decaens, Jimenez *et al*. 2006). One square metre of land surface may contain some ten thousand species of soil organisms, whereas aboveground biodiversity is some orders of magnitude lower. Microorganisms such as algae, bacteria and fungi form the majority of the soil biomass (Figure 1.2). One teaspoon of soil contains several thousands of microbial species, several hundred meters of fungal hyphae, and more than one million individuals. Indeed microbial species are still largely unknown. This is one of the major differences between aboveground and belowground biodiversity. Soils also comprise a large variety of invertebrates, such as earthworms, mites, spiders, beetles, ants and termites, as well as litter-inhabiting arthropods such as millipedes, centipedes and wood lice. But the best-known soil inhabitants may well be the small mammals, such as moles and voles which can show fantastic adaptations to living in a dark belowground world.

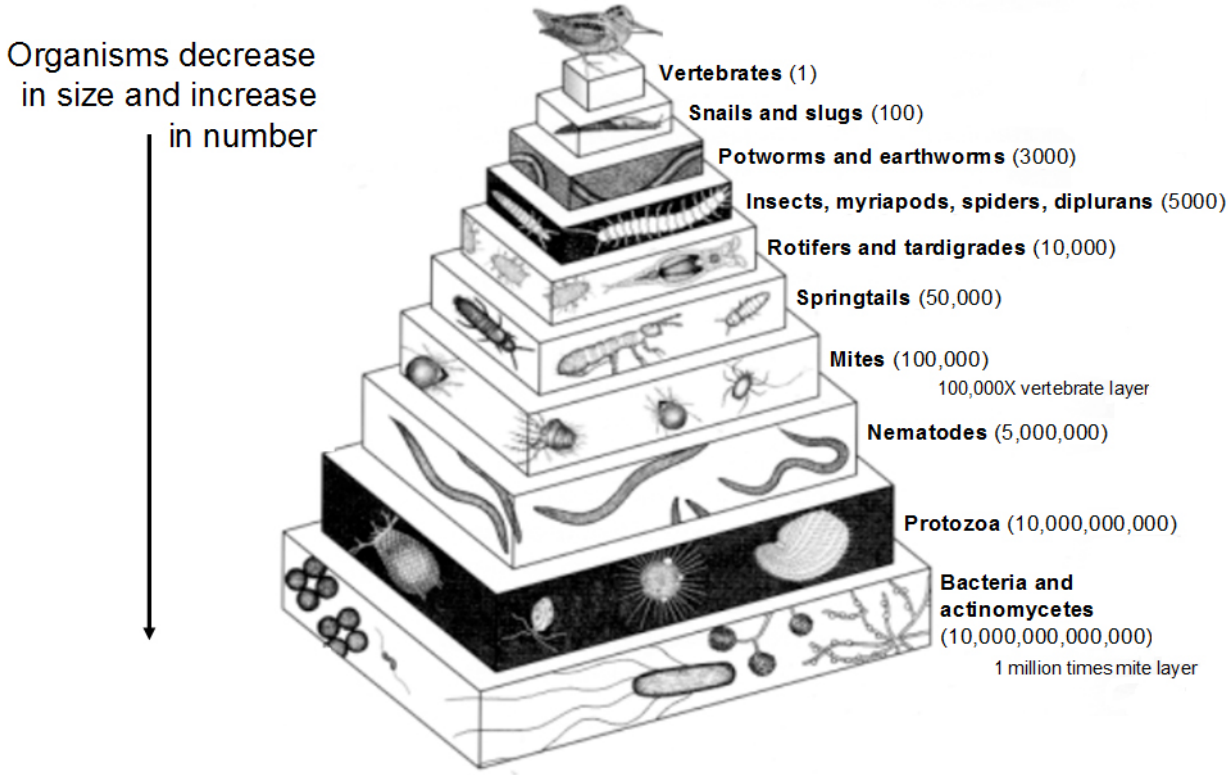

Figure 1.2 - Organisms in one square meter of soil.

Biodiversity is considered to comprise all biological variation from genes to species, up to communities, ecosystems and landscapes (MEA 2005). Soil biodiversity is the variation in soil life, from genes to communities, and the variation in soil habitats, from micro-aggregates to entire landscapes. As many species have overlapping functions, there is less functional biodiversity than taxonomic diversity.

Most of the phenomena that are observed in the visible, aboveground world are steered directly or indirectly by species, interactions, or processes in the soil (Wardle 2002; Bardgett, Bowman *et al*. 2005). With the exception of fish, all the food that we eat, the air that we breathe, clothes that we wear, and construction materials that we use, are directly or indirectly linked to soil. This is why soil biodiversity is so important for life on earth. Soil biota can regulate the structure and functioning of aboveground individuals and communities directly, by stimulating or inhibiting certain plant species more than others. Alternatively, soil organisms can regulate aboveground communities indirectly by altering the dynamics of nutrients available to plants. These indirect effects tend to involve less specific interactions and occur over longer durations than the direct regulations (Van Der Putten 2003, Wardle *et al*. 2004).

When soil organisms eat, grow, and move, they perform essential services for ecosystems, as well as for human society. Among the key ecosystem services mediated by soil biota are the

transfer, storage, and provision of clean ground water, the storage of carbon and the prevention of trace gas emissions crucial for climate control, as well as the provision of nutrients and pest and pathogen regulation, supporting plant growth and aboveground biodiversity. In fact, soil biota are involved in the provision of all the main supporting and regulating services, and the current rate of soil destruction, sealing and other threats due to the misuse of soil by humans, is threatening the sustainability of human life on earth. The responsible management of soil and its biodiversity is pivotal to sustaining human society.

1.2 Soil microbial communities and their interactions with plants

Microorganisms, Bacteria, Archaea, viruses, protists and fungi, are vital to the function of all ecosystems. This is largely because they exist in enormous numbers (there are roughly 5×10^{30} bacteria alone worldwide) and so have immense cumulative mass and activity. They are also probably more diverse than any other organisms, so it is easy to see why the structure of microbial communities, that is, the different kinds of organisms and their abundances, is so important to the way in which ecosystems function.

Understanding ecosystem function, and predicting Earth's response to global changes, calls for much better knowledge than we have today about microbial processes and interactions. Although microorganisms are perhaps the most diverse (Torsvik *et al*., 2002) and abundant type of organism on Earth, the distribution of microbial diversity at continental scales is poorly understood. With the advent of ribosomal DNA-analysis methods that permit the characterization of bacterial communities without culturing, it is now possible to examine the full extent of microbial diversity and describe the biogeographical patterns exhibited by microorganisms at large spatial scales (Norman and Pace, 1997). Scientific understanding of microbial biogeography is particularly weak for soil bacteria, even though the diversity and composition of soil bacterial communities is thought to have a direct influence on a wide range of ecosystem processes. Much of the recent work in soil microbial ecology has focused on cataloging the diversity of soil bacteria and documenting how soil bacterial communities are affected by specific environmental changes or disturbances. As a result, we know that soil bacterial diversity is immense and that the composition and diversity of soil bacterial communities can be influenced by a wide range of biotic and abiotic factors (Fuhrman, 2009).

Most bacteria live in the top 10cm of soil where organic matter is present. Some species of them are very fragile and can be killed by slight changes in the soil environment. Other species are extremely tough, able to withstand severe heat, cold or drying. Some can lie dormant for decades waiting for favorable conditions. Others can extract nitrogen directly from the air or break down some toxic substances. Populations of microbes can boom or bust in the space of a few days in response to changes in soil moisture, soil temperature or carbon substrate. To gain advantage in this process, many microbes release antibiotic substances to suppress particular competitors. In this way some species can suppress other disease-causing microorganisms.

1.2.1 Functional groups

Soil belowground diversity reflects a wide range of functional roles. A functional group is composed by different species which have similar or identical ecosystem functioning. Soil community can be classified according to different functional organization levels (Lavelle 1997; Swift *et al.*, 2004; Barrios, 2007; Kibblewhite, 2008). According to Turbè *et al* 2010, soil organisms can be divided in three functional groups (Figure 1.3):

- **Ecosystem Engineers:** organisms responsible for maintaining the structure of soil by the formation of pore networks and bio-structures, and aggregation, or particle transport. They also regulate the availability of resources for other soil organisms since soil structures become hotspots of microbial activities and affect the water infiltration and distribution in soil (e.g. earthworms, ants, termites and some small mammals).
- **Biological Regulators** organisms responsible for the regulation of population dynamics of other soil organisms, through grazing, predation or parasitism, including soilborne pests and diseases. (e.g. small invertebrates, such as nematodes, pot worms, springtails, and mites).
- **Chemical engineers** (main bacteria and fungi) responsible for organic matter decomposition and nutrient cycling readily available for plants and they regulate 90% of energy flux in soil. They play a key role in bioremediation, by accumulating pollutants in their bodies, degrading pollutants into smaller, non-toxic molecules, or modifying those pollutants into useful metabolic molecules.

It is important to highlight that the classification into functional groups is indicative of the most characteristic role of an organism, but is not rigid. For example, some biological regulators or chemical engineers (e.g. through the secretion of sticky proteins) can also act as ecosystem engineers.

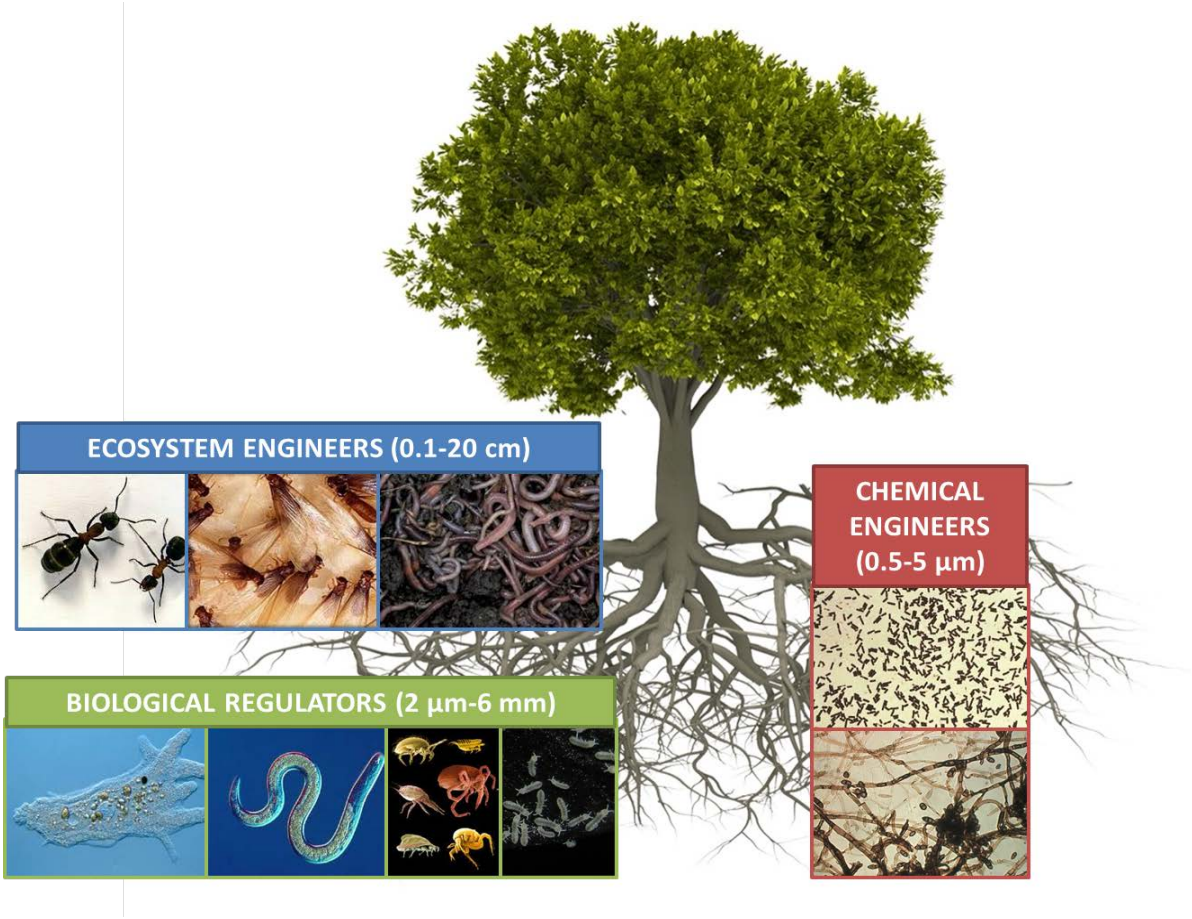

Figure 1.3 – Soil microorganisms functional groups

Table 1.1: Summary of characteristics of the three soil functional groups (modified from Turbè *et al.*, 2010)

	CHEMICAL ENGINEERS	BIOLOGICAL REGULATORS	ECOSYSTEM ENGINEERS
Main organisms	Bacteria, fungi	Protists, nematodes, mites, springtails	Ants, termites, earthworms, plants roots
Function	Organic matter decomposition, mineralization and nutrients release, pest control, toxic compounds degradation	Regulation of microbial community dynamics, faecal pellet structures, mineralization, nutrient availability regulation, litter transformation and organic matter decomposition	Creation and maintenance of soil habitats, transformation of physical state of both biotic and abiotic material, accumulation of organic matter, compaction of soil, decompaction of soil, soil formation
Body size	0.5-5 µm (bacteria) 2-10 µm (fungal hyphae diameter)	2-200 µm (protists) 500 µm (nematodes) 0.5-2 mm (mites) 0.2-6 mm (springtails)	0,1-5 cm (ants) 0.3-7 cm (termites) 0.5-20 cm (earthworms)

1.2.2 Soil bacteria

All Bacteria are decomposers playing an important role in decomposition of organic materials, especially in the early stages of decomposition when moisture levels are high. In the later stages of decomposition, fungi tend to dominate.

Nitrogen-fixing bacteria, as *Rhizobium bacteria*, live in special root nodules on legumes such as clover, beans, medic, wattles etc. They extract nitrogen gas from the air and convert it into forms that plants can use. This form of nitrogen fixation can add the equivalent of more than 100kg of nitrogen per hectare per year. *Azotobacter, Azospirillum, Agrobacterium, Gluconobacter, Flavobacterium* and *Herbaspirillum* are all examples of free-living, nitrogen-fixing bacteria, often associated with non-legumes.

A number of bacteria have been commercialized worldwide for disease suppression. *Bacillus megaterium* is an example of a bacterium that has been used on some crops to suppress the disease-causing fungus *Rhizoctonia solani*.

Aerobic bacteria need oxygen, so where soil is well drained aerobes tend to dominate. Anaerobes do not need oxygen and may find it toxic. This group includes very ancient types of bacteria that live inside soil aggregates. Anaerobic bacteria favor wet, poorly drained soils and can produce toxic compounds that can limit root growth and predispose plants to root diseases.

Actinobacteria help to slowly break down humates and humic acids in soils. They prefer non-acidic soils with pH higher than 5.

Sulfur oxidisers bacteria can covert sulfides into sulfates, a form of sulfur which plants can use.

Although molecular surveys provide essential information on the composition of soil bacterial communities, they are only the first step towards understanding the ecology of such communities. At present, we are largely unable to interpret the taxonomic survey data in an ecologically meaningful manner nor do we know why certain taxa are more abundant in some soils than in others. A select number of bacterial taxa have been well studied and their ecological characteristics are reasonably well defined. This is the case for those taxa with specific physiological capabilities, such as the ammoniaoxidizing *Nitroso-* genera, the N2-fixing *Rhizobium*, and the methane-oxidizing *Methylo-* genera. However, these taxa are the exceptions: the majority of soil bacterial taxa, even those that are numerically dominant, have not been extensively studied and their ecological characteristics remain largely unknown. For example, the phylum *Acidobacteria* is one of the most abundant taxonomic groups of soil bacteria but we know next to nothing about their physiological capabilities, habitat preferences, and life history attributes (Tringe at al., 2005; Fierer *et al*., 2007).

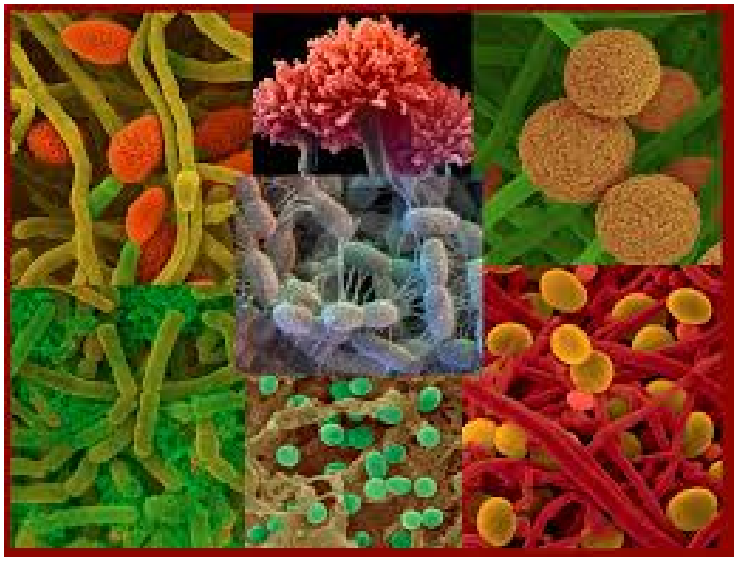

Figure 1.4 - Examples of soil bacteria (body size: 0.5-5 μm)

1.2.3 Rhizosphere bacteria

The term 'rhizosphere' was coined by Hiltner in 1904 to describe the portion of soil where microorganism-mediated processes are under the influence of the root system. The well-studied rhizosphere effect describes the phenomenon that, in comparison with bulk soil, the biomass and activity of microorganisms is enhanced as a result of exudation of compounds by the root (Sørensen, 1997; Raaijmakers *et al*., 2009). The enormous importance of plant–microorganism interactions in the rhizosphere for carbon sequestration, ecosystem functioning and nutrient cycling in natural ecosystems as well as in agricultural and forest systems (Singh *et al*., 2004), it is crucial to understand the factors influencing the microbial communities in this habitat (Figure 1.5) .

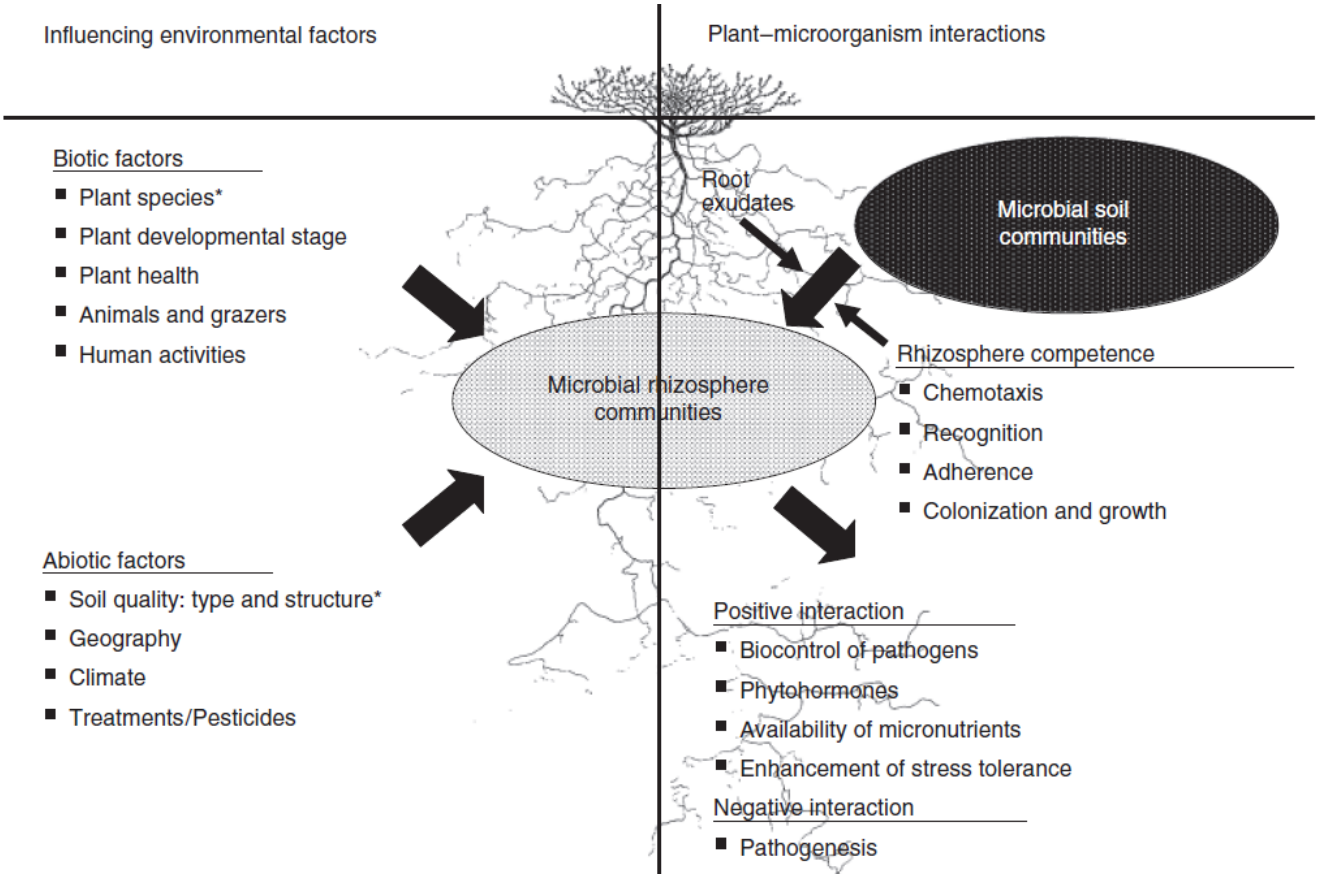

Figure 1.5 - Influencing factors of rhizosphere microbial communities and model how microbial communities were selected from soil: by root exudates and their rhizosphere competence (from Berg & Smalla 2009).

Each plant species is thought to select specific microbial populations. Root exudates are a driving force in this process, but researchers are only beginning to understand the role of single compounds in mediating belowground interactions (Bais *et al*., 2006; Haichar *et al*., 2008). The composition of root exudates varies from plant to plant and affects the relative abundance of microorganisms in the vicinity of the root (Somers *et al*., 2004). Plants not only provide nutrients for microorganisms, but some plant species also contain unique antimicrobial metabolites in their exudates. The existing huge diversity of plant species with an estimated range from 310 000 to 422 000 species (Pitman & J¨orgensen, 2002) and corresponding secondary metabolites of plants (Buchanan *et al*., 2000) affects below-ground diversity.

The composition of the Rhizosphere should be considered as resulting from a hierarchy of events. First, the soil can be considered as a microbial seed bank, and the physico-chemical properties of the soil, together with biogeographical processes, structure this community. Then, the location where plants are grown determines which indigenous biota the plant roots are exposed to. Finally, the plant species and genotype determine which members of this reservoir of microorganisms can grow and thrive in the rhizosphere.

Plant species can strongly influence the composition and activity of the rhizosphere microbiota, and differences in root morphology, as well as in the amount and type of rhizodeposits, between plants contribute greatly to this species-specific effect (Ladygina & Hedlund, 2010; Kowalchuk *et al*., 2002).

Specific metabolites released into the rhizosphere can trigger multiple responses in different soil microorganisms. For example, plant flavonoids can attract not only symbionts but also pathogens. Flavonoids also stimulate mycorrhizal spore germination and hyphal branching, and influence *quorum sensing*, as has been shown for the flavonoids naringenin from legumes and catechin from the medicinal tree *Combretum albiflorum* (Perez-Montano *et al.*, 2011). Similarly, constitutive secondary defence metabolites, such as pyrrolizidine alkaloids, can affect the rhizosphere by favouring resistant or tolerant microorganisms or, in some cases, microorganisms that metabolize these compounds (Kowalchuk & van Veen, 2006).

Many of the current insights into interactions and processes in the rhizosphere have emerged from studies on agricultural or horticultural crop plants and model species such as *Arabidopsis thaliana* and *Medicago truncatula*. However, considerable progress is also being made in understanding the microbial ecology of the rhizosphere of non-cultivated plant species in natural ecosystems and how microorganisms influence resource allocation, biodiversity and above-ground interactions with herbivores and their natural enemies. To better understand the players and processes that operate in the rhizosphere, a variety of molecular techniques, have been applied over

the past decade. At the plant community level, substantial progress has been made in studying intermingled root systems from different plant species.

Bacteria, fungi (including arbuscular mycorrhizal fungi (AMF)), oomycetes, viruses and archaea that live in the rhizosphere are attracted by and feed on rhizodeposits (nutrients, exudates, border cells and mucilage released by the plant root). Numerous studies have shown species-specific effects of plants on the composition and relative abundance of microbial populations in the rhizosphere.

Many independent studies have depicted *Proteobacteria* as dominant members of the rhizosphere microbiota. This is in line with *Proteobacteria* being generally fast-growing *r*-strategists with the ability to utilize a broad range of root-derived carbon substrates. Although a reduction of microbial diversity is often reported in the rhizosphere compared with in the bulk soil, providing a general description of the rhizosphere is difficult owing to large discrepancies between different studies, which might be due not only to biological variability, but also to practical issues related to the actual sampling of the rhizosphere (Berg & Smalla, 2009).

1.3 Ecosystem services provided by soil microbial communities

Many of the functions performed by soil organisms can provide essential services to human society. Most of these services are supporting services, or services that are not directly used by humans but which underlie the provisioning of all other services. These include nutrient cycling, soil formation and primary production. In addition, soil biodiversity influences all the main regulatory services, namely the regulation of atmospheric composition and climate, water quantity and quality, pest and disease incidence in agricultural and natural ecosystems, and human diseases. Soil organisms may also control, or reduce environmental pollution. Finally, soil organisms also contribute to provisioning services that directly benefit people, for example the genetic resources of soil microorganisms can be used for developing novel pharmaceuticals. More specifically, the contributions of soil biodiversity can be grouped under the six following categories:

- **Soil structure, soil organic matter and fertility**: soil organisms are affected by but also contribute to modifying soil structure and creating new habitats. Soil organic matter is an important 'building block' for soil structure, contributing to soil aeration, and enabling soils to absorb water and retain nutrients. All three functional groups are involved in the formation and decomposition of soil organic matter, and thus contribute to structuring the soil. It's important to underline that soil organic matter as humus can only be produced by the diversity of life that exists in soils – it cannot be man-made. When the soil organic matter recycling and fertility service is impaired, all life on earth is threatened, as all life is either directly or indirectly reliant on plants and their products, including the supply of food, energy, nutrients (e.g. nitrogen produced by the rhizobium bacteria in synergy with the legumes), construction materials and genetic resources. This service is crucial in all sorts of ecosystems, including agriculture and forestry.
- **Regulation of carbon flux and climate control**: soil is estimated to contain about 2,500 billion tonnes of carbon to one metre depth. The soil organic carbon pool is the second largest carbon pool on the planet and is formed directly by soil biota or by the organic matter (e.g. litter, aboveground residues) that accumulates due to the activity of soil biota. Land-use change, through the conversion of grasslands to agricultural lands, is responsible for the largest carbon losses from soils. Although planting trees is often advocated to control global warming through CO2 fixation, far more organic carbon is accumulated in the soil. Therefore, besides reducing the use of fossil fuels, managing soil carbon contents is one of the most powerful tools in climate change mitigation policy. The loss of soil biodiversity, therefore, will reduce the ability of soils to regulate the composition of the atmosphere, as well as the role of soils in counteracting global warming.

- **Regulation of the water cycle**: soil ecosystem engineers affect the infiltration and distribution of water in the soil, by creating soil aggregates and pore spaces. Soil biodiversity may also indirectly affect water infiltration, by influencing the composition and structure of the vegetation, which can shield-off the soil surface, influence the structure and composition of litter layers and influence soil structure by rooting patterns. The diversity of microorganisms in the soil contributes to water purification, nutrient removal, and to the biodegradation of contaminants and of pathogenic microbes. Plants also play a key role in the cycling of water between soil and atmosphere through their effects on (evapo-) transpiration. The loss of this service will reduce the quality and quantity of ground and surface waters, nutrients and pollutants (such as pesticides and industrial waste) may no longer be degraded or neutralised. Surface runoff will increase, augmenting the risks of erosion and even landslides in mountain areas, and of flooding and excessive sedimentation in lowland areas. Each of these losses can result in substantial costs to the economy. These costs can be linked to the need for building and operating more water purification plants, remediation costs, and ensuring measures to control erosion and flooding (e.g. the need to increase the height of dikes in lowland areas).
- **Decontamination and bioremediation**: chemical engineers play a key role in bioremediation, by accumulating pollutants in their bodies, degrading pollutants into smaller, non-toxic molecules, or modifying those pollutants into useful metabolic molecules (e.g. taking several months in the case of hydrocarbons, but much more for other molecules). Humans often use these remediation capacities of soil organisms to directly engineer bioremediation, whether *in situ* or *ex situ*, or by promoting microbial activity. Phyto-remediation, which is indirectly mediated by soil organisms, is also useful to remove persistent pollutants and heavy metals. Microbial bioremediation is a relatively low-cost option, able to destroy a wide variety of pollutants and yielding non-toxic residues. Microbial remediation differs from phyto-remediation in a way that it transforms the pollutant instead of accumulating it in a different compartment. The loss of soil biodiversity would reduce the availability of microorganisms to be used for bioremediation.
- **Pest control**: soil biodiversity promotes pest control, either by acting directly on belowground pests, or by acting indirectly on aboveground pests. Pest outbreaks occur when microorganisms or regulatory soil fauna are not performing efficient control. Ecosystems presenting a high diversity of soil organisms typically present a higher natural control potential, since they have a higher probability of hosting a natural enemy of the pest. Interestingly, in natural ecosystems, pests are involved in the regulation of biodiversity.

Ensuring efficient natural pest control avoids having to use engineered control methods, such as pesticides, which have both huge economic and ecological costs. Loss of this ecosystem service will cause loss of biodiversity in entire natural ecosystems.

- **Human health**: soil organisms, with their astonishing diversity, are an important source of chemical and genetic resources for the development of new pharmaceuticals. Many antibiotics used today originate from soil organisms. Given that antibiotic resistance develops fast, the demand for new molecules is unending. Soil biodiversity can also have indirect impacts on human health. Land-use change, global warming, or other disturbances to soil systems can release soil-borne infectious diseases and increase human exposure to those diseases. Loss of soil biodiversity, therefore, could reduce our capacity to develop novel antibiotic compounds, it could enhance the risk of infectious diseases.

1.4 Main causes of soil degradation and biodiversity threats

As soil formation is an extremely slow process, soil can be considered essentially as a non-renewable resource. Soil provides us with food, biomass and raw materials. It serves as a platform for human activities and landscape and as an archive of heritage and plays a central role as a habitat and gene pool. It stores, filters and transforms many substances, including water, nutrients and carbon. In fact, it is the biggest carbon store in the world (1,500 giga tonnes). These functions, guaranteed by biodiversity, must be protected because of both their socio-economic and environmental importance.

Soil's structure plays a major role in determining its ability to perform its functions. Any damage to its structure also damages other environmental media and ecosystems.

Different threats can act directly on soil biodiversity (e.g. chemical pollution) or indirectly, through one of the soil degradation processes (e.g. land-use change can affect soil biodiversity by favouring erosion). Soil degradation can alter productivity, soil functioning, and all related services. The serious problem of soil degradation is driven or exacerbated by human activity such as inadequate agricultural and forestry practices, industrial activities, urban and industrial sprawl and construction works. These activities have a negative impact, preventing the soil from performing its broad range of functions and services to humans and ecosystems and this results in loss of soil fertility, carbon and biodiversity, lower water-retention capacity, disruption of gas and nutrient.

The main soil degradation processes that impact on soil biodiversity are erosion, salinization, compaction, sealing, organic matter depletion and local and diffuse contamination, detailed below.

Soil **erosion** is normally a natural process occurring over geological time scales. Natural soil erosion can be significantly accelerated by anthropogenic activity. Practices that involve deforestation, exposing bare soil to water and wind, the use of deep tillage or mineral fertilisation enhance water run-off and wind action, which triggers erosion (Lal and Kimble 1997; Heisler *et al*. 1998). Factors such as soil characteristics and climate (e.g. long drought periods followed by heavy precipitation), can also favour the acceleration of human-driven erosion. The direct effect of erosion is the degradation of the upper layer of the soil and the decrease of soil organic matter content. As a result, the nutrient availability for soil organisms is diminished, their biomass is reduced, and probably their diversity also (Pimentel *et al*. 1995). Erosion can also have indirect effects on soils and their services, through reducing plant diversity, standing biomass and productivity. In addition, erosion leads to enhanced carbon emissions due to breakdown of soil structure and exposure of carbon in aggregates. In turn, erosion-induced reductions in plant diversity and abundance can reduce soil biodiversity. The Mediterranean region is particularly sensitive to erosion because of its climatic conditions and the nature of its soils.

Salinisation is the accumulation of water-soluble salts in the soil. This process can be natural or human-induced. In general, inappropriate irrigation practices, such as use of

saline water and soil characteristics which inhibit salt washing are at the origin of the problem. Soil salinisation can also be triggered by the over-exploitation of groundwater in coastal areas, which leads to the infiltration of salty marine water. Moreover, marine storms can potentially increase soil salinisation in coastal areas. Salinity is a global threat for soils. In Europe, between 1 and 3 million hectares are affected by this degradation process and in the Mediterranean region, Spain, the Caspian Basin and the Carpathian Basin are the most affected areas.

Soil **compaction** is a type of physical degradation due to the reorganisation of soil micro and macro aggregates, which are deformed or even destroyed under pressure. Compaction results in poor drainage, sub-surface gleying, etc. Soils can be naturally compacted at various degrees, and their natural compaction rate can be further increased by trampling or heavy machinery. The sensitivity of soils to compaction depends on soil properties, such as texture and moisture, organic carbon content, and on several external factors such as climate and land use. The first direct impact caused by soil compaction and the consequent reduction of soil porosity is the reduction of available habitats for soil organisms. This affects in particular soil organisms living in surface areas, such as earthworms. Soil compaction areas are continuously increasing in Europe due to the use of heavier wheel pressure in agriculture.

Soil **sealing** is the process through which soils are covered by an impermeable layer, which impedes exchanges between aboveground and belowground worlds. This phenomena naturally occurs when fine particles form a surface crust that may impede water infiltration in the deeper soil layers. Sealing can lead to a slow death of most soil organisms. Soil biota can initially survive on the moisture and organic matter that was present in the soil before sealing, until these resources are exhausted. Then, soil bacteria enter an inactive state and larger soil fauna may either disperse or, when sealing covers vast areas, die off. Human-driven sealing often concerns soils that are rich in nutrients and valuable for agricultural production, because rich soils are close to urban areas and, therefore, more readily subject to the pressure of expanding urban areas. Moreover, sealing contributes to the elimination of ‘buffer zones’. These are the semi-natural zones between urban and agricultural areas which connect natural ecosystems. Thus, soil sealing and degradation by urbanisation may also affect surrounding natural areas through habitat fragmentation.

1.4.1 Organic matter depletion

Soil organic matter is the fraction of the soil that includes plant and animal residues at various stages of decomposition, plant roots, cells and tissues of soil organisms and substances synthesised by the soil population. Organic matter is largely made up of the elements carbon, hydrogen, oxygen, and nitrogen and also phosphorus and sulphur. It is expressed as a percentage of the soil mass less than 2 mm in diameter. Soil organic matter is a dynamic mixture that reflects the balance between additions of new organic matter and losses of organic matter already in the soil. Soil organic matter exists in various forms which differ in their biodegradability or resistance to decomposition. It is generally divided into three pools: active, intermediate or slow, and recalcitrant or resistant. The active pool includes microbial biomass and labile organic compounds that make up less than 5 percent of the soil organic carbon. The slow pool usually makes up 20 to 40 percent These three pools have different rates of turnover. The active pool ranges from months to years, the slow pool in decades, and the recalcitrant pool in hundreds to thousands of years. The active and part of the intermediate pools are involved in nutrient supply and in the binding of small soil particles together to form larger structural units called aggregates. Aggregation is important for water infiltration, aeration and drainage, and reduces the soil's susceptibility to erosion. On the other hand, the recalcitrant pool or humus possesses a large quantity of negative charges and contributes largely to the nutrient holding capacity (cation exchange capacity) of the soil. It also imparts a dark colour to topsoil.

Organic matter decomposition is a microbiological process which releases inorganic forms of nutrients such as nitrogen (N) phosphorus (P) and sulphur (S) which slowly becomes available for plant use. The humus which develops as an intermediate product of this decomposition also acts as a store for nutrients. Soil organic carbon is generally highly correlated with total nitrogen. Therefore, the amount of N mineralisation (i.e. conversion of organic N compounds to ammonium-N) increases as soil organic carbon increases. Microorganisms associated with soil organic matter may also rapidly decompose soil-applied organic chemicals. Adding organic matter to the soil contributes a certain level of sequestration of carbon from the atmosphere.

The critical quantity of organic matter ensuring an optimal soil fertility has not yet been defined (Korschens *et al*. 1998) but it is fundamental for soil health. These thresholds will be highly context dependent. Many natural processes determine the quantity of soil organic matter, for example temperature, precipitation, wind or rain intensity can contribute in the distribution of soil organic matter in the landscape, but also land cover and/or vegetation type and topography are factors that have an influence on organic matter accumulation.

Organic matter depletion may also be affected by anthropogenic processes, such as:

- Conversion of (semi-)natural ecosystems to agriculture and changes in land use (the conversion of natural to agricultural ecosystems usually causes depletion of 50 to 75% of the previous soil carbon pool).
- Deep ploughing leads to organic matter dilution within soil. Agricultural ecosystems generally contain less soil organic carbon (SOC) than their potential capacity because of the severe losses due to accelerated erosion and leaching (Lal 2005) and because of the increased respiration rate in ploughed soils, due to the enhanced aerobic status of deeper soil layers.
- Soil erosion
- Leaching of nutrients from soil to water (e.g. due to excessive irrigation)
- Artificial removal or decrease of litter due to land conversion (e.g. deforestation)
- Forest fires
- Over-grazing

As an important source of soil fertility and soil structure, SOM depletion leads to a decrease in soil fertility and in soil biota biomass with significant consequences for biodiversity. A large fraction of European soils (45%) has very low organic matter content, between 0 and 2% (Citeau *et al.*, 2008), as shown in Figure 1.6 .

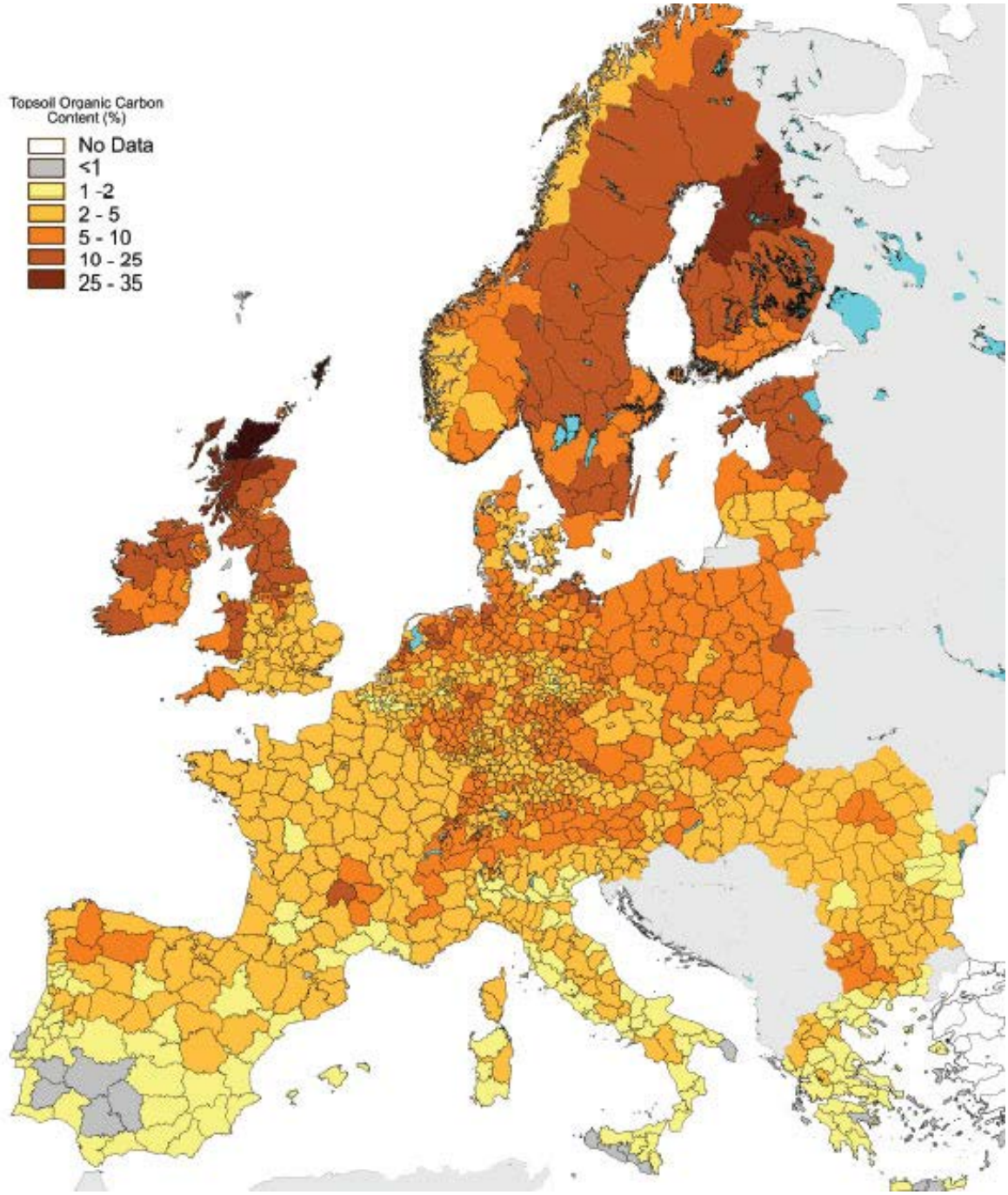

Figure 1.6 - Organic carbon content in European soils (from Turbè *et al*., 2010)

1.4.2 Anthropogenic contamination

A large range of chemical pollutants can reach the soil through various routes (direct application, atmospheric fall out, waste disposal, etc.) and influence the functioning of soils on a wide spatio-temporal scale, from individual organisms to landscapes. The deleterious impacts of pollutants on soils depend on their chemical characteristics, their persistency and toxicity that can act directly or indirectly. In addition, some molecules can bio-accumulate, which means that they can be concentrated in the bodies of soil organisms and taken up into higher levels of the food chain. A number of industrial chemicals can pollute the land near their manufacturing sites or be transported as gaseous emissions or through water to other soils (TGD 2003). The industrial chemicals which can constitute a threat for soil biodiversity include, for example, heavy metals, inorganic gaseous emissions (e.g. NO2), persisting oil and fats (e.g. petroleum) and the polychlorinated biphenyls (PCBs) which can be bio-accumulated by soil organisms.

The pollution of European soils is mostly a result of industrial activities and of the use of fertilizers and pesticides. Toxic pollutants can destabilize the population dynamics of soil organisms, by affecting their reproduction, growth and survival, especially when they are bio-accumulated. In particular, accumulation of stressing factors is devastating for the stability of soil ecosystem services. Pollutants may also indirectly affect soil services, by contaminating the belowground food supply and modifying the availability of soil organic matter. Microorganisms, which have a very short reproduction time, can develop fast resistance to toxic chemicals.

Holistic approaches, that investigate the impacts of chemical pollutants on soil ecosystem functioning as a whole are still lacking and only recently started to be covered in ecological risk assessments. Chemical pollutants can strongly alter the ecology and the physiology of bacteria and fungi. Several studies have demonstrated the effects of pollutants on microbial survival and growth, on microbial respiration and on the enzymatic activity.

Moreover, the characteristics of a chemical pollutant in soils can be altered by the action of soil organisms and by the presence of other pollutants. For example, Cadmium can be present as an impurity in certain phosphate fertilizers, and can be captured by hyperaccumulator bacteria species like *Thlaspi caerulescens* or by fast-growing plants, such as *Salix* and *Populus* spp. that accumulate above-average concentrations of only a smaller number of the more mobile trace elements, including Cadmium.

In some cases contaminants, such as pesticides, can surprisingly favour microbial growth. This occurs for example in the case of Fosthiazate, which, being an organophosphate, may serve as an energy source for microorganisms (Eisenhauer *et al*. 2009; Sturz and Kimpinski 1999; Cycon *et al*. 2006).

When considering the impacts of chemical pollution on soil microorganisms, some important aspects should be taken into account: a single chemical can have different effects on different soil microbial species and communities, disturbing the interactions within and among functional groups; microbial organisms have a very short reproduction time (e.g. an average of 20 minutes for bacteria in optimal conditions), thus an exposition to some toxic chemical could rapidly lead to a resistant microbial population. Chemical resistance evolves via natural selection acting upon random mutation. Thanks to this process, heritable traits (genes) codifying for such resistance and making it more likely for a microbial organisms to survive and successfully reproduce, become more common in a population over successive generations. Furthermore, bacteria have an additional mechanism that can facilitate the development of a chemical resistant population. Once a gene carrying the information for the resistance is generated, bacteria can transfer the genetic information in a horizontal fashion (between individuals) by DNA exchange.

On the other hand, the positive aspect of chemicals-microorganisms interaction is that some chemicals can be transformed by soil microorganisms into non- or less toxic compounds (Bioremediation).

Soil pollution is a major and acute problem in many areas of the EU, and all alternatives to bioremediation (physical removal, dilution, and treatment of the pollutants) are both technically complex and expensive. Microbial bioremediation is a relatively low-cost option, able to destroy a wide variety of pollutants and yielding non-toxic residues. Moreover, the microbial populations regulate themselves, such that when the concentration of the contaminant declines so does their population. However, to date, microbial bioremediation cannot be applied to all contaminants and remains, in some case, a long-term solution. Microbial remediation differs from phyto-remediation in a way that it transforms the pollutant instead of accumulating it in a different compartment. The loss of soil biodiversity would reduce the availability of microorganisms to be used for bioremediation.

2 Recovery strategies for degraded soils

Soil degradation is defined as a change in the soil health status resulting in a diminished capacity of the ecosystem to provide goods and services for its beneficiaries. Degraded soils have a health status such, that they do not provide the normal goods and services of the particular soil in its ecosystem.

Mitigation is intervention intended to reduce ongoing degradation. This comes in at a stage when degradation has already begun and the main aim is to halt further degradation and to start improving resources and their functions. Mitigation impacts tend to be noticeable in the short to medium term: this then provides a strong incentive for further efforts. Mitigation of degraded soils include sustainable recovery strategies that aim to restore the functions of healthy ecosystems and that can contribute to maintaining, supporting and enhancing natural systems and the essential services they provide.

The addition of organic matter, through animal or biosolid waste composted, and the restoration of vegetation are optimal choices to restore and improve soil condition. The utilization of compost is a common sustainable practice able to increase directly carbon content of soil and to affect positively the microbiological and biochemical parameters (microbial biomass C, basal respiration and different enzymatic activities). Together with the application of compost, the establishment of appropriate plant species is an important way to stabilize and/or recover soil degraded by erosion or contamination. This occurs not also directly, respectively through the root system or the well-known mechanisms of phytoremediation, but mainly through the improvement of the soil microbial community and increasing the soil biodiversity.

2.1 Application of organic compost

After the Kyoto Protocol (1997) and the management of organic waste regulations of the European Union (2002), the theme of environmental protection and human and animal health has become imperative. Along with this awareness, has been fundamental to develop economic and environmental sustainable solutions for the treatment and utilization of animal wastes and other organic residues, such as municipal solid waste. For this reason the application of different kind of compost to recover degraded soils is becoming a common practice (Bustamante *et al*., 2010). The positive effects of the application of compost on soil are attributable to the contribution of minerals and organic matter humified that improve the state of aggregation and soil porosity and increase the microbial activity and biomass (Ros *et al*., 2003; Tejada *et al*., 2006, Bastida *et al*., 2008). The biological treatment processes of organic waste consist in the decomposition of the organic fraction of the waste by different groups of microorganisms, with the consequent mineralization of organic components more easily degradable, the mass reduction and the partial sanitizing. The anaerobic treatment is one of the most efficient biological methods for the exploitation of organic waste and it is a potential source of both energy and matter. The composting of organic waste is a biomass decomposition process operated by microorganisms in aerobic conditions, with the consequent partial mineralization and humification of organic substance, that led to a final biologically stable product, the compost, with agricultural suitable property (Zucconi and de Bertoldi, 1987).

Examples of organic materials typically used to produce compost include yard waste, manure, and biosolids. Mature compost, which has gone through a time and temperature dependent process, is made of stable organic matter. Further decomposition by microbes after the compost has been applied to the soil releases nutrients slowly and makes the nutrients available for plant uptake. In the Figure 2.1 is shown how compost interacts with the soil in several ways.

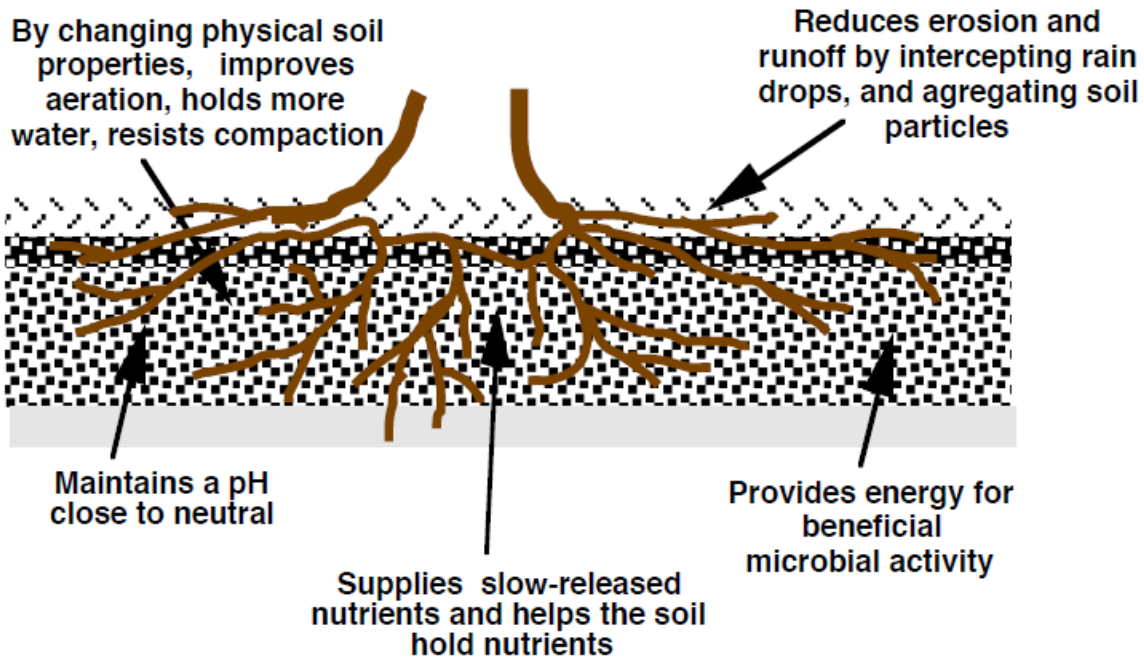

Figure 2.1 - Soil-Compost interaction

- **Compost improves the physical soil characteristics:** the addition of organic matter to soil has been shown to improve water-holding capacity, cation exchange capacity, aggregation and bulk density, buffers pH changes, and increase microbial diversity and activity (Hudson 1994, Brady and Weil 2000, Singer and Munns 2002). In clay textured soils, compost reduces the bulk density and increases the porosity of soils, thus improves the exchange of air and water through the soil. In soils that are predominantly sand, compost will increase the water holding capacity and soil aggregation.
- **Compost supplies macro and micronutrients:** a mature compost can supply virtually every nutrient needed for plant growth in an available form, especially nitrogen. Composting alters the availability of nutrients from the raw materials: during the process, available nutrients (or those that become available due to decomposition) are used by microbes decomposing the carbon-rich bulking agent (e.g., sawdust or yard trimmings). This can be considered a positive attribute of compost, however, as it allows a higher application rate - more organic matter can be incorporated as part of the renovated soil. The nutrients available for plant growth are dependent upon the characteristics of the bulking agent and the stability of the compost. Sawdust has virtually no nitrogen, and in decomposition of it, a significant amount of nitrogen is needed. In contrast, yard trimmings (especially if grass clippings are present) have a fair amount of nitrogen, usually need no nitrogen from other

materials, and in some cases release some of their nitrogen into the soil. A truly stable compost has reached an equilibrium between carbon and nitrogen. That is, there will be neither great demand for nitrogen, nor considerable nitrogen released from the compost. The time required to reach this equilibrium and for a compost to become mature varies for different composts.

- **Compost contributes organic matter:** compost adds organic matter to the soil that stores and slowly releases nutrients; it has a high moisture holding capacity; it enhances movement of water through the soil; and it has a high cation exchange capacity (i.e., it attracts and retains cations - positive charged nutrients). Additionally, the organic matter in compost greatly influences aggregate formation and stability. Where organic matter is present, the soil particles are "bound" together, i.e., aggregated, and voids are present in the aggregated soil compared to the soil low in organic matter. These voids are the "pipes" for water flow (percolation). Aggregation also decreases erosion. Sediment movement by rainwater is reduced when soil particles are larger. Organic matter which binds soil particles together, greatly reduces the potential for movement of these particles into streams. Organic matter not only retains (absorbs) more water than soil, but can also increase permeability of soil by increasing pore space in the soil. This means that the water will pass through the soil rather than flow over the surface of the soil.
- **Compost supplies beneficial microorganisms to soil:** organic matter is the energy source for soil microorganisms and the population of fungi, actinomycetes, and bacteria increases with the addition of compost. They also compete with soil pathogens. Moreover, in a number of cases it has been shown that use of compost suppresses plant diseases as described in (Henry 1991; Hoitink *et al*., 1991)

The long-term goal of ecosystem restoration is best achieved through soil treatments that favor the succession and maintenance of native communities. Because application of organic residues is a cheap, efficient and sustainable treatment, this is considered one method for recovering degraded soils in semiarid regions, in order to improve soil characteristics, thereby enhancing biogeochemical nutrient cycling.

In recent years, the application of organic wastes with high organic matter content, such as fresh and composted urban wastes (Garcia *et al*., 2000; Ros *et al*., 2003) and sewage sludge (Moreno *et al*., 1999) to semiarid soils has become a common environmental practice for maintaining soil organic matter, reclaiming degraded soils, and supplying plant nutrients. However, the influence of organic matter on soil properties depends on amount, type and size of added organic materials

(Nelson and Oades, 1998; Barzegar *et al*., 2002).The effect of each organic material on soil properties depends on its dominant component (Tejada *et al*., 2006).

2.2 Phyto-assisted bioremediation

Phyto-assisted bioremediation is a promising technique for in situ treatment of contaminated soils, based on the complex interactions that occur in the rhizosphere between plant roots and microorganisms (Wenzel *et al*., 2009). The complexity and heterogeneity of multiply polluted "real world" soils will require the design of integrated approaches of rhizosphere management. Bioremediation, i.e. the use of living organisms to manage or remediate polluted soils, is an emerging technology. It is defined as the elimination, attenuation or transformation of polluting or contaminating substances by the use of biological processes. Initially, bioremediation employed microorganisms to degrade organic pollutants, but since the use of green plants was proposed for in situ soil remediation (Baker *et al*. 1991; Salt *et al*. 1995), phytoremediation has become an attractive topic of research and development, based on the following different processes (Salt *et al*. 1995; Wenzel *et al*. 1999):

- Phytostabilisation (and immobilisation) is a containment process using plants—often in combination with soil additives to assist plant installation—to mechanically stabilising the site and reducing pollutant transfer to other ecosystem compartments and the food chain;
- Phytoextraction is a removal process taking advantage of the unusual ability of some plants to (hyper-)accumulate metals/metalloids in their shoots;
- Phytovolatilisation / rhizovolatilisation are removal processes employing metabolic capabilities of plants and associated rhizosphere microorganisms to transform pollutants into volatile compounds that are released to the atmosphere;
- Phytodegradation / rhizodegradation refer to the use of metabolic capabilities of plants and rhizosphere microorganisms to degrade organic pollutants.

Plant-assisted bioremediation is a green recovery strategy that combine bioremediation and phytoremediation but puts attention on belowground soil and on the big capabilities that soil microorganisms develop in presence of specific plants. This technique has great potential because exploits the natural capability of the autochthonous microbial communities in soil, adapted to the contaminant, to resist and degrade pollutants. This capability is selectively enhanced by the intense activity of the plant in soil.

Plant-assisted bioremediation is considered as an environmentally friendly, gentle management option for polluted soil as it uses solar-driven biological processes to treat the pollutant. In contrast to most other remediation technologies, it is not invasive and, in principle, delivers intact, biologically active soil.

The selection of plants focus on pollutant tolerance and root architecture, morphology and biomass, because the extension of root system, both superficially and in depth, is fundamental to create a deep and effective rhizosphere (Siciliano and Germida, 1998*)*.

In addition, it is well established that bioavailability is one of the most limiting factors in bioremediation of persistent organic pollutants in soil. Soils are heterogeneous environments (Hinsinger *et al*. 2005) where microsites and niches hosting microorganisms with degradation capabilities are often separated from micropores containing the pollutant. Frequently, a substantial proportion of the pollutant is not accessible for the degrader community. Therefore, interconnected soil factors such as porosity, water content and diffusivity controlling the transport of water and solutes are important controls of pollutant bioavailability. In Phyto-assisted bioremediation, this problem is generally exceeded by the presence of a root system. Roots create pores which can improve connectivity and diffusivity of the soil (Young and Crawford 2004) thus facilitating mass flow and diffusion of water and pollutants. Roots induce transpiration-driven mass flow towards the rhizosphere, delivering dissolved pollutant compounds to sites of increased microbial activity (Ferro *et al*. 1994). Roots can also serve as a carrier of (degrader) microorganisms through the soil, thus extending the contact between degraders and the pollutant (Gilbertson *et al*. 2007). Some microorganisms may also actively fetch the pollutant via chemotaxis (Valenzuela *et al*. 2006). The exudation of biosurfactants, i.e. small molecules with a hydrophilic head and a hydrophobic tail, by roots (Read *et al*. 2003) and, perhaps more importantly, associated microorganisms (Bento *et al*. 2005) may mobilise hydrophobic pollutants from soil particle surfaces, enabling their transport to sites of high degradation activity.

Plant– degrader interactions that are thought to be most relevant for the success of phyto-assisted bioremediation are depicted in Figure 2.2 and described in the below.

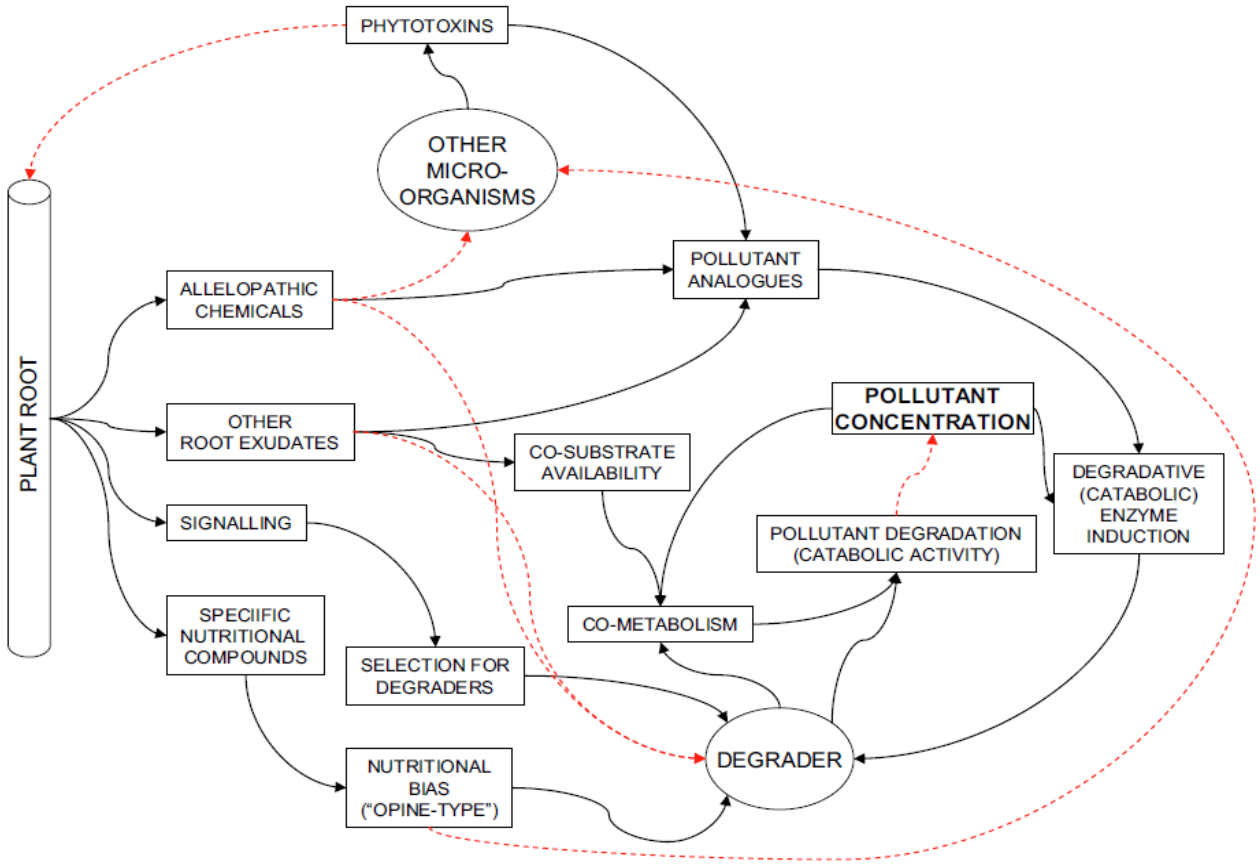

Figure 2.2 – Plant degrader interactions that are thought to be most relevant for the success of phyto-assisted bioremediation

Mechanisms of non-specific stimulation potentially involved in pollutants degradation include exudates that serve as analogues or co-metabolites of organic pollutants (Siciliano and Germida 1998). Pollutant analogues include various known root exudates and allelopathic chemicals excreted by plants in response to pathogen attack in the rhizosphere. Allelopathic chemicals released by plant roots can induce catabolic enzymes in degrader microorganisms and thus induce/enhance degradation of structurally similar pollutants. Allelopathic compounds include flavonoids, as well as other chemicals, e.g. salicin, hirsutin, 2 (3H)-benzoxazolinone or cyanide (Dzantor 2007; Siciliano and Germida 1998). Other pollutant analogues may be derived from unspecific exudation of compounds such as acetylene, biphenyl, p-coumaric acid, morin or palmatic acid (Siciliano and Germida 1998). For instance, it has been demonstrated that polychlorinated biphenyl (PCB) degradation and growth of PCB-degrading bacteria was enhanced by the flavonoids apigenin and naringin (Fletcher and Hedge 1995). Plant roots exude compounds that can serve as cometabolites in microbial pollutant degradation (Hedge and Fletcher 1996). This is important especially where microorganisms cannot utilize the pollutant as a sole carbon source as for instance in the aerobic degradation of trichloroethylene (Hyman *et al*. 1995).

2.3 European legislation on soil protection

The EU thematic strategy for soil protection puts forward measures to protect soil and to preserve its capacity to perform its functions in environmental, economic, social and cultural terms. The strategy includes setting up a legislative framework for the protection and sustainable use of soil, integrating soil protection into national and EU policies, improving knowledge in this area and increasing public awareness. The proposal for a Directive is a key component of the strategy, which enables Member States to adopt measures tailored to their local needs. It provides for measures to identify problems, prevent soil degradation and remediate degraded soil. The measures included in the proposal for a Directive include obligatory identification by Member States of areas at risk of erosion, organic matter decline, compaction, salinisation and landslides, or where the degradation process is already underway. This will be done on the basis of criteria set out in the proposal. Member States must then set objectives and adopt programs of measures to reduce these risks and to address the effects they have. They must also take steps to limit soil sealing, notably by rehabilitating brownfield sites and, where sealing is necessary, to mitigate its effects. The proposal for a Directive also provides for Member States taking appropriate measures to prevent soil contamination by dangerous substances. They must draw up a list of sites polluted by dangerous substances when concentration levels pose a significant risk to human health and the environment, and of sites where certain activities have been carried out (landfills, airports, ports, military sites, activities covered by the IPPC Directive, etc.).

The proposal contains a list of these potentially polluting activities. When these sites are sold and the transaction is made, the owner or potential buyer must submit a report to the competent national authorities and the other party on the state of the soil. This report is produced by a body of person authorized by the Member State. Member States must then remediate the polluted sites in line with a national strategy setting out the priorities. Where it is not possible for the person responsible to sustain the cost of remedying the site, the Member State concerned must make provisions for the appropriate financing.

Below are shown some paragraphs of the "**Thematic Strategy for Soil Protection"** (Commission of The European Communities, Brussels, 22.9.2006)

"Different Community policies contribute to soil protection, particularly environment and agricultural policy. Since the adoption of the 2002 Communication (COM(2002)179), an effort has been made to ensure that recently adopted environment policy initiatives on waste, water, air, climate change, chemicals, flooding, biodiversity and environmental liability will contribute to

improving soil protection. In particular, the Directive on environmental liability (Directive 2004/35/EC (OJ L 143, 30.4.2004, p. 56)) creates a harmonized framework for the liability regime to be applied across the EU when land contamination creates a significant risk of human health. However, it does not apply to historical contamination or to damage prior to its entry into force. There are a variety of approaches to soil protection in the Member States. Nine Member States have specific legislation on soil protection. However, these laws often cover only one specific threat, such as soil contamination and do not always provide a coherent protection framework."

"The Protocol on Soil Protection under the Alpine Convention, seeks to preserve the ecological functions of soil, prevent soil degradation and ensure a rational use of soil in that region. The Kyoto Protocol highlights that soil is a major carbon store which must be protected and increased where possible. Carbon sequestration in agricultural soils by some land management practices can contribute to mitigating climate change. The European Climate Change Programme (ECCP) Working Group on Sinks Related to Agricultural Soils estimated this potential at equivalent to 1.5 to 1.7% of the EU's anthropogenic CO2 emissions during the first commitment period7 under the Kyoto Protocol. The Convention on Biological Diversity (CBD) identified soil biodiversity as an area requiring particular attention. An International Initiative for the Conservation and Sustainable Use of Soil Biodiversity has been established. Several countries, including the USA, Japan, Canada, Australia, Brazil and several developing countries have established soil protection policies which include legislation, guidance documents, monitoring systems, identification of risk areas, inventories, remediation programs and funding mechanisms for contaminated sites for which no responsible party can be found. Such policies ensure a comparable level of soil protection to the approach endorsed by this strategy."

"Ensuring sustainable use of soil Against this background, the Commission considers that a comprehensive EU strategy for soil protection is required. This strategy should take into account all the different functions that soils can perform, their variability and complexity and the range of different degradation processes to which they can be subject, while also considering socio-economic aspects. The overall objective is protection and sustainable use of soil, based on the following guiding principles:

(1) Preventing further soil degradation and preserving its functions:

– when soil is used and its functions are exploited, action has to be taken on soil use and management patterns, and

– when soil acts as a sink/receptor of the effects of human activities or environmental phenomena, action has to be taken at source.

(2) Restoring degraded soils to a level of functionality consistent at least with current and intended use, thus also considering the cost implications of the restoration of soil."

"To achieve these objectives, action is required at different levels – local, national and European. Action at European level is a necessary addition to the action by Member States, given that:

- Soil degradation affects other environmental areas for which Community legislation exists. Failure to protect soil will undermine sustainability and long term competitiveness in Europe. Indeed, soil is interlinked with air and water in such a way that it regulates their quality. In addition soil functions enormously contribute to areas such as biodiversity and marine protection, coastal management, and to the mitigation of climate change.
- Distortion of the functioning of the internal market – the wide differences between national soil protection regimes, in particular as regards soil contamination, sometimes impose very different obligations on economic operators, thus creating an unbalanced situation in their fixed costs. The absence of such regimes and the uncertainty as regards the extent of soil degradation can, in some cases, also hinder private investment.
- Transboundary impact – soil, though generally immobile, is not completely so and therefore degradation in one Member State or region can have transboundary consequences. Losses of soil organic matter in one Member State impair achievement of the EU's Kyoto Protocol targets. Dams are blocked and infrastructure is damaged downstream by sediments from massive erosion further upstream in another country. Groundwater in bordering countries is polluted by contaminated sites on the other side of the border. Therefore it is of outmost importance to act at source to prevent damage and subsequent remedial actions, otherwise costs to restore environmental quality may be borne by another Member State.
- Food safety – uptake of contaminants in the soil by food and feed crops and some food producing animals can have a significant impact on the safety of feed and food, which are traded freely within the internal market, by increasing their level of contaminants, hence posing a risk to human or animal health. Acting at source and at European level, by preventing soil contamination or reducing its level, are a necessary complement to the strict EU measures and controls performed to ensure feed and food safety.
- International dimension – soil degradation is receiving increasing attention in international agreements and charters. By establishing an appropriate and coherent

framework which will translate into better knowledge and management of soil, the EU can play a leading role internationally, facilitating the transfer of know-how and technical assistance whilst at the same time ensuring the competitiveness of their economies.

In addition, action at EU level will also have an added value by contributing to the protection of the health of European citizens that can be impaired in different ways by soil degradation, for instance because of exposure to soil contaminants by direct ingestion (children in playgrounds) or indirect intake (through contaminated food or drinking water). Equally, casualties may occur in the event of landslides."

"Having examined different options, the Commission proposes a Framework Directive as the best means of ensuring a comprehensive approach to soil protection whilst fully respecting subsidiarity. Member States will be required to take specific measures to address soil threats, but the Directive will leave to them ample freedom on how to implement this requirement. This means that risk acceptability, the level of ambition regarding the targets to be achieved and the choice of measures to reach those targets are left to Member States. This recognizes that certain threats, such as erosion, organic matter decline, compaction, salinisation and landslides, occur in specific risk areas which must be identified. For contamination and sealing, a national or regional approach is more appropriate. The proposal sets up a framework for adopting, at the appropriate geographical and administrative level, plans to address the threats where they occur."

"On the basis of a common definition of contaminated sites (i.e. sites which pose significant risk to human health and the environment), its application by the Member States, and a common list of potentially polluting activities, Member States will be required to identify the contaminated sites on their territory and establish a national remediation strategy. This strategy will be based on sound and transparent prioritisation of the sites to be remediated, aiming at reducing soil contamination and the risk caused by it and including a mechanism to fund the remediation of orphan sites. This is complemented by the obligation for a seller or a prospective buyer to provide to the administration and to the other party in the transaction a soil status report for sites where a potentially contaminating activity has taken or is taking place. The Directive also addresses prevention of contamination via a requirement to limit the introduction of dangerous substances into the soil.

PART II
ORGANIC MATTER DEPLETION: EXPERIMENTAL CASE

Changes in microbial community structure and functioning of a semiarid soil due to the use of anaerobic digestate derived composts and rosemary plants

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3.1 Introduction

The loss of organic matter and biodiversity are among the main threats to soil quality, as identified by the EU Soil Thematic Strategy COM 2006 (231) and in the Policy report on its implementation, COM 2012 (46). Soil Organic Matter (SOM) depletion and soil erosion are caused by inappropriate agricultural practices, clearing of vegetation, increased levels and frequency of drought or flooding and forest fires. It has been observed, for example, that land without vegetation can be eroded more than 120 times faster than land covered by vegetation, which can thus lose less than 0.1 tons of soil per ha year−1 (Turbé et al., 2010). The activity and diversity of soil microorganisms are directly affected by the reduction of SOM content, and indirectly by the reduction in plant diversity and productivity. Microbial communities play a key role in organic matter decomposition (Lavelle and Spain, 2001; Chaudhry et al., 2012) and in biogeochemical cycles (Doran and Zeiss, 2000; Paul, 2007; Zhong et al., 2010) and enhance the efficiency of plant nutrient assimilation by promoting their growth and health (Gu et al., 2008; Güneş et al., 2014).

Since most of the soil processes are microbially mediated, soil microorganisms are central to soil ecological functioning, providing several regulation ecosystem services (Millennium Ecosystem Assessment, 2005). Microbial activity and soil fertility are closely related and the soilmicrobiota adapts quickly to environmental constrains by adjusting its biomass, activity rates and community composition. Microbial structure and its functioning can represent accurate indicators of soil quality (Winding et al., 2005; Benedetti et al., 2006; Giacometti et al., 2013). Only recently has more attention been focused on the maintenance of the structural and functional diversity of soil bacterial communities and the ways in which they might respond to various natural or anthropogenic disturbances (Zhang and Xu, 2008; Bouasria et al., 2012).

Studies on microbial biomass carbon and enzyme activities provide information on the biochemical processes occurring in soil and there is evidence that soil biological parameters are early and particularly accurate indicators of soil ecological stress and restoration of soil treated with organic amendments (Sciubba et al., 2014). In Mediterranean areas, intensive agriculture practices, together with adverse climatic conditions (e.g. water scarcity), are among the main causes of soil degradation (Anderson, 2003; Ros et al., 2003; Guerrero et al., 2007) and organic carbon loss (Garcia et al., 1997). As it is an important factor influencing soil structure and nutrient availability for soil biota and plants, SOM depletion leads to a decrease in soil fertility with negative consequences for soil biodiversity and crop production (Pimentel andWilson, 1997; Turbé et al., 2010).

Anaerobic digestion is an efficient biological method for the use of livestock and agro-industrial wastes for producing energy and it transforms organicwastes into two products: a source of renewable energy (biogas) and a potential fertiliser, the digested material (digestate) (Bernal et al., 2009; Poeschl et al., 2012). Composting, a treatment based on the aerobic biological decomposition and stabilisation of organic substrates, can prove a suitable method for improving the properties of the solid fraction of digestate and thus enhance its fertilising value (Bustamante et al., 2012; Bustamante et al., 2013). The application of compost to degraded soils has been found to be a suitable environmental strategy for improving soil physical structure and increasing the amounts of soil organic carbon and other major nutrients such as nitrogen and phosphorous (Filcheva and Tsadilas, 2002; Bustamante et al., 2012). Moreover, the incorporation of compost into soil provides macroand micro-nutrients to plants, favouring their growth and the development of the rhizosphere; the latter generally promotes a further increase in soil carbon content through root exudation, which can in turn stimulate microbial growth (Walsh et al., 2012). In addition, the origin of the raw materials used in compost processing and the characteristics of compost can differently affect soil microbial communities and their use of the carbon contained in these amendments (Martens, 2000), favouring the activity of the microbial groups that are most suited to the amendments used (Bastida et al., 2008a).

Although the effects of long-term amendment with organic wastes on soil microbial characteristics have been reported (Tiquia et al., 2002; Crecchio et al., 2007; Bastida et al., 2008a; Chaudhry et al., 2012), very little is known about the changes in soil microbial structure after an amendment of an agricultural soil with compost during the restoration of degraded soils (Ros et al., 2003; Saison et al., 2006).

In this work we studied the changes in the structure (cell abundance, phylogenetic characterization) and functioning (dehydrogenase activity) of the microbial community in a degraded agricultural soil, in the presence of rosemary plants, after adding two different composts derived from livestock anaerobic digestates, using greenhouse microcosms. Microcosm experiments enabled the studying, under controlled conditions, of the effects of the different treatments (separately or together) on the natural microbial community in order to assess which treatment could be more suitable for restoring soil quality.

The microbial community structure was evaluated by using culture independent molecular methods suitable for identifying microbial populations in their natural environment (Grenni et al., 2009; Barra Caracciolo et al., 2010;Godoi et al., 2014). In particular, the Fluorescence In Situ Hybridization (FISH) method made it possible to characterize the active microbial community using specific fluorescent labelled 16s rRNA targeted oligonucleotide probes. The method detects the

specific sequences of rRNA in single cells, which correspond to the classification of a bacterial population at different phylogenetic levels. Since only viable and highly active cells have a sufficient number of ribosomes for in situ hybridization to be applied successfully with a specific probe, FISH is a valuable tool for determining which microbial populations are really active in soil ecosystem functioning (Kirk et al., 2004; Godoi et al., 2014) .

3.2 Materials and methods

3.2.1 Description of the soil and of the composts used

For this study soil samples were manually collected using a shovel fromthe surface layer (0–20 cm) of a semiarid agricultural area unused for ten years and located in Montelibretti, province of Rome, Lazio (Italy). There had been intensive agriculture there for more than 20 years previously. The soil was left to dry at room temperature and then sieved (b2mm). Itwas highly calcareous (% CaCO3 33.4%), slightly alkaline, with an available phosphorous content of 15.7 mg kg−1, low salinity and poor organic carbon content (7.5 g kg−1). The soil texture was classified as clay loam on the basis of USDA (43% sand, 42% silt and 15% clay) and as Calcaric Cambisol on the basis of the FAOUNESCO Classification (FAO-UNESCO, 1990).

The main characteristics of the soil and the composts are shown in Table 3.1. The two composts used consisted of the solid fraction of the anaerobic digestate of cattle slurry (CS) or pig slurry (PS) mixed with vine shoot pruning at the following rates (on a dry mass basis): compost CS, solid fraction of anaerobically digested cattle slurry (75%) + vine shoot pruning (25%); and compost PS, solid fraction of anaerobically digested pig slurry (75%) + vine shoot pruning (25%). A detailed description of the composting process has been reported elsewhere (Bustamante et al., 2012; Bustamante et al., 2013).

Both composts showed a suitable degree of maturity for use as soil amendments, on the basis of the different criteria suggested by various authors, such as: total organic carbon to total nitrogen ratio (C/N) (Mathur et al., 1993), with values b20 (11.9 and 11.4, in CS and PS, respectively); cation exchange capacity (CEC) N60 cmol kg−*1* SOM (Harada and Inoko, 1980) (CS = 124 and PS = 171 cmol kg−*1*); CEC/TOC N 1.9 (Iglesias-Jiménez and Pérez-García, 1992) with 2.47 and 3.36 for CS and PS, respectively; and the absence of phytotoxicity, in accordance with the germination index (GI) N50% as suggested by Zucconi et al. (1981) (99.6% and 79.8% in CS and PS, respectively).

3.2.2 Microcosm set up

Polyethylene pots were filled with 1 kg of soil (air-dried and passed through a 2 mm sieve) thoroughly mixed with cattle (CS) or pig (PS) anaerobic digestate derived compost at two different doses (expressed on a fresh mass basis): low dose (low), by adding 11.54 g compost per kg soil (corresponding to a dose of 30 t ha^{-1}) and high dose (high), by adding 23.08 g of compost per kg soil (equivalent to a dose of 60 t ha^{-1}).

The doses employed were based on the nitrogen requirements of the rosemary crop, on the assumption that the availability of N to plants is low since most (N90%) of total compost N is bound to the organicN-pool (Amlinger et al., 2003). These doseswere also similar to those used by other authors in rosemary cropping experiments using compost (Cala et al., 2005; Madejón et al., 2009).

The amended soils were compared with soils treated with an inorganic fertiliser (NPK, proportion 100:60:73 obtained adding 192 mg kg^{-1} soil of commercial fertiliser Nitrophoska top 20, 20:5:10, and 26 mg kg^{-1} of Monopotasic phosphate, 0:52:34) and with untreated soil samples, the latter used as microbiological controls. Each treatment was replicated three times.

Table 3.1: Main characteristics of the soil and of the cattle (CS) or pig (PS) anaerobic digestate derived composts.

Parameter	Soil	Compost CS	Compost PS
pH (H_2O)	7.6	6.88	6.53
EC ($dS \cdot m^{-1}$)	0.10	6.19	5.11
Total organic carbon (%)	0.75	34.6	34.5
Total nitrogen (%)	0.19	2.90	3.03
Water-soluble organic carbon (%)	0.0059	0.57	0.81
Water-soluble organic nitrogen (%)	0.0005	0.23	0.61

The experimental treatments were therefore as follows:

- **CS_{low}**: microcosms amended with cattle anaerobic digestate derived compost at low concentration.
- **CS_{high}**:microcosms amended with cattle anaerobic digestate derived compost at high concentration.
- **PS_{low}**: microcosms amended with pig anaerobic digestate derived compost at low concentration.
- **PS_{high}**: microcosms amended with pig anaerobic digestate derived compost at high concentration.
- **NPK**: microcosms treated with inorganic fertiliser.
- **Control**: untreated microcosms.

At the same time, an experimental set with the same treatments and replicates of each treatment was performed and cuttings of rosemary were also planted in each microcosm. Rosemary was used both for its ability to grow in semi-arid regions and because its root system is able to protect soil from erosion. To ensure genetic identity, plants were propagated exclusively by rooted cuttings.

Fifty cuttings (from the same parent plant) 5 cm long and stripped of their lower leaves, were dipped into a hormone containing rooting powder and planted in rooting pots with peat, with the pots being kept in a greenhouse for two months. After this period, plants of the same length were

carefully removed and placed in the pots. All the microcosms (36) were kept in a heated greenhouse under controlled temperature (25 °C) conditions for six months. The pots were watered regularly and the plants maintained gravimetrically at 50% of their field capacity throughout the experiment.

At different times (0, 30, 120 and 180 days), total microbial abundance and dehydrogenase activity, which can be considered good indicators for evaluating the status and functioning of soil microbial communities and reflects the microbial respiration rate providing information on the active portion of the soil microbial community (Rogers and Tate, 2001; Grenni et al., 2009) were measured in the 12 different experimental treatments. Moreover, the main phylogenetic microbial groups were detected in order to evaluate if cattle or pig digestate amendments could, in the presence or absence of rosemary plant, affect the soil microbial community structure.

For this purpose the Fluorescence In Situ Hybridization method was applied 180 days after the treatments. Finally, microbial community diversity was assessed using Shannon and Evenness indexes. The soil organic carbon and nitrogen contentwere also assessed at 0 and 180 days.

3.2.3 Organic carbon and nitrogen analyses

Total organic carbon (OC) and nitrogen (N) analyses were performed in the various treatments both immediately after the soil treatments and at day 180. Two air-dried soil sub-samples (15–20mg) for each replicate microcosm were sieved at 0.5 mm, then acidified with 20 μl 5 M ultrapure HCl and kept at 50–60 °C for 30 min in order to remove inorganic carbon. Finally they were analysed using an elemental carbon analyser (Carlo Erba NA 1500 series 2 C/H/N/O/S). OC and N contents are expressed as percentages (%) of dry weight. Water-soluble organic carbon and water-soluble nitrogen were determined in a water extract of 1:4 (w/v), after filtration through a synthetic filter with a pore diameter of 0.45 μm, by using an automatic analyser for liquid samples (TOC-V CSN Analyzer, Shimadzu) (Bustamante et al., 2011).

3.2.4 Microbial abundance and dehydrogenase activity

Microbial abundance (No. cell g−1 soil) was measured by the epifluorescence direct count method, using DAPI (4’,6-diamidino-2-phenylindole) as the fluorescent agent. The DAPI method is able to detect all the microbial cells in a sample whatever their physiological state and metabolic activity and for this reason is suitable for total microbial counts (Barra Caracciolo et al., 2005a). Briefly, two soil subsamples (1 g) were collected from each replicate microcosm and immediately transferred to a test tube containing 9 ml of a fixing solution (composed of phosphate-buffered saline: 130 mM NaCl; 7 mMNa2HPO4, 3 mM NaH2PO4; 2% formaldehyde (v/v); 0.5% Tween 20

(v/v) and 100 mM sodium pyrophosphate). Moreover, other two soil sub-samples (5 g) were collected from each replicate microcosm in order to analyse microbial dehydrogenase activity, by measuring the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) solution to triphenylformazan (TPF) in line with the method reported in Bending and Rodriguez-Cruz (2007). Thismethod is based on extraction and colorimetric determination of the intensely coloured 2,3,5-triphenyl formazan (TPF) produced from the reduction of colourless 2,3,5-triphenyltetrazoliumchloride in soils after 24 h of incubation at 37 °C in the dark (Grenni et al., 2012). Results were expressed as μg TPF g−1 dry soil. Each microbiological datumis themeans of six values fromthe three microcosm replicates.

3.2.5 Microbial community structure evaluated by FISH in cattle and pig amended microcosms

The effects of the organic amendments and plant occurrence on microbial community structure were evaluated at the end of the experiment (180 days) by applying Fluorescence In Situ Hybridization (FISH). Two soil sub-samples (1 g) were collected from each replicate microcosm and transferred to a test tube containing the above described fixing solution used for measuring microbial abundance.

Prior to FISH an additional cell purification step was performed with a density gradient mediumin order to detach cells from soil particles, as described in detail in Barra Caracciolo et al. (2005b). Fluorescence In Situ Hybridization of the harvested cells, counter stained with DAPI, was performed using published protocols (Pernthaler et al., 2001; Barra Caracciolo et al., 2005a; Barra Caracciolo et al., 2005b).

Fluorescent probes were applied for the identification, under the epifluorescence microscope, of the *Archaea* and *Bacteria* domains. Within the *Bacteria* domain the major bacterial divisions commonly found in soil such as α-, β-, γ-, δ-, ε- *Proteobacteria, Planctomycetes, Cytophaga-Flavobacterium* lineage of the *Bacteroidetes* (CFB group), *Actinobacteria* (High G+C Gram-positive bacteria, HGC) and *Firmicutes* (Low G + C content Gram-positive bacteria, LGC) were investigated. Moreover, some bacterial groups, such as *Enterobacteriaceae* (within γ-*Proteobacteria*) and *Desulfovibrionales* (within δ-*Proteobacteria*), were also studied at more specific hierarchical levels. For this purpose the following oligonucleotide probes were applied: Arch915 (for *Archaea*), EUB338I-III (for *Bacteria*) and inside this domain ALF1b, BET42a, GAM42a, DELTA495a,b,c and EPS710 (for α-, β-, γ-, δ-, ε- *Proteobacteria*, respectively), Pla46 and Pla886 (for *Planctomycetes*), CF319 (for *Cytophaga-Flavobacterium* lineage of the *Bacteroidetes*), HGCa,b,c (for *Actinobacteria*), LGC354a,b,c (*Firmicutes*), SRB385

(*Desulfovibrionales*) and finally EBAC1790 together with its competitor EBCO1790 (*Enterobacteriaceae*). All probes were synthesized by MWG AG Biotech, Germany. Further details of these probes are available at http://www.microbial-ecology.net/probebase (Loy et al., 2007). Hybridizations were carried out with Cy3-labelled oligonucleotide probes.

The slides were mounted with drops of VECTASHIELD Mounting Medium (Vector Laboratories) and the preparations were examined and counted with a Leica DM LB 30 epifluorescence microscope at 1000 magnification, counting a minimum of 300 cells per section. The estimation of cells binding to the fluorescent probes is calculated as a percentage of the total DAPI positive cells.

Moreover, the diversity of the bacterial community, considering the 12 taxa listed above, was calculated using the following indexes:

$$\text{Shannon}\left(H'\right) = \sum_{i=1}^{11} p_i \log p_i$$

where p_i is the relative abundance of taxa i within the bacterial community

$$\text{Evenness}(E) = H/H_{max}$$

The number for each microbial taxa was obtained by multiplying the percentage from each probe and the total microbial abundance obtained by DAPI counts.

3.2.6 Rosemary plant biomass

At the end of the experiment, all the rosemary plants were carefully removed from the pots, washed with distilled water to remove any particles attached and weighed (aerial parts + roots) to determine their fresh weight. The plants were then dried at 60 °C in an air-forced oven and 72 h after their dry weights were determined.

3.2.7 Statistical analysis

The statistical analysis of dehydrogenase activity, bacterial abundance and FISH data was done using an analysis of variance (ANOVA), with significant differences at the $p < 0.05$ level. We applied the two way ANOVA analysis considering time always as variable 1 and the plant or compost occurrence as variable 2. The PC Program used was SIGMASTAT.

3.3 Results

3.3.1 Total and water-soluble soil organic carbon and nitrogen contents

The total soil organic carbon (% OC) and nitrogen (% N) contents of the soil at the initial and final samplings (0 and 180 days) in the different treatments in the absence and presence (Plant) of the rosemary plants are reported in Figure 3.1. The initial total organic carbon content in the soil amended with the composts (CSlow, CShigh, PSlow and PShigh) was higher than that found in the Control and NPK soils (Figure 3.1a). Although at the end of the experiment the carbon contents decreased in all treatments, their values remain higher in the amended soils.

The initial total nitrogen content (% N) was higher in all amended soils and also in the NPK ones, as expected in the case of a mineral fertiliser (Figure 3.1b). Similarly to the total OC, the water-soluble organic carbon concentration (expressed in mg kg−1) was higher in the soils amended with the cattle and pig composts than in the Control and NPK soils, both at the start (Control: 65.58, NPK: 57.31, CSlow: 75.66, PSlow: 107.42, CShigh: 100.79, PShigh: 108.73) and at the end of the experiment (Control: 52.64, Control + Plant: 55.92; NPK: 56.43, NPK + Plant: 53.68; CSlow: 73.26, CSlow + Plant: 70.83; PSlow: 70.88, PSlow + Plant: 100.12; CShigh: 62.01, CShigh + Plant: 98.87; PShigh: 76.07, PShigh + Plant: 140.77).

In addition, the concentrations of water-soluble nitrogen were also higher in the compost-amended soils both at the beginning (Control 4.92, NPK 5.29, CSlow 4.96, PSlow 8.42, CShigh 19.53, PShigh 34.41), and at the end (Control 3.92, Control + Plant 4.70; NPK 5.14, NPK + Plant 4.89; CSlow 4.30, CSlow + Plant 4.53; PSlow 6.39, PSlow + Plant 7.44; CShigh 14.98, CShigh + Plant 9.6; PShigh 30.99, PShigh + Plant 21.41), with the highest values in the soils amended with pig slurry digestate compost (PS) at both doses (low and high). This effect was observed in the all soils,with andwithout plants, presumably owing to the higher nitrogen contents found in the PS compost (Table 3.1).

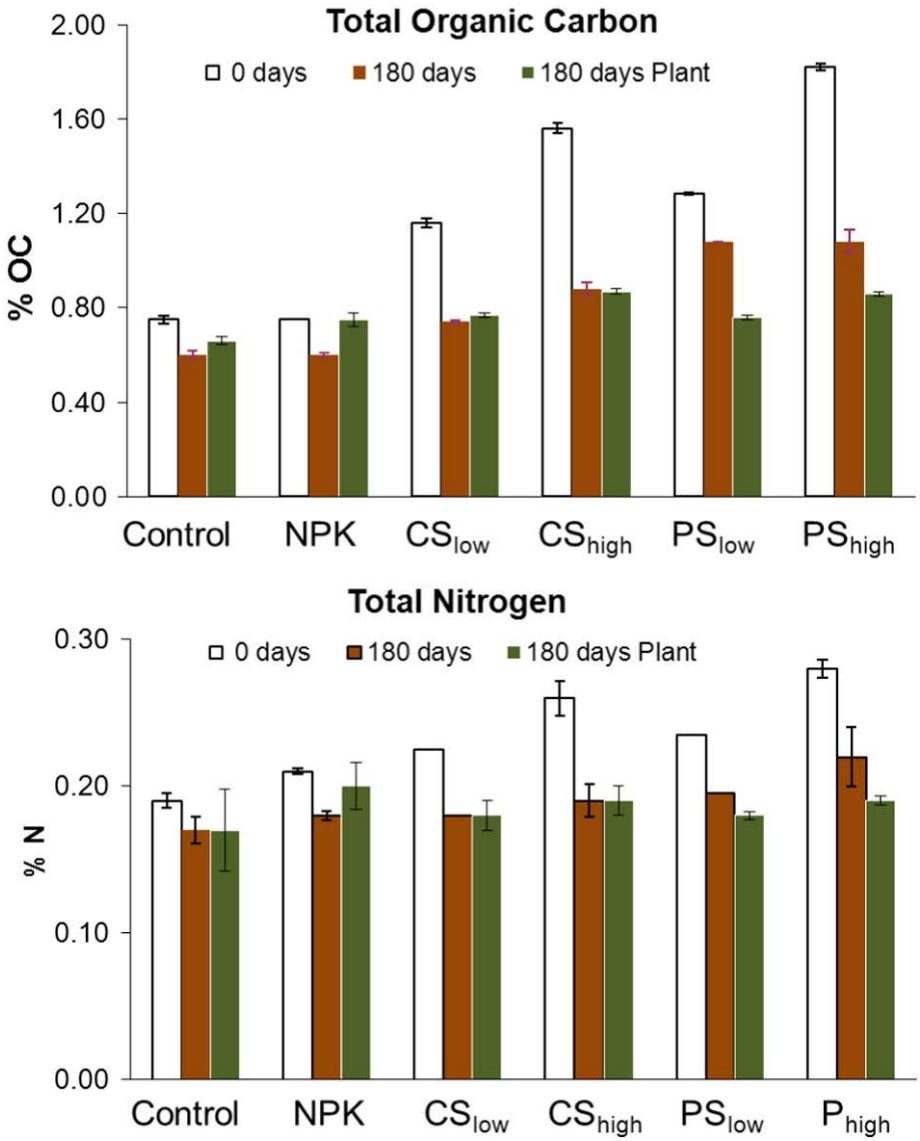

Figure 3.1 - Total organic carbon (% OC) and b) nitrogen (% N) at days 0 and 180, in the absence and presence (Plant) of rosemary in the various experimental treatments. Control: untreated microcosms; NPK: microcosms treated with inorganic fertiliser; CSlow and CShigh: microcosms amended with cattle anaerobic digestate derived compost at respectively low (30 t ha−1) and high (60 t ha−1) concentrations; PSlow and PShigh: microcosms amended with pig anaerobic digestate derived compost at respectively low and high concentrations. The vertical bars represent the standard errors of six values.

3.3.2 Microbial abundance

The microbial abundance (No. cells g^{-1} dry soil) in the soil microcosms over time for the experimental treatments CSlow, CShigh, PSlow, PShigh, NPK and Control and in the absence/presence of the rosemary plants is reported in Fig. 3.2a and b, respectively. The microbial abundance in the microcosms without plant (Figure 3.2a) was positively affected by the addition of the CS and PS composts. In fact, after day 30, the average abundance values in the CSlow, CShigh, PSlow and PShigh treatments were significantly higher (two-way analysis of variance, $p < 0.01$) than those in the control soil (Control) and the fertilised one (NPK). At day 180, the highest values for microbial abundance were observed in CShigh ($1.07 \cdot 108$ cells g^{-1} soil), PShigh ($1.10 \cdot 108$ cells g^{-1} soil) and PSlow ($1.13 \cdot 108$ cells g^{-1} soil), respectively. A certain, but smaller increase in abundance was also observed in the Control and NPK microcosms, but this trend was presumably due merely to the more favourable experimental conditions (i.e. constant soil watering and a temperature of 25 °C) compared to the initial ones.

The microbial abundance in the treated microcosms in the rosemary plant presence (Figure 3.2b) was positively influenced both by the pig and the cattle slurry anaerobic digestate derived composts and by the fertiliser. Between 30 and 120 days, the highest value was observed in the soil treated with the mineral fertiliser (NPK). However, at day 180 the cell number was similar in all the experimental treatments (Figure 3.2b) and significantly lower ($p < 0.01$) than that observed in the absence of the plant in the CShigh, PShigh and PSlow treatments (Figure 3.2a). The increase in microbial abundance at day 30 in the NPK microcosms is ascribable to a synergic and direct positive effect ascribable to the fertiliser and the rhizosphere.

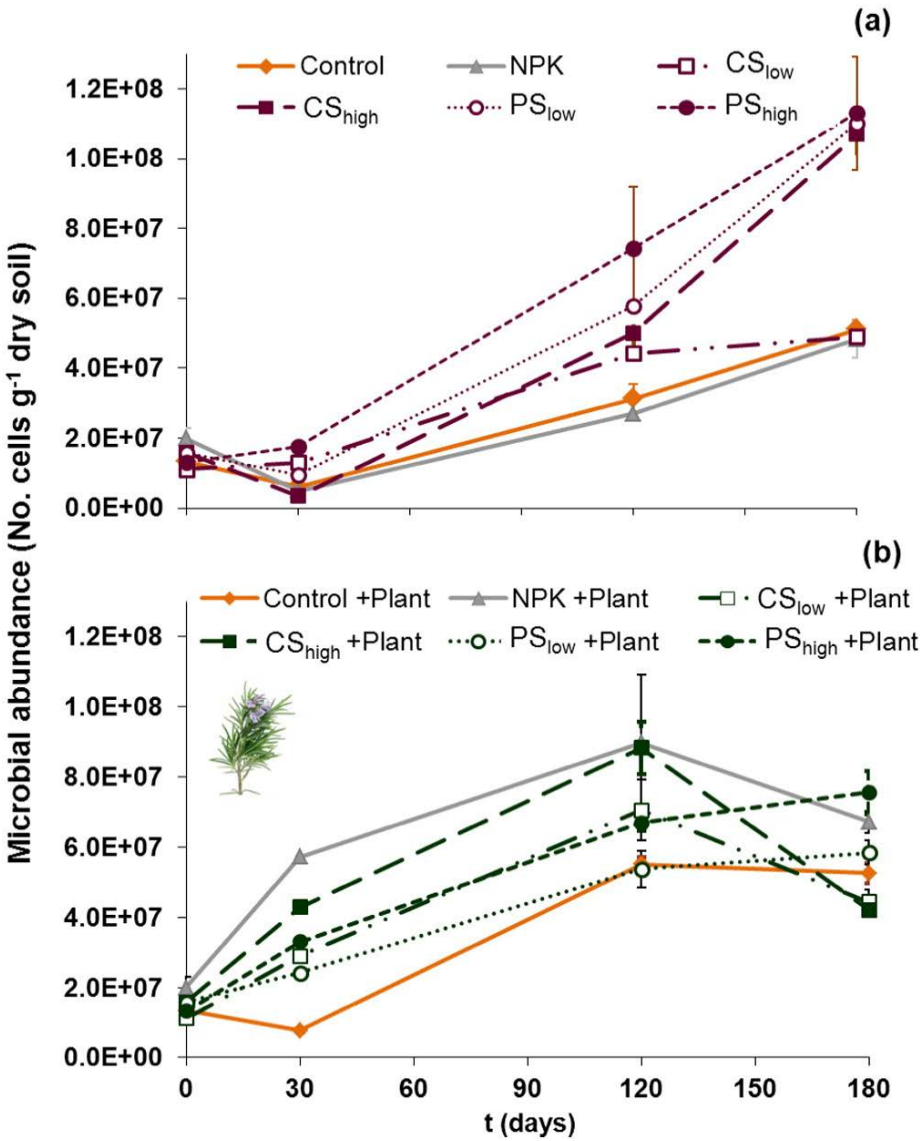

Figure 3.2 - Microbial abundance (No. cell g−1 dry soil) over time in the Control soil and in soils treated with inorganic fertiliser (NPK), or cattle or pig anaerobic digestate derived compost at low (CSlow, PSlow) or high (CShigh, PShigh) concentrations. a) Microcosms without plant; b) microcosms with the rosemary plant. The vertical bars represent the standard errors of six values.

3.3.3 Dehydrogenase activity

Soil dehydrogenase activity (μg TPF g^{-1} dry soil) over time for the various experimental treatments of the soil microcosms in the absence and presence of the rosemary plants is reported in Figure 3.3a and b, respectively. Organic amendments in the microcosms without the plant (Figure 3.3a) did not significantly affect soil dehydrogenase activity. In fact, after 30 days dehydrogenase activity increased in the amended soil treatments compared to the control (Control) and fertilised (NPK) ones, but at 180 days enzymatic activity values were similar in all treatments (117.77 ± 2.6 μg TPF g^{-1} dry soil) and were not affected by either the type or amount of amendment used.

On the contrary, soil dehydrogenase activity was significantly affected by the presence of the plant as shown in Figure 3.3b. In particular, the dehydrogenase values in all treatments with plant were significantly higher (two-way analysis of variance, $p < 0.01$) than those without plant both at 30 days (average values of 145.50 ± 4.1 μg TPF g^{-1} dry soil vs 122.75 ± 7.0 μg TPF g^{-1} dry soil in presence and absence of rosemary, respectively) and 180 days (average values of 159.70 ± 3.9 μg TPF g^{-1} dry soil vs 117.70 ± 2.6 μg TPF g^{-1} dry soil in the presence and absence of rosemary, respectively).

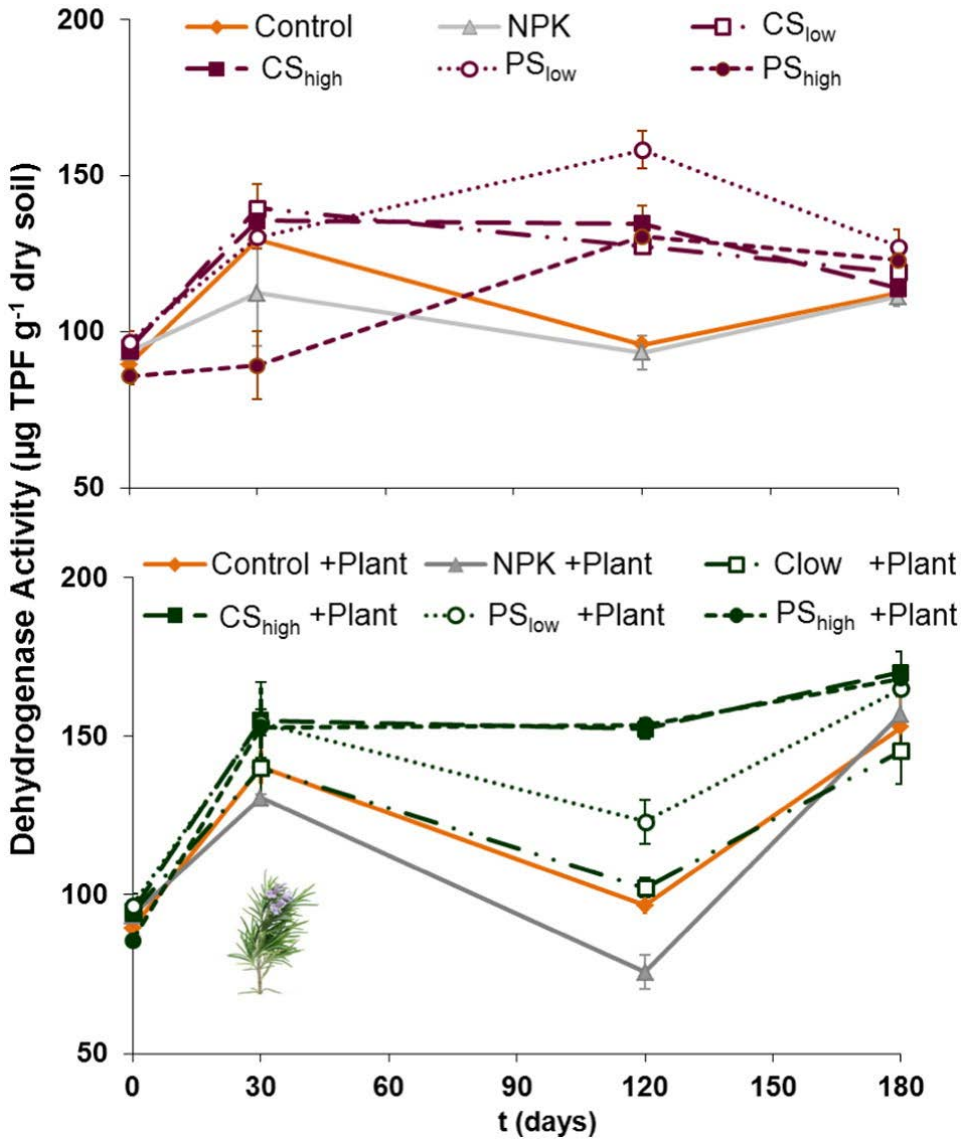

Figure 3.3 - Microbial Dehydrogenase activity (µg TPF g−1 dry soil) over time in the Control soil and in soils treated with inorganic fertiliser (NPK), or cattle or pig anaerobic digestate derived compost at low (CSlow, PSlow) or at high (CShigh, PShigh) concentrations. a) Microcosms without plant; b) microcosms with the rosemary plant. The vertical bars represent the standard errors of six values.

3.3.4 Microbial community structure and diversity 180 days after compost addition

The Fluorescence In Situ Hybridization (FISH) technique made it possible to determine the structure of the active microbial community of the soil at the end of the experiment (180 days) and the results are shown in Figures 3.4 and 3.5. The number of *Archaea* detected by FISH was significantly higher ($p < 0.01$) both in the Control and in the amended CShigh and PShigh soils (Figure 3.4, left side) and their average abundance value was $3.0 \cdot 106 \pm 6.7 \cdot 105$ cells g^{-1} soil, corresponding to about 4% of the overall microbial community. On the contrary, the number of *Archaea* collapses in CSlow and PSlow and in all the treatments where the rosemary plant is present (Figure 3.4, right side), with an average value of $3.26 \cdot 105 \pm 8 \cdot 104$ cells g^{-1} soil (corresponding to about 0.5% of the overall microbial community). The percentage of *Bacteria* detected in the Control

was 50.2 ± 3.2% (corresponding to 2.5 · 107 cells g^{-1} soil),whereas it increased to an average value of 66±2.3% (corresponding to 5.0 · 107 cells g^{-1} soil) in all treated soils.

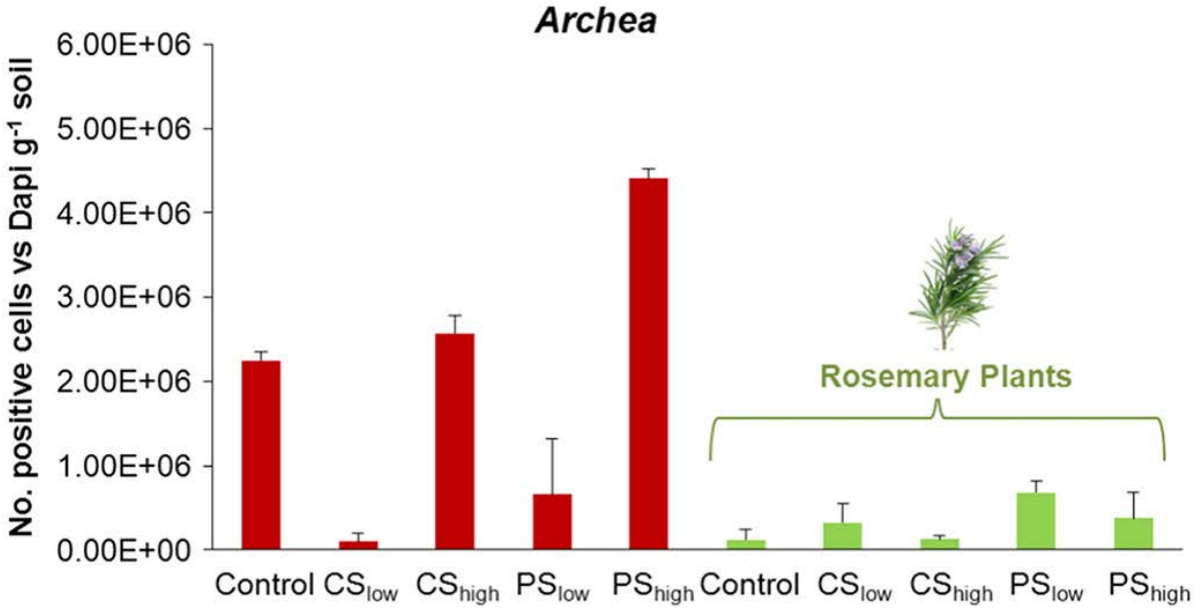

Figure 3.4 - Archaea detected by FISH at day 180 in the Control and soils treated with cattle (CSlow, CShigh) or pig (PSlow, PShigh) anaerobic digestate derived compost at low (30 t ha−1) or high (60 t ha−1) concentrations, in the absence (left side) and presence (right side) of rosemary. The vertical bars represent the standard errors of six values.

Inside the *Bacteria* domain a significant shift ($p < 0.01$) in the dominance of several bacterial groups was observed when the rosemary plant was present or absent (Figure 3.5a and b). In particular, when the plant was present α-, β-, and γ-*Proteobacteria* generally increased while *Planctomycetes* (Pla), *Bacteroidetes* (CF) and *Firmicutes* (LGC) decreased (Figure 3.5 b), with the highest values of α-, β-, and γ-*Proteobacteria* being found in CSlow and PSlow. Finally, neither *Enterobacteriaceae* (EBAC1790) nor *Desulfovibrionales* (SRB385) were found in any treatments.

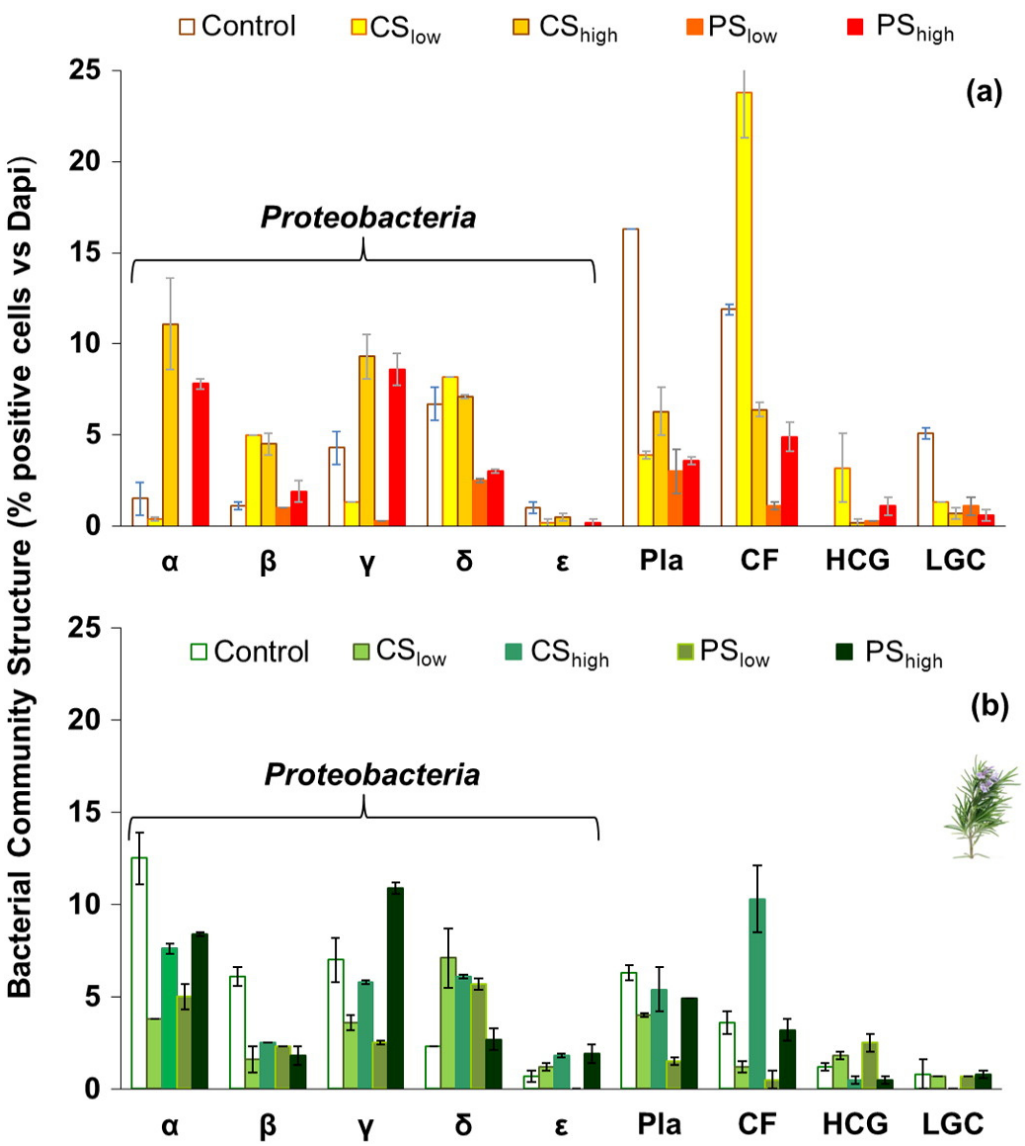

Figure 3.5 - Bacterial community structure detected by FISH at day 180 in the Control and soils treated with cattle (CSlow, CShigh) or pig (PSlow, PShigh) anaerobic digestate derived compost at low (30 t ha−1) or high (60 t ha−1) concentrations. a) Microcosms without plant; b) microcosms with the rosemary plant. α = α-*Proteobacteria*; β = β- *Proteobacteria*; γ = γ-*Proteobacteria*; δ = δ-*Proteobacteria*; ε = ε-*Proteobacteria*; Pla = Planctomycetes; CF=Cytophaga-Flavobacterium lineage of the Bacteroidetes (CFB group); HGC = Actinobacteria; LGC=Firmicutes. The vertical bars represent the standard errors of six values.

In order to have a general overview of the differences in microbial community diversity between the various treatments, the Shannon and Evenness indexes were calculated. The results are shown in Figure 3.6a and b. The highest diversity values were found in the soils amended with the composts at low concentrations when the rosemary plant was present (CSlow + Plant and PSlow+ Plant).

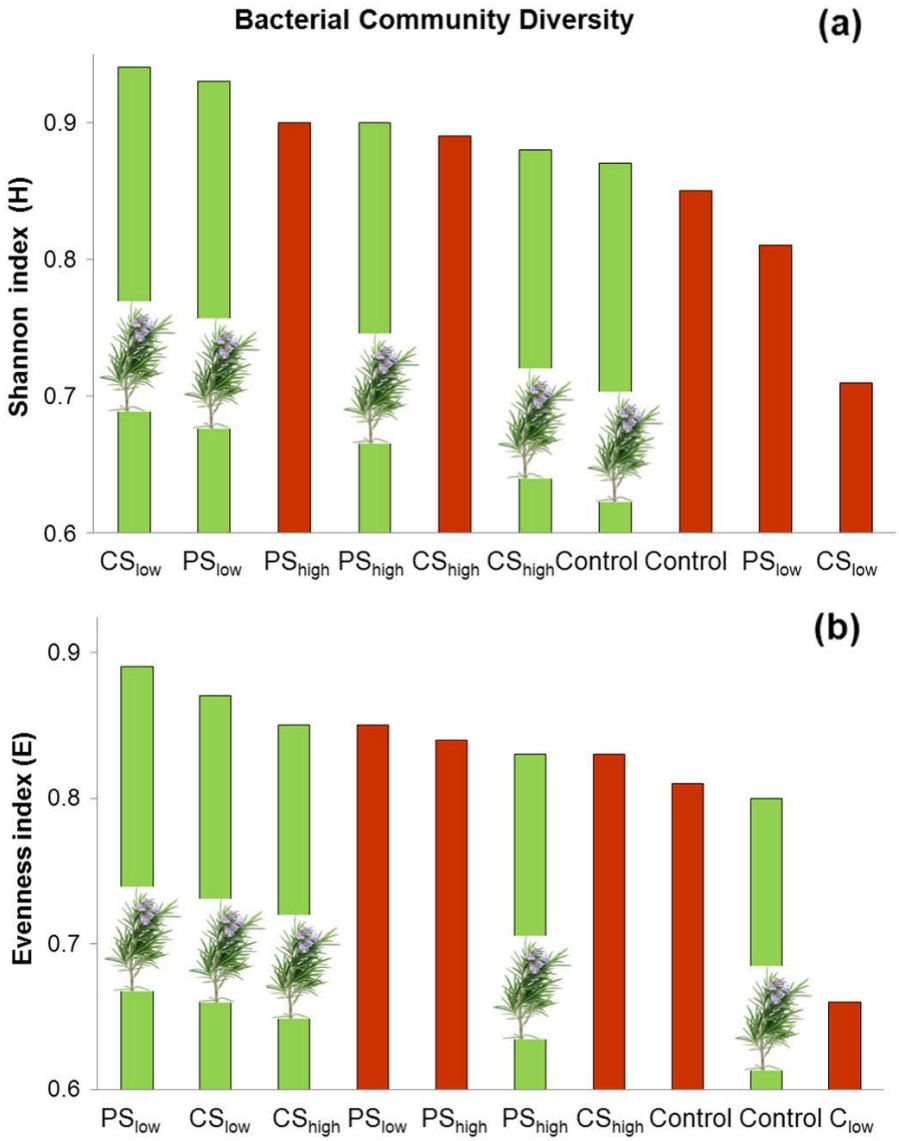

Figure 3.6 - Bacterial community diversity calculated by Shannon (a) and Evenness (b) indexes in the presence (rosemary image) and absence of the rosemary plant in microcosms treated with cattle (CSlow, CShigh) or pig (PSlow, PShigh) anaerobic digestate derived compost at low (30 t ha−1) or high (60 t ha−1) concentrations.

3.3.5 Rosemary plant biomass

The average values of rosemary plant dry biomasswere significantly lower (t test p b 0.01) in the Control than in the NPK and amended soils and the amendments applied to soil increased (about twice) the plant biomass in a similar way to fertiliser (Control: 2.2 ± 0.16 g; NPK: 4.2 ± 0.085 g; PShigh: 3.4 ± 0.2 g; PSlow: 3.9 ± 0.19 g; CShigh: 3.00 ± 0.15 g; CSlow: 3.45 ±0.3 g). The biomass value closest to the NPK treatment is in PSlow and this result is in accordancewith the higher content of water-soluble N of in the compost from pig anaerobic digestate (Table 3.1).

3.4 Discussion

In this experiment, we tested the addition of two composts and the presence/absence of the rosemary plants on the microbial community of a degraded agricultural soil. OC was relatively lower in treatments where the amendments were added at low concentrations in line with the lower amount of initial carbon added with the amendments. However, at day 180, a notable decrease in OC concentrations was observed in the soils amended with PS (both doses: 30 t ha−1 or 60 t ha−1) when the plant is present, probably because in these conditions the rhizosphere particularly stimulated microorganisms into using the most available C fraction (water-soluble C), which was higher in pig the anaerobic digestate derived compost (PS) than in cattle one (CS; Table 3.1).

The enhancement of microbial activity with the input of easily-mineralisable organic matter with an addition of compost has been reported in several studies (Guerrero et al., 2007; Said-Pullicino and Gigliotti, 2007; Bustamante et al., 2010). Moreover, it is well known that organic amendments enhance nutrient content and in particular provide a better habitat for microorganism development owing to the increase in soil porosity, water holding capacity and aggregate stability, factors that influence soil quality (Van-Camp et al., 2004; Bastida et al., 2008b).

Despite the significant stabilisation of organic materials during composting, which results in a substantial reduction of the most labile organic matter pools, stable compost can nonetheless contain a significant proportion of readily available organic compounds (e.g. microbial-derived hydrolysable sugars) (Said-Pullicino et al., 2007). This organic matter fraction serves as a readily available source of C and energy for the microbial biomass (Bustamante et al., 2010). Moreover, the origin of the initial materials used in the production of compost and the latter's properties may influence soil microbial communities and their use of the C in organic materials, enhancing the activity of the microbial groups that are most suited to the amendments used (Martens, 2000; Bastida et al., 2008a). Several authors (García et al., 2000; Tejada et al., 2006; Bastida et al., 2008b) have also reported that the presence of a stable plant cover is important for increasing soil carbon input and for the development of soil microbial communities, both of which contribute to improve soil fertility.

On the other hand, the increase in microbial abundance observed in the absence of plants (Figure 3.2a) may be ascribable both to the addition of allochthonous bacteria present in the compost and to the carbon content of the compost itself; in fact the higher abundance values were matched by higher values for both the total organic carbon and soluble carbon found in the treated soils. However, when plant is present (Figure 3.2b), this result is not so evident, because other factors related to the rhizosphere microhabitat stimulated the cell abundance in all soils, including the NPK-treated ones. The general relative increase in microbial abundance in all treatments with plants

is in fact ascribable to the rosemary roots which may have stimulated microbial growth in a different way by producing root exudates, as found by Gu et al. (2008). Moreover, the higher microbial dehydrogenase activity found in the microcosms with the rosemary plants, and in particular at day 180 (Figure 3.3b) supports the fact that, whatever the cell number, microbial activity is significantly higher in the rhizosphere because of the presence of the root system (Crecchio et al., 2004; Garcia et al., 2005; Berg and Smalla, 2009; Bini et al., 2013; Jorge-Mardomingo et al., 2013). In fact, the dehydrogenase activity reflects the microbial respiration rate and provides information on the active portion of the soil microbial community (Rogers and Tate, 2001).

In the absence of the rhizosphere (Figures 3.2a and 3a), the microbial number does not reflect microbial activity. This is because many inactive cells can survive in soil and the DAPI count method that we used is able to detect all the microbial cells occurring in a soil sample, whatever their cultivability and activity. The fact that similar studies, using cattle manure as the amendment and two different plant species, found a positive relation between microbial abundance and activity (Giacometti et al., 2013) is due to the use of a different microbiological method, such as the total phospholipid fatty acid (PLFA) content, which is an indicator of the viable microbial biomass.

Regarding the structure and diversity of the microbial community analysed at the end of the experiment, the application of the amendments and/or the presence of a rosemary plant affected it in different ways.

The overall results of the FISH method, which made it possible to detect active microbial populations and to evaluate significant differences among the various treatments at day 180, highlight that the rosemary plants and the addition of the amendments at low concentrations positively influenced microbial community biodiversity, as confirmed through the two diversity indexes applied (Figures 3.5 and 3.6).

The occurrence of the *Archaea* group, which is generally higher in extreme or stressed conditions (high or low temperature, nutrient deficiency, oxygen deprivation, etc.), in lower soil layers and is associated with a lower organic carbon content (Valentine, 2007; Hansel et al., 2008; Buée et al., 2009), was relatively high in the Control soil, if compared to other agricultural soils where its occurrence has generally found to be quite scarce (Barra Caracciolo et al., 2005a; Barra Caracciolo et al., 2010; Barra Caracciolo et al., 2011; Godoi et al., 2014). This fact may be ascribable to the low organic C content of the soil studied. Moreover, in accordance with the fact that the composting process naturally involve *Archaea*, their numbers increase in the soils amended with the cattle and pig slurry anaerobic compost in high concentrations, as was also found after addition of biosolids or manure to soil in several studies (Thummes et al., 2007; Schauss et al.,

2009) (Figure 3.4); it is in fact known that many *Archaea*, given their great resistance capacity once they have reached soil, can be reactivated when conditions are favourable to them (Thummes et al., 2007; Kelly et al., 2011).

The decrease in *Archaea* was observed in all microcosms where rosemary plant was present. This can be attributed to an improvement in soil quality (see for example at day 180 the enzyme activity, which is one of the most important parameters that is used for the quality of soil, Bastida et al., 2008b) and their low average percentage (0.5%) is in line with their low presence and activity in soils with a good quality state, as found in other studies (Barra Caracciolo et al., 2011; Godoi et al., 2014).

In a similar way, a general increase in the Bacteria percentage in the amended soils can be linked to the soil quality improvement, mainly owing to the increase in soil organic matter with the incorporation of the organic amendments (e.g. the most labile organic fraction, the water-soluble organic C, increased from 0.8 to 2.5 fold if compared to the control soil). Moreover, within the Bacteria domain (Figure 3.5) *Proteobacteria* generally increased, while *Planctomycetes* (*Pl*a), *Bacteroidetes* (CF) and *Firmicutes* (LGC) decreased when the rosemary plant was present. This result was particularly evident in the soil amended with pig and cattle anaerobic digestate derived-compost at low concentrations (PSlow and CSlow respectively, Figure 3.5 b).

The shift in *Proteobacteria* dominance observed is in line with those reported in other experiments for a good quality state soil (Barra Caracciolo et al., 2011; Mocali et al., 2013; Godoi et al., 2014) and with rhizosphere formation (Singh et al., 2007; Buée et al., 2009). In fact, at day 180, the root system of each rosemary plant completely occupied each microcosm (bulk soil from the rhizosphere could not be separated). *Proteobacteria*, which include most bacterial species involved in the main biogeochemical cycles are typically the most abundant phylum found in a good quality state soil. For an example, *Rhizobiaceae*, which include both nitrogen-fixing and nitrifying bacteria such as *Nitrobacter*, belong to α-*Proteobacteria* (Brock et al., 2007). In line with our results Chaudhry et al. (2012) report that organic compost amendments promote both microorganism diversity and *Proteobacteria* dominance in soil if compared with the same soil conventionally treated with synthetic chemical fertilisers.

The decrease in *Planctomycetes* in the presence of rosemary is in line with the fact that they are affected by oxygen presence and their occurrence is therefore limited in an aerobic rhizosphere (Buckley et al., 2006). In a similar way, the decrease in *Firmicutes* (LGC), bacteria occurring in stressed conditions (e.g. limited carbon source) by forming spores or cysts, is in line with the increase in *Proteobacteria* in the rhizosphere, as also reported by Singh et al. (2007). The fact that microbial diversity is dependent on the amount and quality of organic carbon and that the

Proteobacteria group in soil plays an important role in organic matter use was found by other authors (Berg and Smalla, 2009; Chaudhry et al., 2012; Mocali et al., 2013). On the other hand, Bacteroidetes (CF) are a heterogeneous group of aerobic and anaerobic species (Janssen, 2006) and their variations in abundance were not clearly linked with the experimental treatments. Other authors have also studied the microbial community of organically amended soils. As an example, Sciubba et al. (2014) studied the microbial community of an agricultural soil before and after treatment with biosolids using DGGE profiles. In this experiment, the authors did not find significant differences between DGGE profiles in the control and treated soils and, thus, they affirmed that the method used was not sufficiently accurate to detect small variations in community composition, especially in stable and active populations characterized by high species diversity such as those in soil (Neilson et al., 2013; Sciubba et al., 2014).

However, there are few or even no experiments on the effects of anaerobic digestate derived composts together with plants on soil microbial community structure using the FISH method. This method is particularly useful and reliable for identifying an overall active microbial community in situ. However the use of other molecular methods may also be advisable depending on the specific purpose of a study (e.g. identification of specific microbial species). Consequently, although microbiological communities and activity are nowadays recognised to be accurate indicators of soil quality, it is very important to use methods capable of identifying truly active microbial populations and consider different measures of their activity and diversity simultaneously .

3.5 Conclusions

Different effects on soil characteristics depending on the amendment doses were observed. The application of the amendments and/or the presence of the rosemary plant affected the structure and functioning of the microbial community studied in different ways. The soil microbial community was clearly affected both by the compost intrinsic characteristics (e.g. initial amount of carbon and nitrogen content) and by the dose applied (low or high).

The microbial community composition was presumably the result of different processes (e.g. competition, exclusion, duplication) among both the compost microbial populations and the autochthonous ones, which adapted to the specific experimental treatments. Rhizosphere formation due to the presence of rosemary acted as a driving force favouring a more active microbial community in all treatments, whatever the availability of allochthonous organic carbon from the compost amendments.

The overall increase in activity was in fact ascribable to the root exudations, which can produce OC that is readily usable by microorganisms. Moreover, the rhizosphere promoted the shift in dominance to the *Proteobacteria* class. The fact that the highest percentages of *Proteobacteria* and the highest diversity values were found in the co-presence of rosemary plants and pig or cattle compost at low concentrations suggest that these combined treatments can be considered the best for improving the quality of a degraded soil.

Finally, the simultaneous use of anaerobic digestate derived composts together with rosemary plants has several advantages as it:

- ✓ reduces the costs of livestock waste disposal
- ✓ uses a species suited to the semi-arid Mediterranean climate
- ✓ improves the soil organic carbon content of a degraded soil
- ✓ increases the diversity of the microbial community, which is the main promoter of soil fertility and quality and therefore responsible for several ecosystem services.

PART III
ANTROPHOGENIC CONTAMINATION: EXPERIMENTAL CASE

Effects of Municipal Solid Waste compost and the leguminous *Medicago sativa* on a soil microbial community historically contaminated from PCBs

4.1 Introduction

Polychlorinated biphenyls (PCBs) are persistent pollutants (POPs), as classified in the Stockolm Convention in 2001, exclusively generated from human sources. They are a family of 209 compounds called congeners, which have a molecular weight ranging from 188 to 439.7. They have a chemical formula of $C_{12}H_{10}$-nCln and their structure consists of two phenyl (C6H5) rings single-bonded together, with between 1 to 10 chlorine atoms, substituted at different positions in the biphenyl rings. Depending on their position, the chlorine atoms in the biphenyl rings of PCBs can be classified as ortho (2,2' and 6,6' positions), meta (3,3' and 5,5' positions) or para (4) substituted. The notation can be seen in Figure 4.1 (Meggo and Schnoor, 2013).

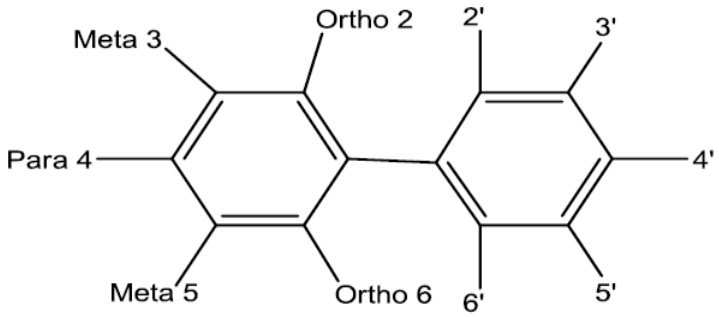

Figure 4.1- Nomenclature for PCBs. The 12 carbon positions in the biphenyl are numbered using carbon 1 for the phenyl- phenyl bond and then numbers 2-6 for the first ring and 2'6' for the second ring. Thus, there are four *ortho* (i.e. 2, 2', 6 and 6'), four *meta* (i.e. 3, 3', 5 and 5') and two *para* (i.e. 4 and 4') positions. Short notations used for halogen-substituted biphenyls use either the notation pictured above, giving the substituted position in order of increasing numbers and using the prime notations for the second ring (e.g. for a given hexa-chlorobiphenyl = 2,2',3,3',5,6'-CB) (Wiegel and Wu 2000).

Depending on the chlorine atoms number and their position, PCB congeners differs in chemical, physical and toxicological properties (Passatore et al., 2014). The high chemical and physical stability and high dielectric constant of PCBs made them useful for a range of industrial applications, including lubricants, dielectric fluids, and plasticizers. Commercial production of PCBs started in 1929 until the recognition of their toxicity and persistence in the environment led to their interdiction in most countries in the late 1970s. In the meanwhile, an estimated amount of 1.5 mega tons of PCBs was produced worldwide, of which a significant fraction is being released into the environment. They are continuously being released into the environment through spills, leaks from electrical and other equipment, and improper disposal and storage. Consequently every environmental compartment, including air, water, soil, sediments, and living organisms have been exposing to PCBs for several years (Tehrani and Van Aken, 2014). PCB mixtures found in environmental matrices usually do not resemble the commercial PCB mixtures, since the most

abundant PCBs in commercial mixtures are ortho-substituted congeners that are more easily degradable. However, smaller amounts of the so-called “dioxin-like" PCBs, namely the coplanar (non-ortho substituted) and mono-ortho substituted congeners, are present in the commercial mixtures as well. The latter are very stable and resistant to biodegradation and metabolism. Moreover, it is well known that lower chlorinated PCBs can volatilise and are, thus, more susceptible to atmospheric removal processes (Fielder, 1997; Mackay et al. 1992).

It is generally recognized that the microbial metabolism is potentially capable to degrade persistent organic pollutants, including PCBs (Pieper and Seeger, 2008). Laboratory studies have been identified bacterial strains able to transform several PCBs congeners through metabolic and co-metabolic pathways. However, the degradative pathway and the environmental parameters involved in PCBs transformation remain to be still clarified (Sylvestre e Toussaint 2011). Although their defiance to natural degradation, there is evidence that microorganisms are capable of degrading PCBs, even if rather slowly. Consequently, the next major focus of PCB research has been on maximizing the potential of these natural degraders in order to accelerate the degradation process (Meggo et al., 2012).

Biodegradation of PCB mixtures requires broad specificity and multiple types of attack. PCBs are known to biodegraded in 3 general ways:

- **Anaerobically by reductive dehalogenation:** replacement of chlorines by hydrogen in anaerobic sediments.
- **Aerobically as a growth substrate:** Less chlorinated PCB degraded faster
- **Aerobically by cometabolism.** Either by bacteria that grow on other PCBs or by whiterot fungi (e.g., *Phanerochaete chrysosporium*)

Higher chlorinated congeners (with four or more chlorine atoms) are susceptible to reductive dechlorination, an energy yielding process, mediated by anaerobic bacteria. In this process, PCBs serve as electron acceptor for the oxidation of organic carbon (Field e Sierra-Alvarez 2008, Sylvestre e Toussaint 2011). Dehalogenation of PCBs has been observed in soil, sewage sludge, and estuarine and marine sediment, under different redox conditions. Higher halogenated PCBs are usually reductively dehalogenated to less halogenated molecules under anaerobic conditions in the presence of organic compounds (Song et al., 2015). The reductive dehalogenation of highly and moderately chlorinated PCBs by anaerobic microorganisms generally involves selective dechlorination from para and meta positions. Nevertheless, ortho dechlorination of PCBs has also been described. Bacterial strains belonging to Dehalococcoides and Dehalobium genera have been associated to halogenation of PCBs (Seeger at al., 2010). In figure 4.2 are shown different Bacterial

reductive dehalogenation pathways of selected polychlorinated biphenyls by Dehalococcoides and related bacteria and by an enrichment culture, described in Bedard et al., 2006; Cutter et al., 2001; Fennell et al., 2004. Environmental factors such as temperature, H2 partial pressure, pH, electron donors and electron acceptors, carbon source availability, can affect microbial dechlorination rate (Wiegel and Wu, 2000, Zhang et al., 2013). The addition of compost to PCB contaminated soil has been attracting increasing attention, because organic fertilizers can significantly promote dechlorination (Semple et al., 2004).

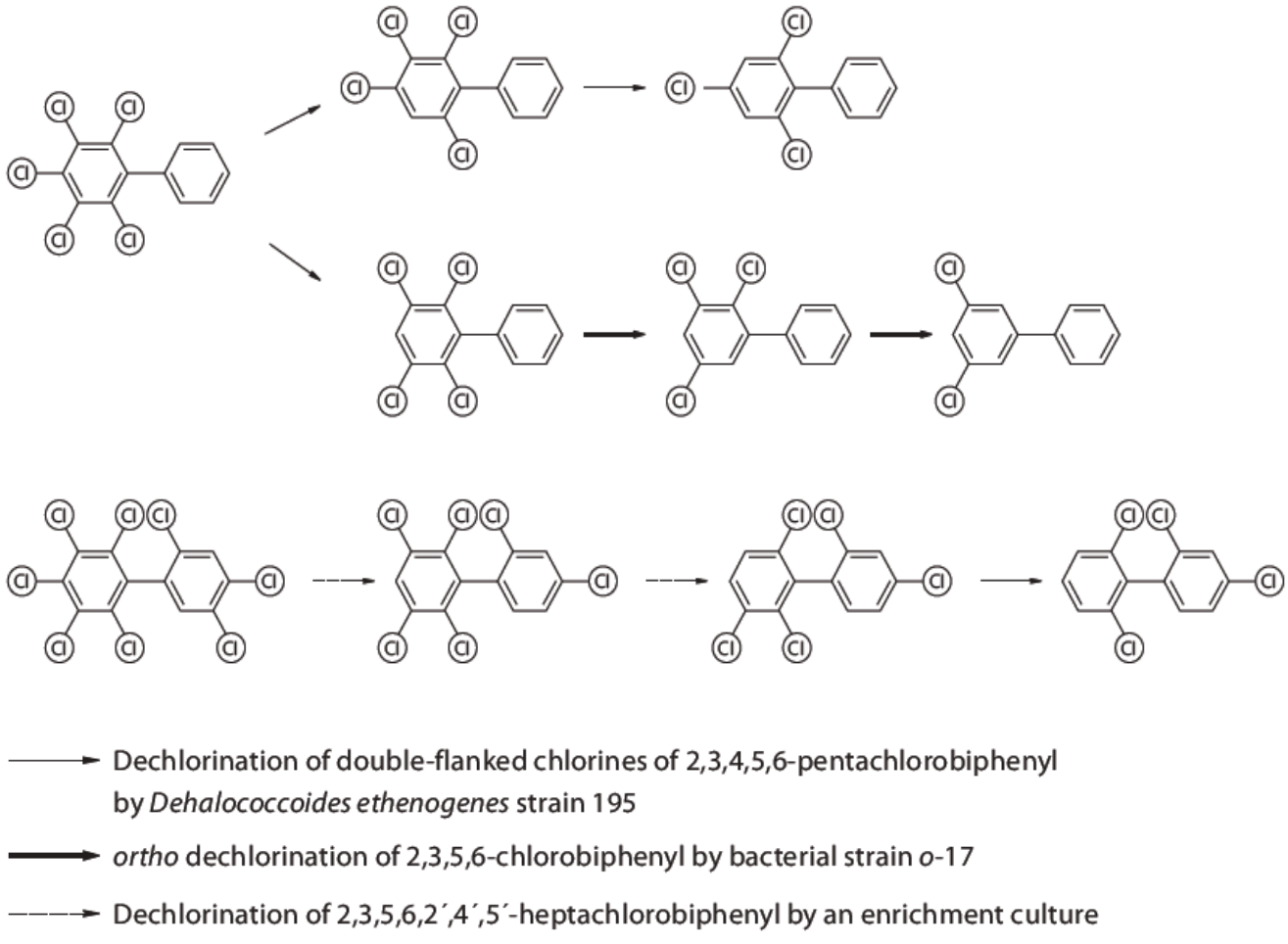

Figure 4.2 - Bacterial reductive dehalogenation of selected polychlorinated biphenyls by *Dehalococcoides* and related bacteria and by an enrichment culture (modified from Pieper and Seeger, 2008).

Lower chlorinated PCB congeners (with four or less chlorine atoms), resulting from the dechlorination of higher chlorinated ones, undergo aerobic oxidative degradation. Diverse aerobic bacteria capable of oxidizing PCBs have been reported (Pieper and Seeger, 2008). Bacterial strains of *Pseudomonas*, *Burkholderia*, *Comamonas*, *Cupriavidus*, *Sphingomonas*, *Acidovorax*, *Rhodococcus*, *Corneybacterium* and *Bacillus* genera have been characterized (Furukawa and Fujihara, 2008; Seeger and Pieper, 2009). *Burkolderia xenovorans* LB400 is able to degrade a broad

range of PCBs and is a model bacterium for PCB degradation. *Rhodococcus jostii* RHA1 is another potent PCB-degrading soil bacterium (Seeger at al., 2010). This aerobic pathway is catalysed by dioxygenases and primarily leads to the formation of catechol-like dihydroxylated metabolites, which generally undergo breakdown of the biphenyl core by opening one of the aromatic rings (Tehrani and Van Aken, 2014). PCB can be aerobically degraded also by cometabolism, either by bacteria or by whiterot fungi (e.g. *Phanerochaete chrysosporium*) (Gomes at al., 2013). In the figure below is reported an example of aerobic degradative pathway (Figure 4.3).

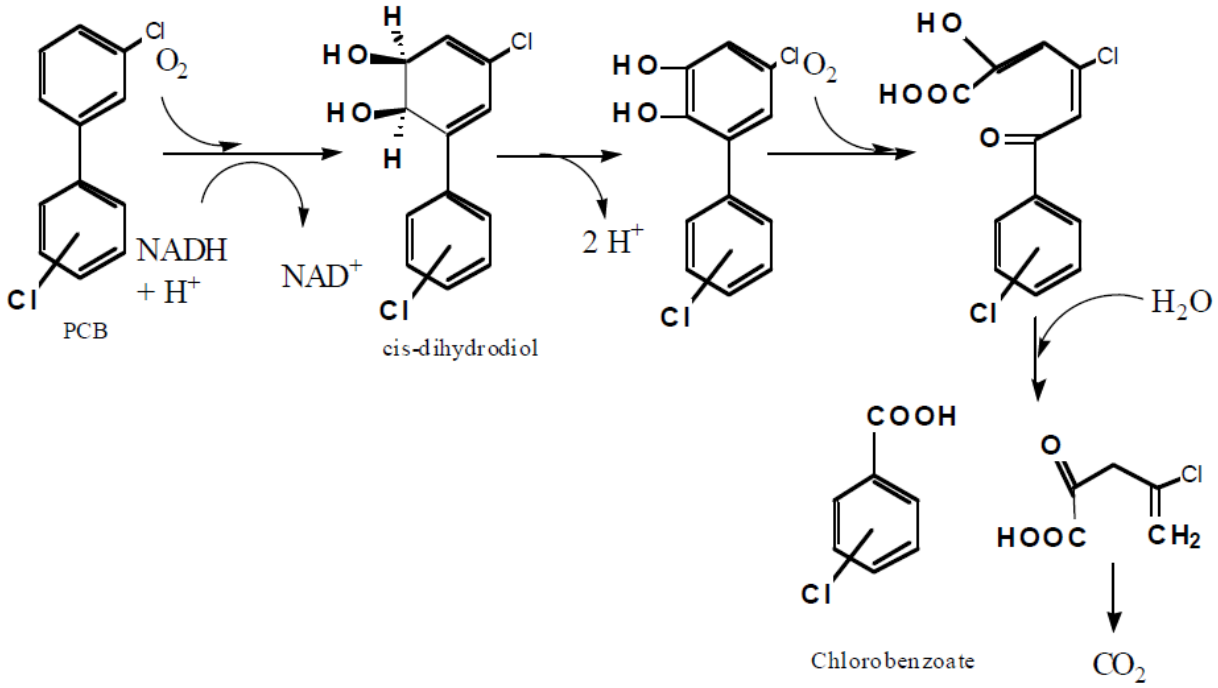

Figure 4.3 - Mono-, di-, tri-, and some tetra-chlorinated PCBs could be degraded by meta-cleavage of unchlorinated 2,3-carbons by many bacterial strains (Strand 1990).

The association plant-microorganisms can improve PCBs degradation in rhizosphere because it exploits the synergic action between roots and soil microbial community (Mackova 2009, Xu et al 2010, Glick 2010, Sylvestre e Toussaint 2011, Meggo at al., 2013). Plant can assist natural microbial communities to transform, remove and contain contaminants in a soil restoring process, called phyto-assisted bioremediation (Wenzel , 2009). Many plant species are capable of thriving on PCB contaminated soils and some species can promote indigenous populations of PCB-degrading microorganisms that harbor degradative abilities as found in some field studies (Leigh 2006; Passatore et al., 2014). Plant roots tend to transfer contaminants from the bulk soil to the rhizosphere where the microbial activity is promoted by root exudates. Some exudates can contain plant secondary metabolites (PSMs) that may act as signal chemicals to promote or induce the

bacterial enzymes involved in PCB degradation (Sylvestre 2013; Meggo at al., 2013, Qin et al., 2014). In return, degrading bacteria can produce plant growth stimulators or can suppress pathogens through competition and antibiotics production (Doty, 2008; Meggo et al., 2012). Moreover, plants by exploring soil with oxygen-transporting roots, offer the possibility of exposing the anoxic portion of soil to aerobic conditions, favouring the oxidation of low-chlorinated PCBs. A wide range of plant genera have been proven to enhance the dissipation of PCBs in soil, from trees like *Populus* and *Salix* to different forages, both grasses and legumes (Dzantor et al. 2000; Chekol et al. 2004; Mackova et al. 2009; Ding et al. 2011) such as *Medicago sativa L.* The latter is the most widely grown perennial legume in the world and has been found to selectively support the growth of not only symbiotic nitrogen fixing bacteria (Xu et al. 2010), but also PCB-degrading bacteria (Li et al. 2013; Teng et al., 2011).

The aim of this study was to assess the effect of different treatments (i.e. biostimulation and/or plant-assisted bioremediation) on the biodegradation of PCBs in a greenhouse microcosms experiment using soil samples from an historically contaminated soil. In particular, MSW compost was added in order to stimulate microbial activity, Medicago sativa plant was used for its potential to enhance both soil quality and PCB dissipation and Apirolio as an additional PCB source. Some soil samples were amended with Apirolio in order to simulate an accidental spill of this oil in the environment as it can frequently occur from electronic wastes.

The PCB-contaminated and degraded soil was collected from an experimental area in which a phytoremediation strategy, using as the main detoxifying plant the poplar clone Monviso (Populus generosa x Populus nigra), is in progress (Figure 4.4); a possible seeding of Medicago sativa between poplar tree rows was projected to improve soil recover both in term of fertility and decontamination and a microcosm experiment was set-up in order to verify, under controlled conditions, this possibility.

The microcosm experiment was maintained under the eight different conditions for 224 days and at fixed times 6 PCB markers and 12 dioxin-like PCBs were searched for and the changes in the structure (cell abundance, phylogenetic characterization) and functioning (cell viability, dehydrogenase activity) of the autochthonous microbial community were evaluated.

Figure 4.4 - Experimental area, in Taranto, in which the phytoremediation strategy is in progress.

4.2 Material and Methods

4.2.1 Description of soil, compost and plant

Soil samples were collected from the surface layer (0-15 cm) of the PCB-contaminated area called ex-campo Cimino-Manganecchia, (Taranto), located in front of the Mar Piccolo (Figure 4.5). The northern part of the contaminated area is bordered to a small power station, with electrical transformers. Uncontrolled spill and improper disposal of dielectric fluids (oil containing polychlorinated biphenyls) from electric transformers, have resulted in PCBs pollution over a 30-year period. The area of about 5000 mq^2, consisted of originating limestone soil mixed with backfill (also of marine origin); a mix of organic and inorganic wastes as well as plastics, bulky waste, various debris because this area has been used for a long time as an uncontrolled landfill.

Figure 4.5 - Location of the contaminated area in Southern Italy (Taranto).

The experimental contaminated area has been processed before planting poplars through weeding, milling, plowing, composting (25,78 t/ha). Firstly, some soil samples were collected and analyzed for a preliminary characterization of the amount of PCBs and metals present and of soil quality in terms of natural microbial community abundance and activity.

Secondly, some soil samples were collected from the three different points in which PCB contamination was found to have the highest values and were taken to the lab for the microcosm experiment set-up. The soil pH (in water) was mildly alkaline (8); the organic carbon content was

1.6% and about 3% the organic matter content; the nitrogen and phosphorus (evaluated by determination of assimilable) content were quite scarce (0.20 g/kg and to 8.5 mg/kg, respectively). The soil was classified as a sandy loam according to the USDA soil classification system (sandy 51%, silt 21%, clay 20%).

Municipal Solid Waste compost (MSW compost) was added to soil in amount of about 26 t/ha. It was produced and supplied by CIC (Italian Composters Consortium) and its quality was certified according to ISO/IEC 17025. The main characteristics of MSW compost are shown in Table 4.1.

Table 4.1: Characteristics of municipal solid wastes compost (dry basis).

	Units	**Value**	**Limit (D.Lgs 75/2010)**
Total Phosphorus	% P_2O_5	1,5	
Total Potassium	% K_2O	1,8	
pH		8,0	6 – 8,5
Electrical conductivity	dS/m	4,43	
Moisture	% m/m	15,4	Max 50
Organic Carbon	% C	26,3	Min 20
Humic and Fulvic C	%	10,7	Min 7
Total Nitrogen	% N	2,17	
Ratio Carbon/Nitrogen		12,12	Max 25
Cadmium	mg/Kg	0,5	Max 1,5
Hexavalent Chromium	mg/Kg	<0,50	Max 0,50
Mercury	mg/Kg	0,35	Max 1,5
Nickel	mg/Kg	9,9	Max 100
Lead	mg/Kg	36,1	Max 140
Cupper	mg/Kg	95,5	Max 230
Zinc	mg/Kg	236,6	Max 500
Sodium	mg/Kg	6143,2	
Inert (glass, plastics, metals) > 2mm	%	0,17	Max 0,5
E. coli	CFU/g	<25	Max 1000
Salmonella	CFU/g	Absent	Absent
Salinity	meq/100g	65,5	
Ammonia nitrogen	% N	0,05	

Medicago sativa L. (Alfalfa) is a deep-rooted perennial plant that belongs to the Leguminosae (pea) family. In some parts of the world, it is also known as lucerne. Alfalfa has small divided leaves, purple clover-like flowers, and spiral pods (Figure 4.6). It has been widely cultivated since ancient times as a livestock feed and cover crop, producing both high-yield and high-quality forage.

Alfalfa is more drought tolerant than grasses; however, it is not well adapted to acidic or poorly drained soils. In addition to being an excellent forage crop, alfalfa enhances the stability of agricultural production systems by improving soil productivity and reducing soil and water losses (Chekol et al., 2001).

Several plants have been shown to enhance the dissipation of PCBs in soil (Dzantor et al. 2000; Chekol et al. 2004; Mackova et al. 2009; Ding et al. 2011) and Alfalfa was found to selectively support the growth of not only symbiotic nitrogen fixing bacteria (Xu et al. 2010), but also of PCB-degrading bacteria, such as *Pseudomonas fluorescens* F113 (Villacieros et al. 2003). Previous papers showed that alfalfa (Mehmannavaz et al. 2002; Xu et al. 2010; Tu et al. 2011) facilitated PCB removal.

Figure 4.6. *Medicago sativa L.* and its different functional elements (stem, inflorescence, seeds).

Before seeding *Medicago sativa*, a germination test (OECD 208 - Seedling Emergence and Seedling Growth Test) was performed using the contaminated soil with and without the addition of the commercial Apirolio (100 mg/kg and 100 mg/kg). The results showed that the plant was able to grow in the collected contaminated soil, even if it was poor in nutrients and organic carbon content.

4.2.2 Microcosms set up

Soil samples for microcosms set up were collected from 3 points of the experimental site where the highest values of PCB were found. Stones and plant root residues in the soil samples were discarded and they air-dried and then sieved through a 2-mm mesh.

Equal proportions of each sample were mixed together and then divided per each microcosm consisting of polyethylene pots (10×11 cm) for plants (1L capacity).

Some pots were filled with 800 g of soil (Historically Contaminated Soil microcosms); some pots with the soil thoroughly mixed with the MSW compost at the final concentration of 26 t/ha, corresponding to an agronomic dose (Compost microcosms) and others were filled with soil treated with the commercial Apirolio* (100 mg/kg).

Finally, to half of the entire experimental set, eight seeds of Alfalfa (*Medicago sativa*) were added. Alfalfa was chosen for both its potential to enhance PCB dissipation and its ability to improve soil quality, being a nitrogen-fixing plant. For each different condition were performed 3 replicates (28 microcosms). The final soil water content was 60 %.

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*Apirolio addition to soil samples

In many studies (Salemi et al., 2012; Correia-Sá et al., 2012; Rashid et al., 2010), to reach a specific PCBs concentration, the solid sample is contaminated by the addition of standard solutions dissolved in volatile organic solvent such as n- hexane or acetone, in order to obtain a uniform distribution of the contaminant within the sample. The solvent is then evaporated at room temperature or in the oven at the temperature compatible with the matrix and with the analytes This type of procedure, however, is not suitable for phytoremediation experiments, because the organic solvent may alter the microbiological characteristics of the soil. Therefore, in this experiment, we distributed 100 mg/kg of Apirolio on a certified sand in order to obtain extremely small solid particles, which were subsequently dispersed in an appropriate amount of soil.

The experimental treatments were therefore as follows (Figure 4.7):

- **HCS**: historically contaminated soil (untreated microcosms);
- **HCS +Plant**: historically contaminated soil microcosms where Alfalfa was planted;
- **HCS+compost**: historically contaminated soil microcosms amended with MSW compost;
- **HCS+compost +Plant**: historically contaminated soil microcosms amended with MSW compost where Alfalfa was planted;
- **Apirolio**: historically contaminated soil microcosms treated with Apirolio;
- **Apirolio +Plant**: historically contaminated soil microcosms treated with Apirolio where Alfalfa was planted;
- **Apirolio+compost**: historically contaminated soil microcosms treated with Apirolio and MSW compost;
- **Apirolio+compost +Plant**: historically contaminated soil microcosms treated with Apirolio and MSW compost where Alfalfa was planted.

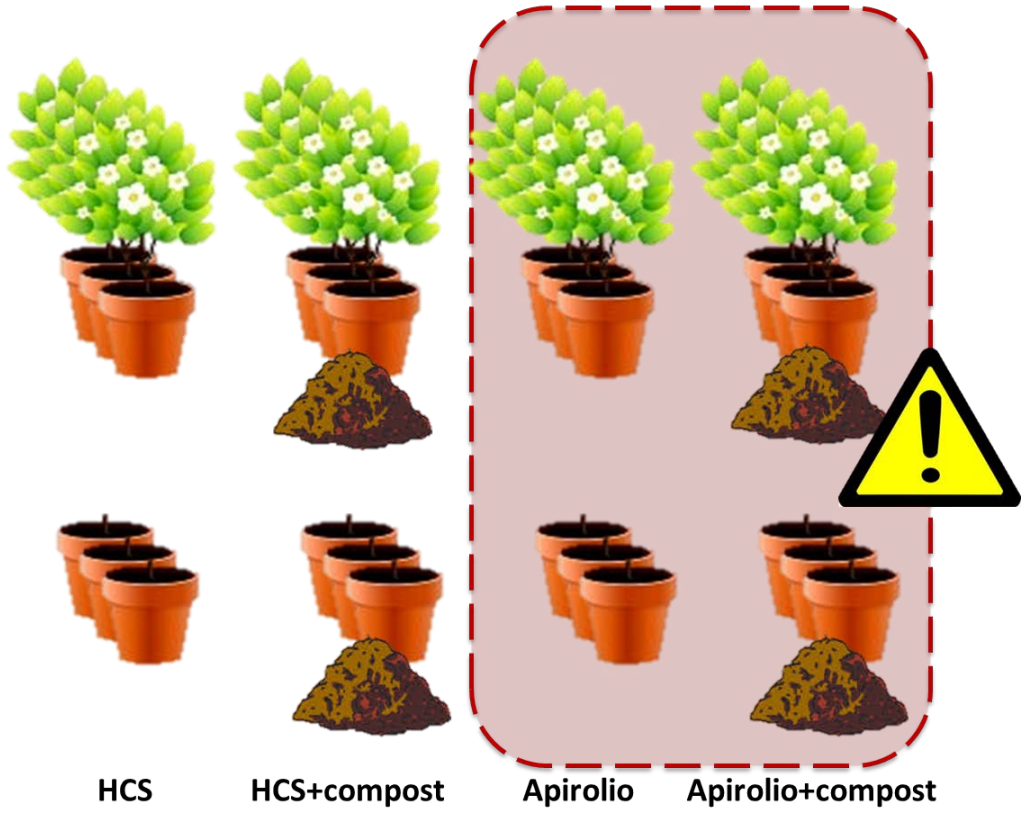


Figure 4.7 – Schematic illustration of the microcosm experiment set up: 8 different conditions were present, 4 with plant and 4 without plant (HCS +Plant, HCS+compost +Plant, Apirolio +Plant, Apirolio+compost +Plant, HCS, HCS+compost, Apirolio, Apirolio+compost). For each condition 3 replicates were present

All the microcosms (24) were kept in a heated greenhouse under controlled temperature (25 °C) conditions for eight months. The pots were watered regularly and the plants maintained gravimetrically at 60% of their field capacity throughout the experiment.

As plant roots fill the whole soil, at each sampling time, all the soils were destructively collected, homogenized, and divided into two sets. One was immediately used for the microbiological analysis such as microbial abundance, and dehydrogenase activity and the other was stored at −20 °C for PCBs and Ester-Linked Fatty Acids (ELFAs) extraction. The total microbial abundance and the dehydrogenase activity were evaluated at the same time of the PCB analysis because they can be considered good indicators for evaluating the status and functioning of soil microbial communities (Rogers and Tate, 2001; Grenni et al., 2009). Moreover, a characterization of the main microbial groups present in soil was performed at the end of the experiment in order to evaluate if the different treatments alone or combined (plant and/or compost and/or apirolio) could affect the soil microbial community structure. For this purpose two methods were used such as the analysis of Ester-Linked Fatty Acids (ELFAs) which makes it possible a phenotypic fingerprinting of soil microbial communities (including Eucaryotic microfungi) and the more specific Fluorescence In Situ Hybridization which identify at different phylogenetical level *Bacteria* and *Archaea*. The identification of microbial groups by these methods is related to their physiological state and activity.

4.2.3 Microbial abundance and dehydrogenase activity

Microbial abundance (No. cell g−1 soil) was measured by the epifluorescence direct count method, using DAPI (4',6-diamidino-2-phenylindole) as the fluorescent agent. The DAPI method is able to detect all the microbial cells in a sample whatever their physiological state and metabolic activity and for this reason is suitable for total microbial counts (Barra Caracciolo et al., 2005a). Briefly, two soil subsamples (1 g) were collected from each replicate microcosm and immediately transferred to a test tube containing 9 ml of a fixing solution (composed of phosphate-buffered saline: 130 mM NaCl; 7 mMNa2HPO4, 3 mM NaH2PO4; 2% formaldehyde (v/v); 0.5% Tween 20 (v/v) and 100 mM sodium pyrophosphate). Moreover, other two soil sub-samples (5 g) were collected from each replicate microcosm in order to analyse microbial dehydrogenase activity, by measuring the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) solution to triphenylformazan (TPF) in line with the method reported in Bending and Rodriguez-Cruz (2007). Thismethod is based on extraction and colorimetric determination of the intensely coloured 2,3,5-triphenyl formazan (TPF) produced from the reduction of colourless 2,3,5-triphenyltetrazoliumchloride in soils after 24 h of incubation at 37 °C in the dark (Grenni et al., 2012). Results were expressed as μg TPF g−1 dry soil. Each microbiological datumis themeans of six values fromthe three microcosm replicates.

4.2.4 Microbial community structure evaluated by FISH

The microbial community structure in the MSW compost applied and the effects of the organic amendment, the contaminant and plant occurrence on microbial community structure in microcosms soil were evaluated at the beginning and at the end of the experiment (1 day; 224 days) by applying Fluorescence In Situ Hybridization (FISH). Two soil sub-samples (1 g) were collected from each replicate microcosm and transferred to a test tube containing the above described fixing solution used for measuring microbial abundance.

Prior to FISH an additional cell purification step was performed with a density gradient mediumin order to detach cells from soil particles, as described in detail in Barra Caracciolo et al. (2005b). Fluorescence In Situ Hybridization of the harvested cells, counter stained with DAPI, was performed using published protocols (Pernthaler et al., 2001; Barra Caracciolo et al., 2005a; Barra Caracciolo et al., 2005b).

Fluorescent probes were applied for the identification, under the epifluorescence microscope, of the *Archaea* and *Bacteria* domains. Within the *Bacteria* domain the major bacterial divisions commonly found in soil such as α-, β-, γ-, δ- *Proteobacteria, Cytophaga-Flavobacterium* lineage of

the *Bacteroidetes* (CFB group) were investigated. For this purpose the following oligonucleotide probes were applied: Arch915 (for *Archaea*), EUB338I-III (for *Bacteria*) and inside this domain ALF1b, BET42a, GAM42a, DELTA495a,b,c (for α-, β-, γ-, δ-, *Proteobacteria*, respectively) and finally CF319 (for *Cytophaga-Flavobacterium* lineage of the *Bacteroidetes*). All probes were synthesized by MWG AG Biotech, Germany. Further details of these probes are available at http://www.microbial-ecology.net/probebase (Loy et al., 2007). Hybridizations were carried out with Cy3-labelled oligonucleotide probes.

The slides were mounted with drops of VECTASHIELD Mounting Medium (Vector Laboratories) and the preparations were examined and counted with a Leica DM LB 30 epifluorescence microscope at 1000 magnification, counting a minimum of 300 cells per section. The estimation of cells binding to the fluorescent probes is calculated as a percentage of the total DAPI positive cells.

4.2.5 Microbial community structure evaluated by ELFA

The use of microbial lipids to identify microorganisms and characterize microbial communities in natural systems constitutes a well-established method. Indeed, fatty acids are the key component (about 40%) of cellular membranes of all living cells. The most widely used extraction and separation method to obtain fatty acids derived from phospholipids is that proposed by Bligh and Dyer (1959) and modified by White et al. (1979). PLFAs are above all useful biomarkers or signatures for fingerprinting the soil microbial community because of relative abundance of certain PLFAs, which differ considerably among the specific group of microorganisms. This approach, based on the variability of fatty acids present in cell membranes of different organisms, allows a phenotypic fingerprinting of soil microbial communities (Table 4.2).

Table 4.2: Fatty acids used as biomarkers

Fatty acids	Biomarkers	References
i15:0, a15:0, 15:0, i16:0, 16:1ω7c, i17:0, a17:0, 17:0, cy17:0, 18:1ω7c, cy19:0	Total bacterial biomass	Frostegård and Bååth (1996), Bailey et al. (2002)
18:2ω6,9c	Fungal biomass	
i15:0, a15:0, i16:0, i17:0, a17:0	Gram-positive bacteria	O'Leary and Wilkinson (1988), Zogg et al. (1997)
16:1ω7; 18:1ω7; cy17:0; cy19:0;	Gram-negative bacteria	Zelles (1999), Waldrop et al. (2000)
18:1ω9c; 16:1ω5	Arbuscular mycorrhizal fungal biomass	Madan et al. (2002), Olsson (1999)
10Me16:0; 10Me17:0; 10Me18:0	Actinomycetes (recently renamed actinobacteria)	Lechevalier (1977)

Recently, microbial fatty acids can be directly extracted from soil by a simple method that reduces the costs and analysis time. It consists of a mild alkaline reagent to lyse cells (KOH in methanol) and release fatty acids from lipids (ester-linked fatty acids; ELFAs) once the ester bonds are broken (Schutter and Dick 2000).

In this study, soil microbial community structure was characterized by ester linked fatty acid (ELFA) profiles of microcosms soil samples. From a 3 g freeze-dried sub-sample, ELFAs were extracted by the method of Schutter & Dick (2000). Briefly, this consists of a mild alkaline methanolysis, neutralization and extraction with the conversion of fatty acids to fatty acid methyl esters (FAMEs). The FAMEs were extracted and dried down for 1 hour in a centrifugal evaporator, resuspended in 100 ml hexane and transferred into vials for gas chromatography (GC) analysis. The FAMEs were analyzed on a Pye Unicam PU4400 gas chromatograph (GC) with a flame ionisation detector. The detector and injector of the GC were set to a temperature of 320 C° and the column was programmed to heat in three stages from 60 C° to a maximum of 310 C°. The eluted peaks were recorded and identified with reference to a qualitative standard of bacterial acid methyl esters.

The fatty acid nomenclature used below is that described by Ranneklev & Bååth (2003): fatty acids are designated as the total number of carbon atoms:number of double bonds with the position closest to the aliphatic end of the molecule indicated with the geometry c for cis and t for trans. The prefixes i and a refer to iso and anteiso branching, respectively. Cyclopropane fatty acids are designated by the prefix cy. The prefix OH is used to designate hydroxyl groups, with the position from the carboxyl groups indicated.

Fatty acids i15:0, a15:0, i16:0, i17:0 and a17:0 were chosen to represent Gram positive bacterial markers (Frostegård et al., 1991; Grayston et al., 2004; Joergensen & Potthoff, 2005). The cyclopropane fatty acids 17cy and 19cy and the monoenoic and cyclopropane unsaturated ELFAs 16:17c and 18:17c were chosen to represent Gram negative bacterial markers (Zogg et al., 1997; Johansen & Olsson, 2005). Fatty acids 18:1w9c 18:2w6,9c were selected as a fungal marker and the ratio of fungal to bacterial markers was used as an indicator of changes in the relative abundance of these two microbial groups (Frostegård & Bååth, 1996). Methyl branched fatty acid, commonly used to represent actinomycetes, were not found. As such, the ELFA data give a qualitative insight into the microbial population and its structure.

The fatty acid C19:0 phosphatidylcholine was added as an internal standard before methylation. Fatty acid peak areas were converted to nmol g_1 soil using internal standards. Lipids were assigned to functional groups according to previous studies (Frostegard et al., 1993; Zelles, 1999; Balser and Firestone, 2005).

4.2.6 Chemical analysis: extraction, identification and quantification of specific PCB congeners

The extraction, identification and quantification of specific PCB congeners was carried out by the Water Research Institute of the National Research Council (IRSA-CNR) in Bari. The quantitative analysis of polychlorinated biphenyl congeners was carried out by the EPA method 1668, optimized for the quantitative determination of seven PCB congeners (28, 52, 101, 118, 138, 153 and 180) although it is possible to measure other congeners. To the detection of PCBs in microcosms soil and in plants, the 6 marker PCBs (28, 52, 101, 138, 153, 180) (Table 4.3), the most bio-accumulated in food chains and the 12 dioxin-like PCBs (77, 81, 123, 118, 114, 105, 126, 167, 156, 157, 169, 189) (Table 4.4), so called for dioxins similar toxicological properties, were analyzed.

The extraction was carried out using ASE technique (Accelerated Solvent Extraction) as reported by the technical Thermo Scientific, Technical Note 210 (2012). The advantage of this technique lies in the procedures for the removal of interference by the use of adsorbents, combining extraction and purification in a single step. Before being packed in the cells of extraction, samples (5 grams each replica) previously lyophilized, were spiked with two internal standards (104 and 194). Soil extracts were analyzed for total PCBs using Gas Chromatography coupled to mass spectrometry (Thermo Scientific Finningan TRACE GC ultra). The lower limit of detection of the instrument is 0.5 µg / kg. According to the conditions set by this standard method, single PCB congeners with concentrations of at least 0.5 µg / kg of dry matter can be determined without interference,

Table 4.3: IUPAC name and molecular structure of 6 marker PCB congeners 28, 52, 101, 138, 153, 180

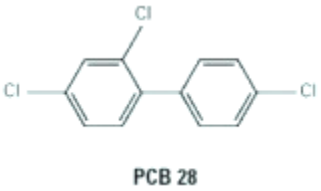
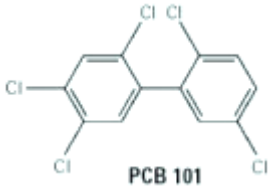
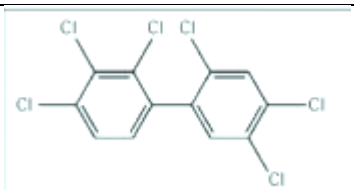
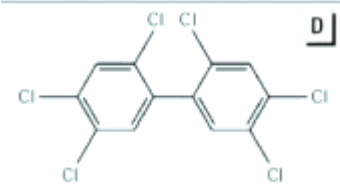
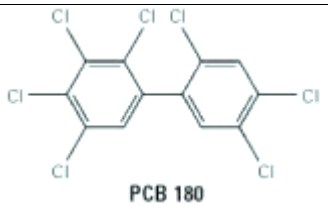
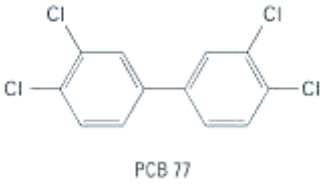
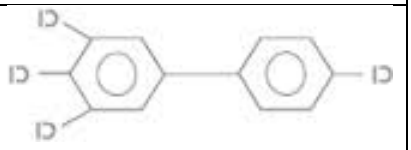
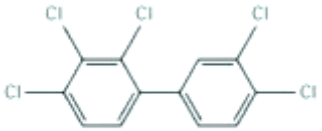
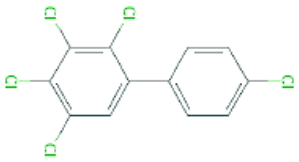
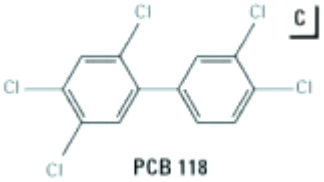
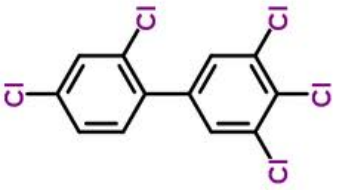
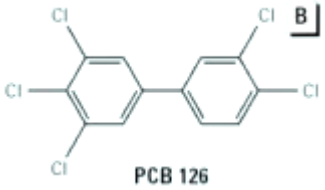
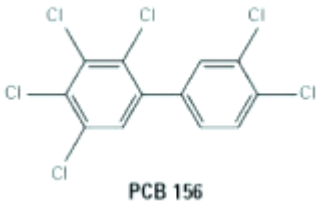
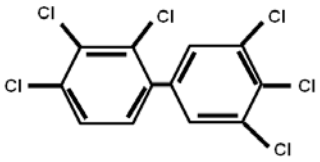
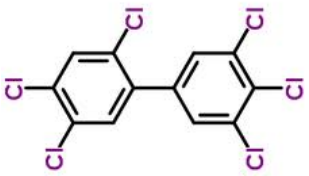
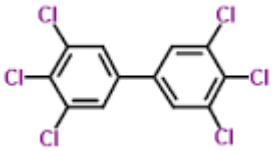
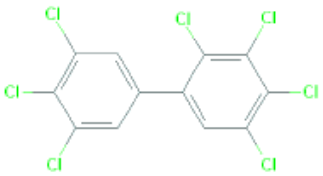
Congener number	IUPAC name	Molecular structure
28	2,4,4'-**Tri**chlorobiphenyl	 PCB 28
52	2,2',5,5'-**Tetra**chlorobiphenyl	 PCB 52
101	2,2',4,5,5'-**Penta**chlorobiphenyl	 PCB 101
138	2,2',3,4,4',5'-**Hexa**chlorobiphenyl	 PCB 138
153	2,2',4,4',5,5'-**Hexa**chlorobiphenyl	 PCB 153
180	2,2',3,4,4',5,5'-**Hepta**chlorobiphenyl	 PCB 180

Table 4.4: IUPAC name and molecular structure of 12 dioxin-like PCB congeners 77, 81, 123, 118, 114, 105, 126, 167, 156, 157, 169, 189

Congener number	IUPAC name	Molecular structure
77	3,3',4,4'-**Tetra**chlorobiphenyl	 PCB 77
81	3,4,4',5-**Tetra**chlorobipheny	
105	2,3,3',4,4'-**Penta**chlorobiphenyl	 PCB 105
114	2,3,4,4',5-**Penta**chlorobiphenyl	
118	2,3',4,4',5-**Penta**chlorobiphenyl	 PCB 118
123	2,3',4,4',5'-**Penta**chlorobiphenyl	
126	3,3',4,4',5-**Penta**chlorobiphenyl	 PCB 126

156	2,3,3',4,4',5-**Hexa**chlorobiphenyl	 PCB 156
157	2,3,3',4,4',5'-**Hexa**chlorobiphenyl	
167	2,3',4,4',5,5'-**Hexa**chlorobiphenyl	
169	3,3',4,4',5,5'-**Hexa**chlorobiphenyl	
189	2,3,3',4,4',5,5'-**Hepta**chlorobiphenyl	

4.2.7 Statistical analysis

The statistical analysis of dehydrogenase activity, bacterial abundance, FISH data, ELFAs data and PCBs data was done using an analysis of variance (ANOVA), with significant differences at the p b 0.05 level. We applied the two way ANOVA analysis considering time always as variable 1 and the plant, Apirolio or compost occurrence as variable 2. The PC Program used was SIGMASTAT.

4.3 Results

4.3.1 Microbial abundance

In soil samples collected for microcosm experiment, an initial microbial abundance of $4.88 \cdot 10^5$ cells g^{-1} dry soil was measured. One day after the microcosms set up, a significantly increase in cells number was observed were compost was added (HCS+compost = $1.86 \cdot 10^6$ No. cells g^{-1} dry soil) comparing to other conditions.

The microbial abundance (No. cells g^{-1} dry soil) in the soil microcosms at the end (224dd) of the experiment in HCS, Apirolio, HCS+compost and Apirolio+compost in the absence/presence of the *Medicago sativa* is reported in Figure 4.8. The microbial abundance in MSW Compost was $8.10 \cdot 10^7$ No. cells g^{-1} dry compost.

At the end of the experiment (day 224) the microbial abundance was lower in HCS< HCS+Plant<Apirolio<Apirolio+Plant<Apirolio+Compost≤HCS+Compost+Plant≤HCS+Compost< Apirolio+Compost+Plant.

The highest values of abundance were due to the compost addition (two-way analysis of variance, $p < 0.05$), however it was also possible to observe a positive effect of Apirolio (Apirolio > HCS) and plant (HCS+plant> HCS; Apirolio+Plant>Apirolio) on the cell number (Figure 4.8)

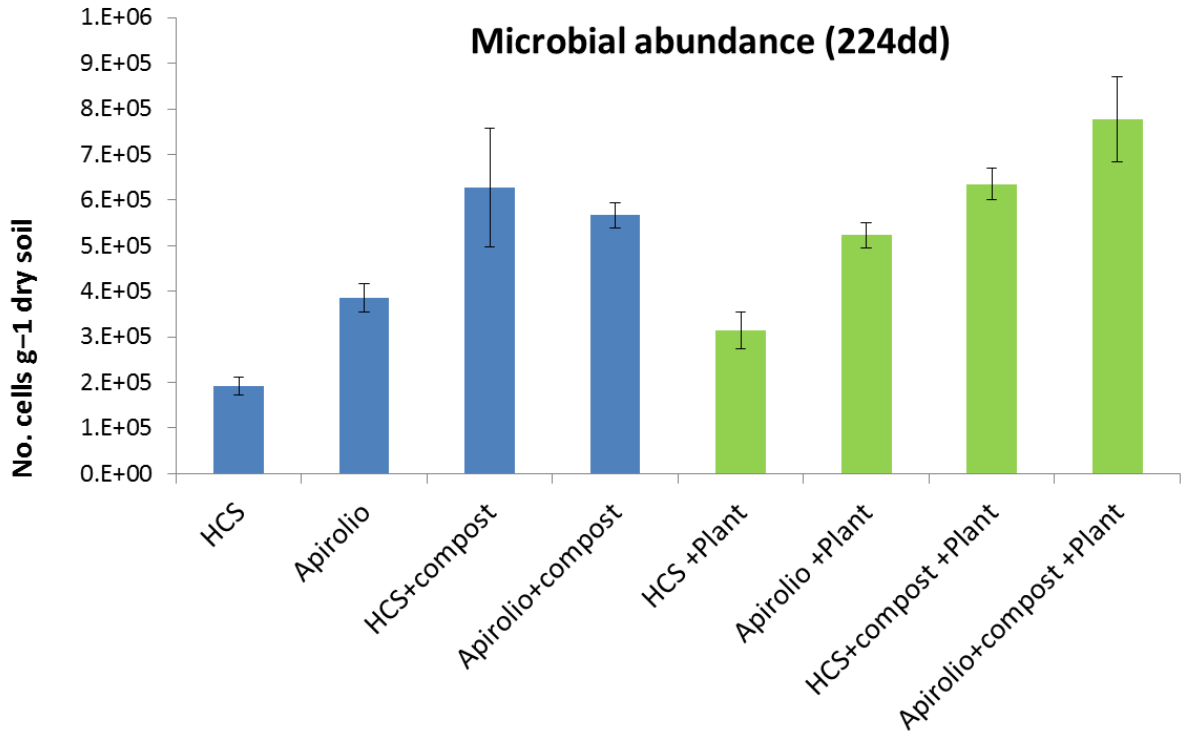


Figure 4.8- Microbial abundance (No. cell g^{-1} dry soil) at 224 days in the HCS soil, in Apirolio soils and in soils treated with MSW compost (HCS+compost; Apirolio+compost). In blue are microcosms without plant; in green are microcosms with *Medicago s.* plant. The vertical bars represent the standard errors of three values.

4.3.2 Dehydrogenase activity

Soil dehydrogenase activity (µg TPF g^{-1} dry soil) over time in various experimental treatments of the soil microcosms in the absence and presence of the *Medicago sativa* is reported in Figure 4.9.

After 1 day from microcosms set up, the compost addition influenced significantly the microbial activity: HCS+compost and Apirolio+compost values were more than twofold higher than those where compost was not added (HCS and Apirolio). Moreover, the dehydrogenase activity was not negatively affected by the Apirolio addition and their values were similar to those measured in the historically contaminated soil (HCS).

At day 224, a significant increase in dehydrogenase activity was observed (t test, $p < 0.01$) in the presence both of the compost and of the plant. The plant and compost acted in a synergic way in increasing microbial activity (Figure 4.9).

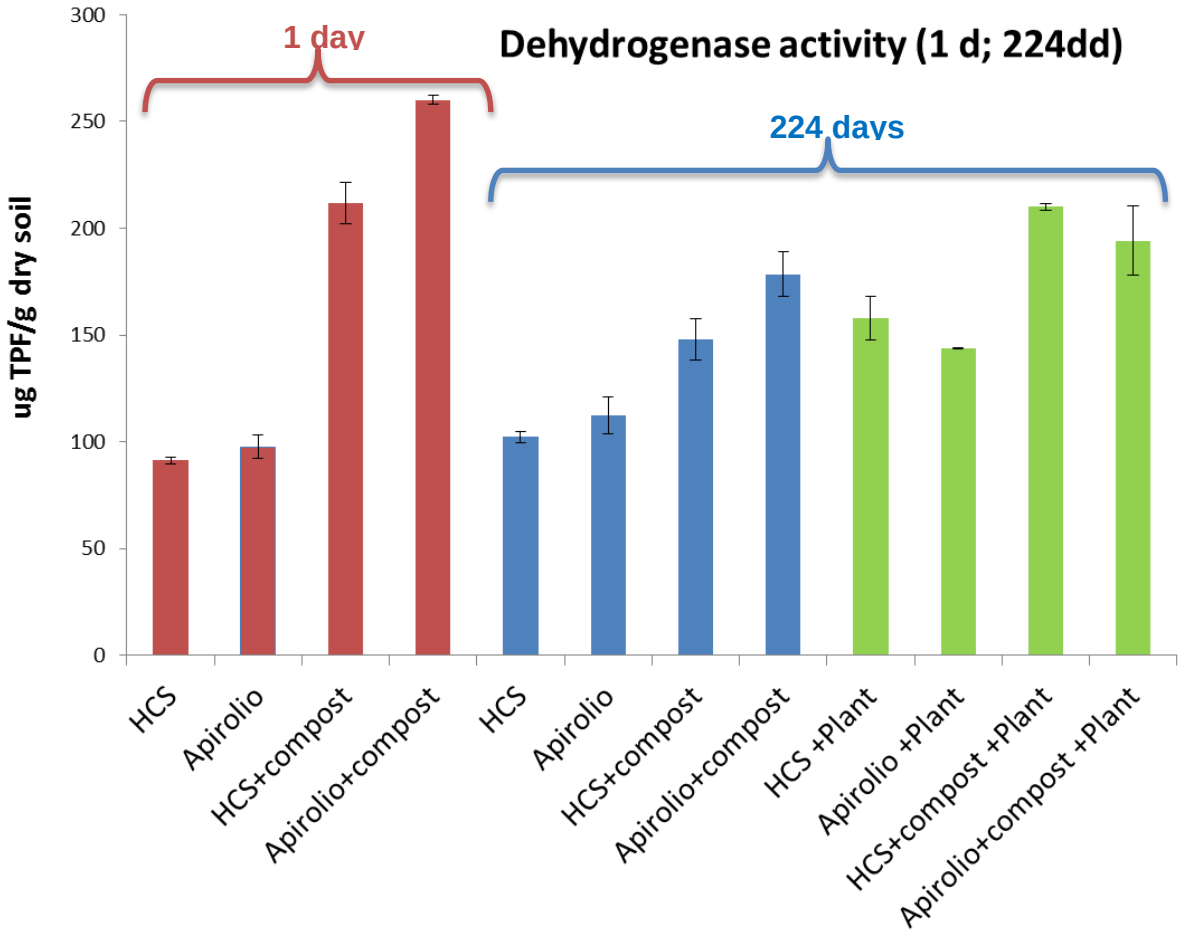


Figure 4.9 - Microbial Dehydrogenase activity (µg TPF g−1 dry soil) over time in the HCS soil, the in Apirolio soil and in soils treated with MSW compost (HCS+compost; Apirolio+compost). In red are 1 day microcosms, in blue are 224 days microcosms without plant; in green are 224 days microcosms with *Medicago s.* plant. The vertical bars represent the standard errors of three values.

4.3.3 Microbial community structure and diversity evaluated by FISH

The Fluorescence In Situ Hybridization (FISH) technique made it possible to determine the structure of the active microbial community of the MSW compost, the initial contaminated soil used to set up the experiment and microcosms soil at the end of the experiment (224 days) and the results are shown in Figure 4.10 and 4.11.

The percentage of *Bacteria* detected in the MSW compost and in the initial soil was 87,7 ± 0.1% (corresponding to $7.1 \cdot 10^7$ cells g^{-1} compost) and 65.6 ± 3.9% (corresponding to $2.2 \cdot 10^5$ cells g^{-1} soil) respectively and inside the Bacteria domain the phylogenetic characterization through FISH method is shown in Figure 4.10.

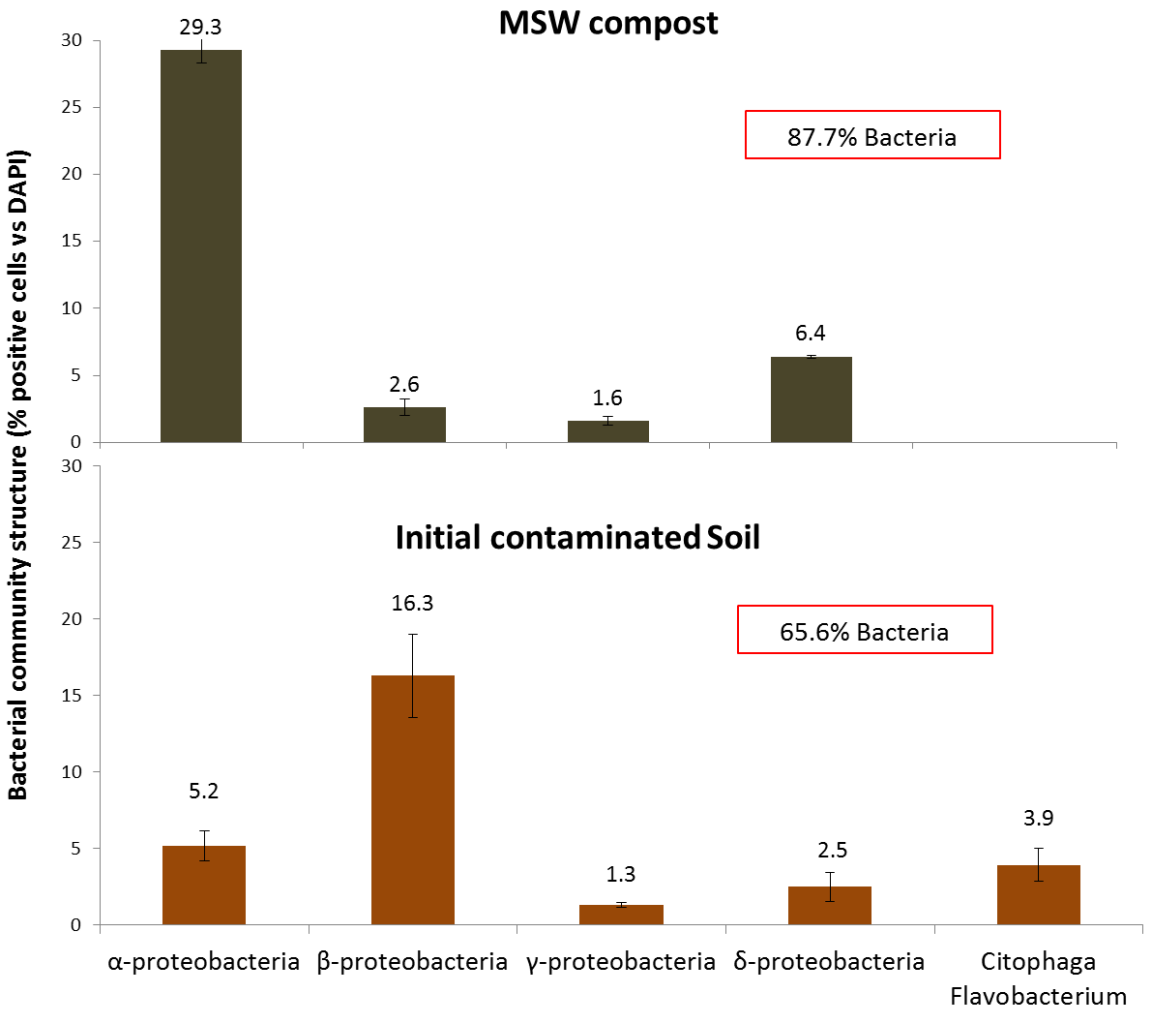


Figure 4.10 - Bacterial community structure detected by FISH in MSW compost (above) and initial soil used used for microcosms set up (below). In horizontal axis are the bacterial taxa detected. The vertical bars represent the standard errors of three values.

At the end of the experiment the bacterial community structure is better characterized in microcosms where Medicago s. was present than in those without plant, mainly because, in the latters, the microbial activity is too low to FISH success. Inside the Bacteria domain a significant shift (p b 0.01) in the dominance of several bacterial groups was observed when plant was present (Figure 4.11). In particular, comparing with initial soil, when the plant was present α-*Proteobacteria* significantly increased, mainly in conditions HCS, HCS+compost and Apirolio+compost. At the same time β-*Proteobacteria* significantly decrease and δ- *Proteobacteria* disappeared.

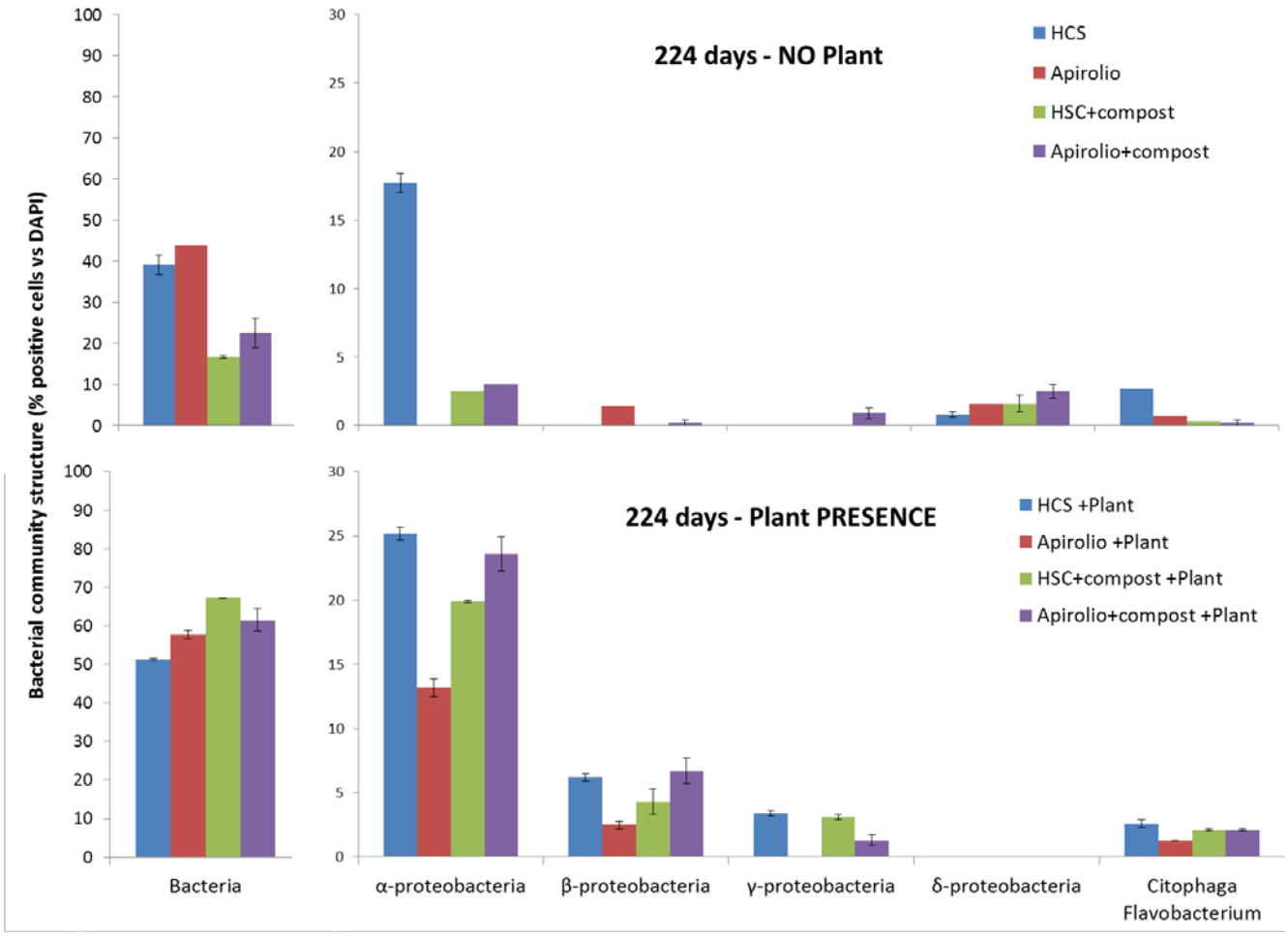


Fig. 4.11 - Bacterial community structure detected by FISH, at day 224, in soil microcosms in plant absence (above) and in plant presence (below). In horizontal axis are the bacterial taxa detected. The vertical bars represent the standard errors of three values.

4.3.4 Changes in microbial community composition evaluated by ELFA

Processed lipid data are expressed as mole percent (Mol % = mole fraction*100, 0-100%). Mole percent was normalized by the total biomass in a sample, and it is a measure of the relative abundance of any specific lipid. In this study 18 individual ELFAs were identified by the instrument, however only 11 were used as biomarker, such as the sum of specific ELFAs for Gram positive bacteria (i15:0, a15:0, i16:0 and i17:0), Gram negative bacteria (cy17:0, cy19:0, 16:1ω7, 17:1ω7c and 18:1ω7c) and Fungi (18:1ω9c and 18:2ω6,9c), Figure 4.13.

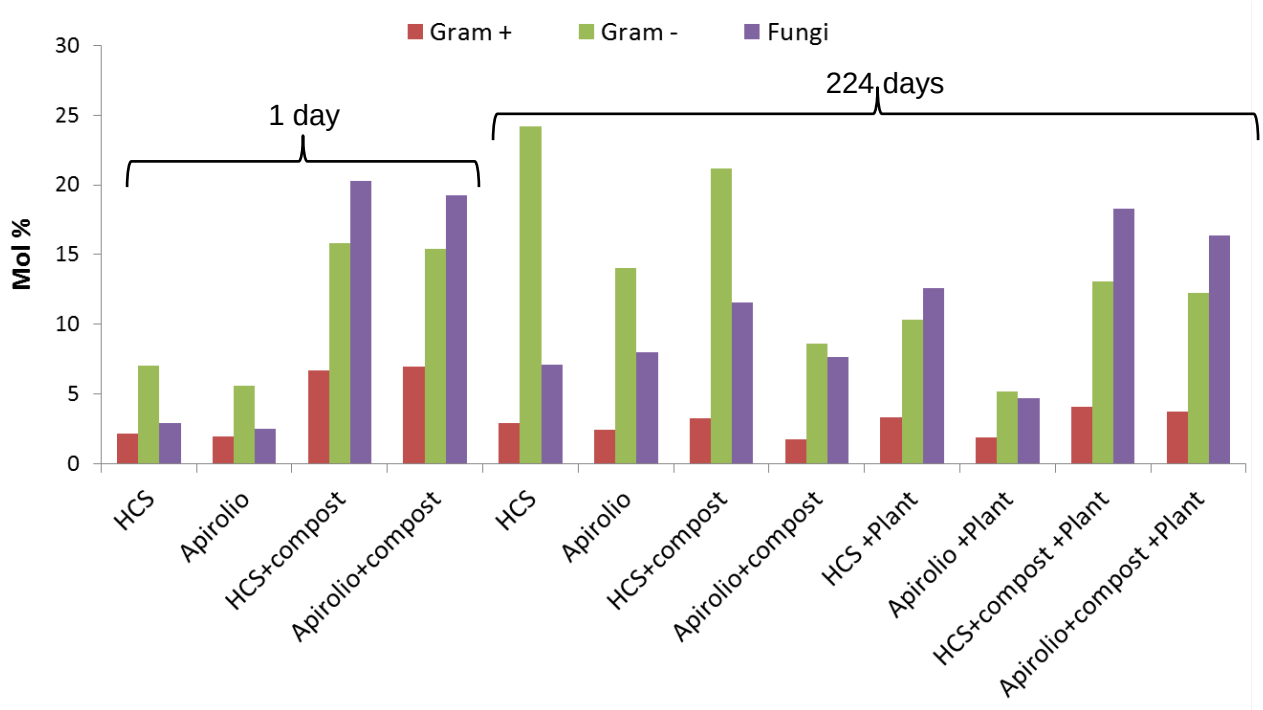


Figure 4.13 - Sum of different ELFAs concentrations, expressed as mole percent, identifying the Gram positive bacteria (Gram +), Gram negative bacteria (Gram -) and Fungi in all microcosms soil at the beginning (1day) and at the end (224days) of the experiment.

At day 1 the compost addition (HCS+compost and Apirolio+compost) affected significantly the increase of the total biomass but in particular Gram positives and negatives became three times higher than the conditions without compost and Fungi ten times higher than non-composted microcosms.

After 224 days, the plant generally influenced the increase in Fungi while in unplanted microcosms a significantly increase in Gram negatives was observed.

The Fungi/Bacteria ratio is generally used to analyze the microbial community status in environmental stressed conditions. In figure 4.14 the Fungi/Bacteria ratio is reported for all the microcosms over the time.

At day 1, the lowest values were measured for non-composted soils.

After 224 days, ratio values in planted soils were generally higher than in those where Medicago s. was absent.

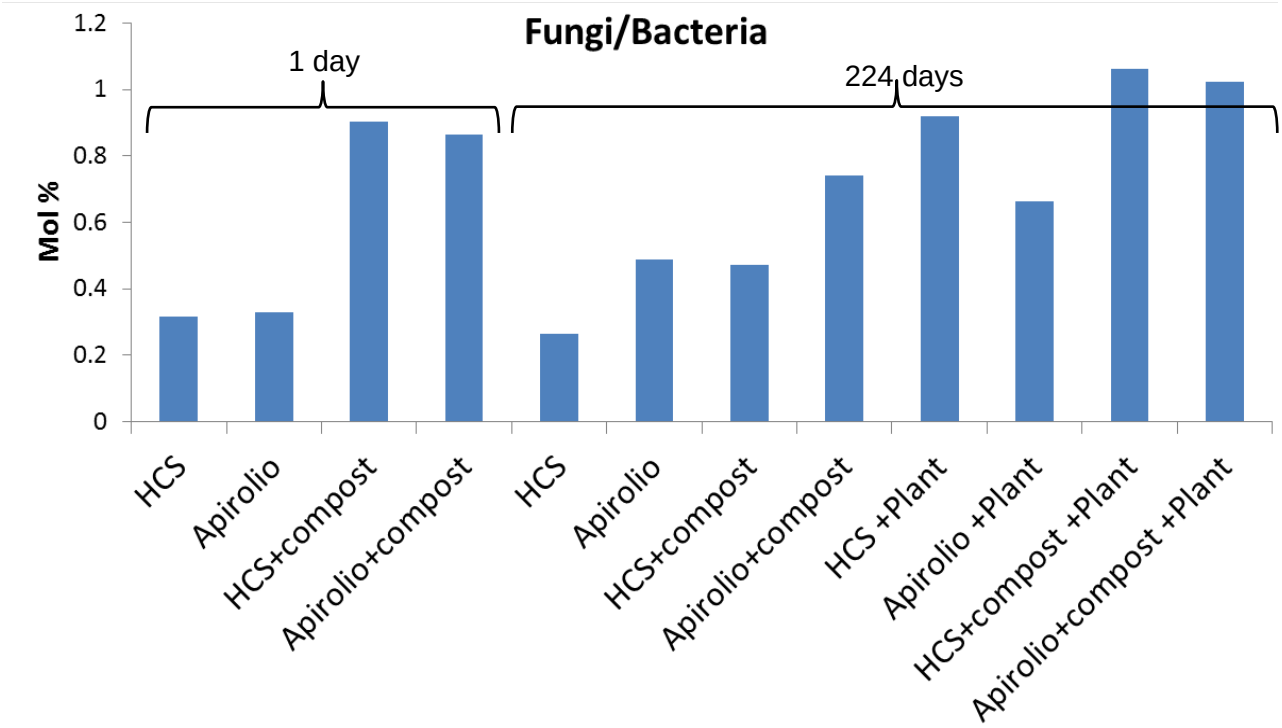


Figure 4.14 - Fungi/Bacteria ratio, expressed as mole percent, in all microcosms soil at the beginning (1day) and at the end (224days) of the experiment.

4.3.5 Concentrations of PCBs in microcosms soil and plant

The results of the 6 PCB markers and 12 dioxin-like PCBs searched for in the historically contaminated soil (HCS, HCS+compost, HCS +Plant, HCS+compost +Plant) are reported in Figure 4.15 A,B,C,D. Some congeners were never found such as PCBs 77, 81, 123, 114 and 169 and 189 presumably because they were not present or below the detection limit of 0.3 µg/L. In the historically contaminated soil (HCS) the most abundant congeners found were PCBs 138, 153, 180 (PCB markers) and among the dioxin-like PCB 118.

Between 0 and day 133 it is possible to highlight a significant plant effect (HCS +Plant) on the occurrence of some congeners with an increase in 138, 153, 180 and 156 (Figure 4.15 B), while in the other microcosms (HCS, HCS+compost, HCS+compost +Plant) a general decrease in most of all the congeners analysed was observed (Figure 4.15 A,C,D) .

In HCS +Plant microcosms all the congeners which previously increased (138, 153, 180) at day 224 decreased and some completely disappeared (e.g. 126). At the same time, in all other microcosms some congeners increased (138, 153, 180) and others decreased (28, 52) following a similar trend in the HCS +Plant microcosms at day 133. PCB126 (penta-Cl) disappeared in all microcosms.

Comparing the amount of all congeners initially found (24h) with those found at the end of the experiment (224 dd) it is possible to highlight a general decrease in all congeners (including those high chlorinated) ascribable to compost, plant and compost+plant presence, while in HCS, PCBs 138, 153, 180 and 118 increased.

In HCS microcosms a significant decrease was observed just in lower-chlorinated congeners such as PCB28 and PCB52, because the end of the experiment the higher-chlorinated 138, 153, 180 and 118 increased.

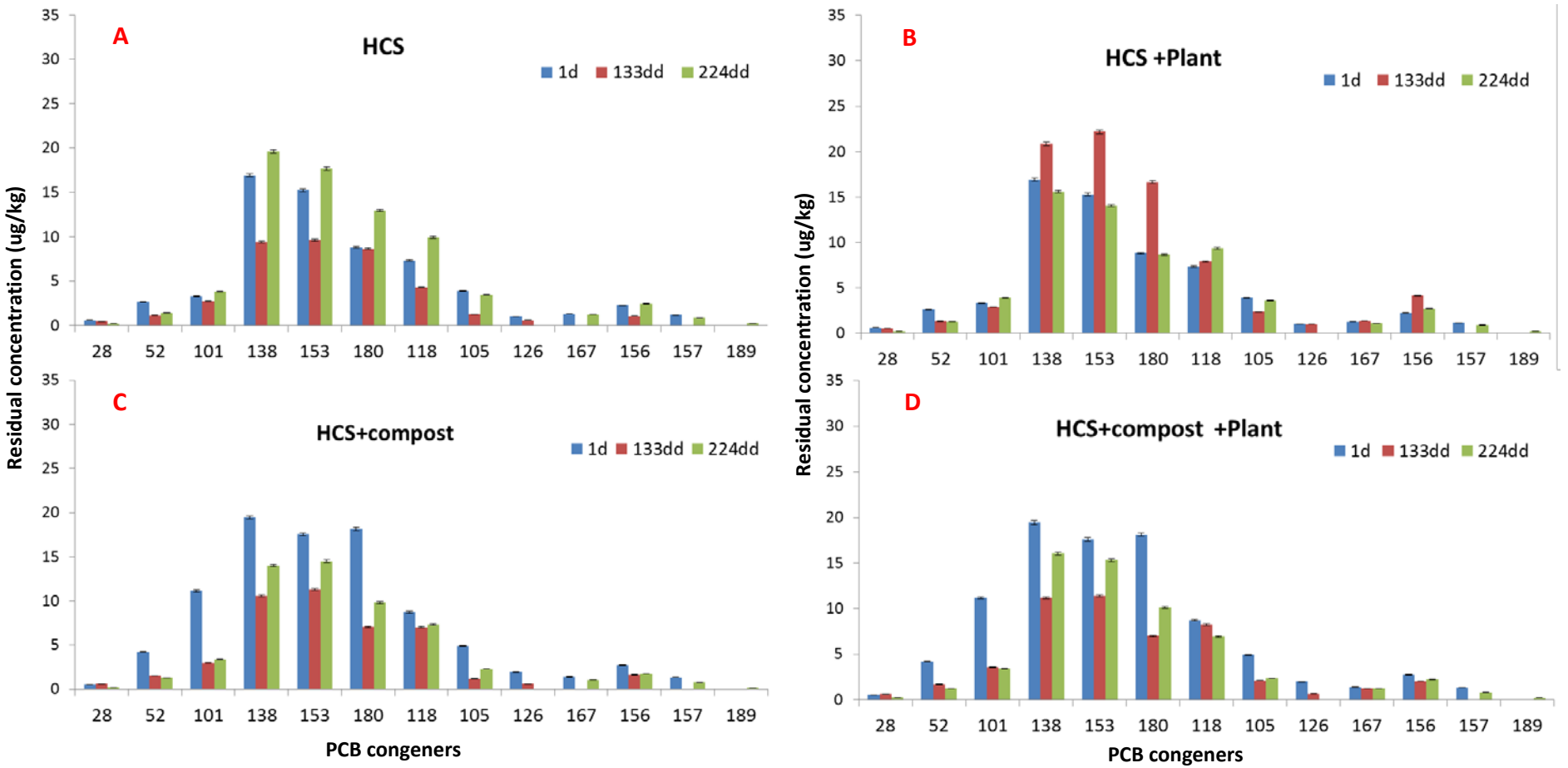


Figure 4.15 (A,B,C,D) – PCB congeners analyzed in historically contaminated soil microcosms, over the time, in presence and absence of *Medicago s.* plant. (**A**: HCS=historically contaminate soil; **B**: HCS +Plant=historically contaminate soil with *Medicago s.* plant; **C**: HCS+compost= historically contaminate soil amended with MSW compost; **D**: HCS+compost +Plant= historically contaminate soil amended with MSW compost, in presence of *Medicago s.* plant. The vertical bars represent the standard errors.

The results of the six PCB markers and 12 dioxin-like PCBs searched for the historically contaminated soil treated with Apirolio (Apirolio, Apirolio+compost, Apirolio +Plant, Apirolio +compost +Plant) are reported in the Figure 4.16 A,B,C,D. Some congeners were never found such as PCB 77, 81, 123, 114, and 169 presumably because they were not present or below the detection limit of 0.3 µg/L.

In the Apirolio contaminated soil an initial higher amount of the congeners PCBs 28, 52 and 167 and 157 were found, suggesting their presence in the commercial products applied. The comparison of transformation PCB congeners between Apirolio and HCS microcosms at the different sampling times does not show the same effect of plant and/or compost presence (Figure 4.16 A). In Apirolio some congeners increased at day 133, such as 101, 138, 153, 180, 118, 105 and 156 and the same trend (except for 180) with higher values was observed in Apirolio+compost +Plant, moreover in the latter condition also PCB 52 increased significantly. As above mentioned, the increase of 101, 138, 153, 180 and that of 105 and 156 can be associated to PCBs 194 and 189 degradation, respectively.

At the end of the experiment, however, lower-chlorinated congeners (28 and 52) and many higher-chlorinated ones (e.g. 167 and 157) decreased in all conditions, although with different percentages comparing to their initial concentration.

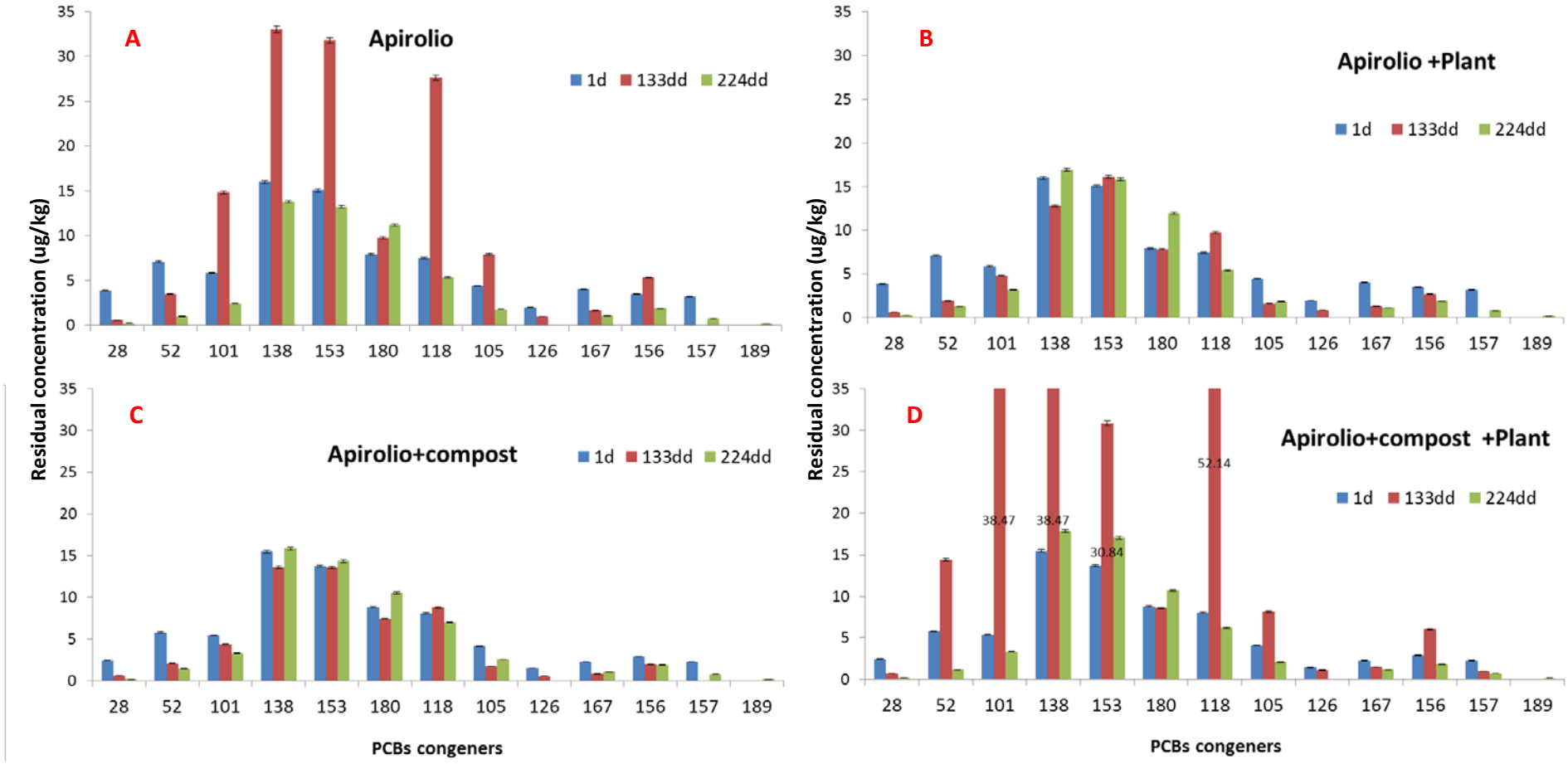


Figure 4.16 (A,B,C,D) – PCB congeners analyzed in historically contaminated soil microcosms amended with the dielectric oil Apirolio, over the time, in presence and absence of *Medicago s*. plant. (**A**: Apirolio=historically contaminate soil amended with Apirolio; **B**: Apirolio +Plant=historically contaminate soil amended with Apirolio and in presence of *Medicago s*. plant; **C**: Apirolio+compost= historically contaminate soil amended with Apirolio and MSW compost; **D**: Apirolio+compost +Plant= historically contaminate soil amended with Apirolio, MSW compost and in presence of *Medicago s*. plant. The vertical bars represent the standard errors.

In Figure 4.17 and 4.18 are shown the amount of specific congeners detected in roots and leaves of some planted microcosms (HCS+compost; Apirolio and Apirolio+compost). It is interesting observe that some congeners moved from roots to leaves, and this is particularly evident where the higher plant biomass was observed (Apirolio+compost).

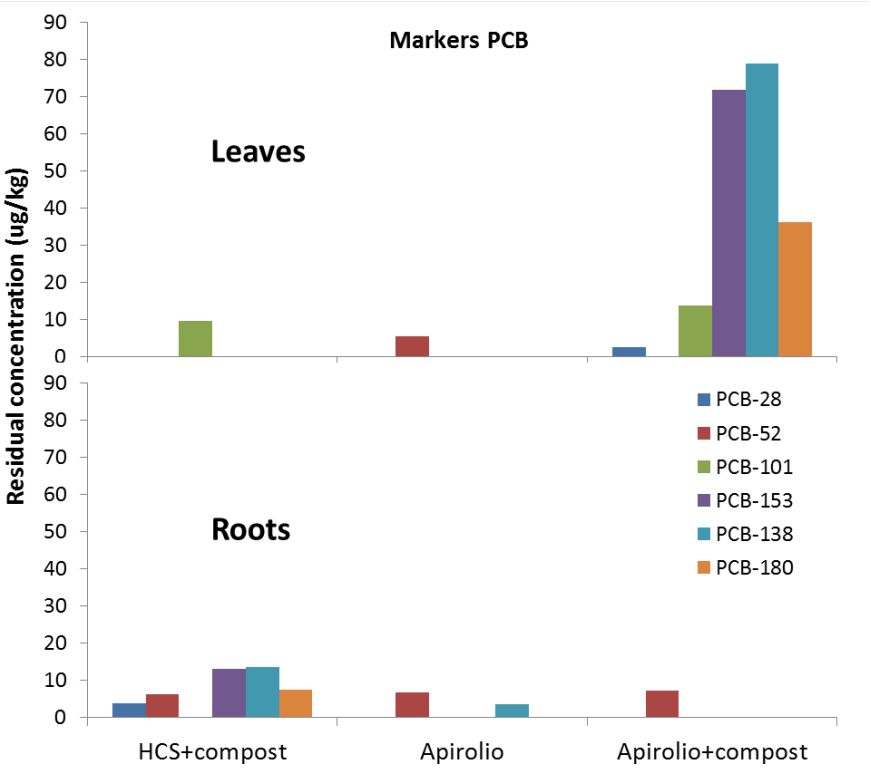


Figure 4.17 – Markers PCB (28, 52,101, 153, 138, 180) detected in leaves (above) and roots (below) at day 224 in the HCS+compost, Apirolio and Apirolio+compost conditions.

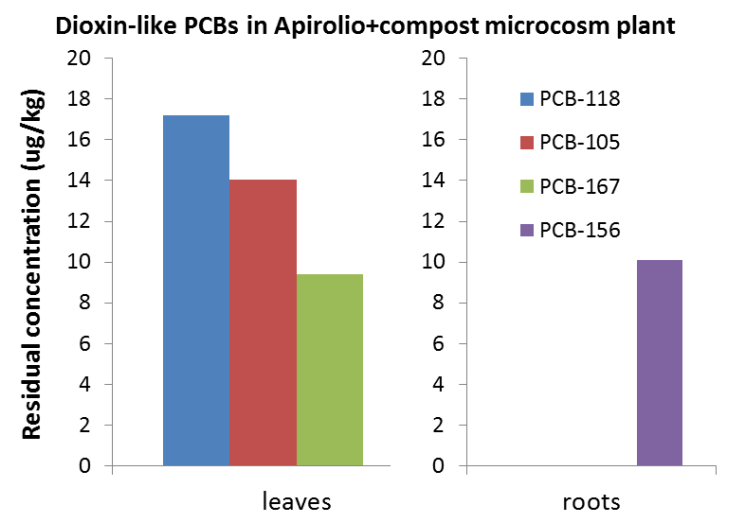


Figure 4.18 – Some Dioxin-like PCB (118, 105, 167, 156) detected in leaves (left) and roots (right) at day 224 in the Apirolio+compost condition.

4.4 Discussion

In this experiment, the addition of Apirolio dielectric oil and/or Municipal Solid Waste compost and the presence/absence of the *Medicago sativa* plant on the microbial community of a PCB historically contaminated soil were tested.

The natural capability of the microbial autochthonous community to transform these molecules was shown, even if is not possible attribute only one and unequivocal degradation pathway for the transformation of all PCB congeners analyzed. PCB biodegradation is the result of many different aerobic and anaerobic pathways which can or not occur in the same environment depending on the possibility that aerobic and anaerobic micro-niche can coexist.

The effects on PCB transformations of *Medicago sativa* and the MSW compost addition were different in the historically contaminated soil or in the Apirolio amended soil.

In any case the microbial abundance and the dehydrogenase activity were positively affected by the compost and plant presence and added together to the soil they increased the overall microbial activity (Figure 4.9). In fact is generally recognized that compost can be a source of carbon (**Grenni et al., 2009; Grenni et al., 2012**) and rhizosphere root exudates can favor the bacterial activity in the long term (**Crecchio et al., 2004**). Moreover, the microbial community structure analysis by the fingerprint method ELFA (ester linked fatty acid) shows how in these conditions an increase in Fungi and Gram-negatives bacteria was observed compared to HCS and Apirolio conditions (Figure 4.13); the lowest Fungi/Bacteria ratio in HCS and Apirolio conditions is a stress indicator showing the low soil quality. The plant addition favored the increase of Gram-negative *Proteobacteria,* as shown by FISH analysis, and improved the soil quality because it is known that they include most bacterial species involved in main biogeochemical cycles (**Barra Caracciolo et al., 2011; Godoi et al., 2014**). In this sense a significant increase in Alpha-*Proteobacteria*, which comprise many genera that are nitrogen fixing bacteria associated to leguminous like *Medicago sativa* was observed.

The bacterial community observed at day 224 was the result of both soil treatments (compost/plant presence) and the PCB transformations which can occur via co-metabolic and metabolic pathways. Inside the Alpha- (e.g. Spingomonas), Beta- (e.g. *Burkholderia, Alcaligenes, Achromobacter, Comamonas, Ralstonia*), and Gamma-*Proteobacteria* (e.g. *Pseudomonas, Acinetobacter*) found by FISH there are species reported to be able to degrade PCBs, and we can suppose the occurrence in the plant-treated microcosm, while in the bulk soil some Gram-positive bacteria could be involved in the degradation pathways (e.g. *Rhodococcus* and *Corynebacterium*).

The high complexity of PCB contamination can be explained by the fact that it is not a single molecule contamination but, as mentioned earlier, it derives from commercial mixtures containing from 60 to 90 different congeners of PCBs and other additives. The study of PCB contamination, therefore, reflects the complexity of these molecules. According to the number of chlorine atoms and their position (ortho, meta, para) on the benzene rings, the several congeners differ in their chemical, physical and toxicological properties as well as in the possibility that they can be more or less easily degraded by microorganisms (**Passatore at al., 2014**).

In this study 6 marker congeners and 12 dioxin-like PCBs were analyzed, according to EU Regulation No. 1259/2011. Therefore, we have to take into account that the 18 congeners analyzed are only a part of those possibly present in the Apirolio mixture used and for this reason the variation of the amount of a specific congener not necessarily corresponds to the increase or decrease of another between those analyzed. Nevertheless some degradative pathways were identified.

The overall results show how autochthonous microbial populations were naturally able to degrade and transform PCBs in the soil from the contaminated area. In fact in the case of HCS microcosms the decrease in PCBs 28 (69%) and 52 (46%) observed could be due to a stimulation of microbial aerobic degradation due to the soil handling (e.g. sieving, watering) before microcosm set up.

The compost and plant addition improved PCB degradation of both lower-chlorinated and higher-ones, acting differently and promoting transformation of the same congeners, but in different times. At the end of the experiment PCB28 decreased by more than 50% of its initial concentration (HCS+Plant 63%, HCS+compost 60%, HCS+Compost+plant 54%) and in the same way PCB52 (HCS+Plant 52%, HCS+compost 68%, HCS+Compost+plant 71%).

PCB transformation from higher-chlorinated to lower-chlorinated (Cl<4) congeners involve different possible degradative pathways where the biphenyl molecule is an electron acceptors. Reductive dechlorination of the higher PCB congeners occurs in anaerobic conditions with an accumulation of lower chlorinated congeners. PCB congeners were mostly attacked in the para and meta positions, resulting in the accumulation of lower congeners with ortho-chlorines (Kimbrough and Goyer, 1985; Safe, 1989; Quensen et al.,1998).

In this study the increase in plant presence (day 133) and subsequent decrease (day 224) in PCBs 180, 138, 153, can be due to the decrease of the hocto-chlorinated PCB194 which was in fact found in a high amount (23 µg/kg soil) in the initial field site characterization. PCB 194 is reported to be transformed in to PCB180 and the latter in turn in to PCBs138 or 153 (hexa-Cl); PCB153 can be transformed into PCB101 (penta-Cl) and the latter into PCB52 (tetra-Cl) (). Although **PCB52** have

2 chlorides in the ortho positions and it is considered quite persistent, it was degraded and this is possible thanks to a 3, 4 (4,5) dioxygenase attack or even a monoxygenase mechanism (Bedard et al., 1987, Komancova et al.,2003). Similarly, **PCB 153** has ortho substituents on both rings, making it more recalcitrant to microbial attack, however microbial degradation has been observed involving the bacteria *Burkholderia* LB 400 and *Alcaligenes eutrophus* (now *Ralstonia eutropha*) H850 (Bedard et al., 1986, Leigh et al. 2006).

Regarding PCBs 118 (penta-Cl) and 156 (exa) they can be transformation products of the higher-chlorinated PCB 189 (epta-Cl);

Finally, degradation of PCB 28 (three-Cl), as well as that of other lower-chlorinated PCBs (<5 Cl) occurs through bacterial dehalogenase and peroxidase, which can also be positively affected by compost and plant presence (Singer et al., 2003; Sylvestre and Toussaint, 2011). A large number of aerobic bacteria are capable of co-metabolizing PCBs with biphenyl as the primary substrate. The biphenyl metabolic enzymes encoded by the bph gene cluster are responsible for the attack of PCB (Abramowicz, 1990; Furukawa, 2000). Aerobic degradation was found in several Gram-negative bacteria, e.g. *Pseudomonas, Burkholderia, Acinetobacter, Alcaligenes, Achromobacter, Comamonas, Ralstonia, Sphingomonas*, and some Gram-positive ones such as *Rhodococcus, Bacillus* e *Corynebacterium* (Field e Sierra-Alvarez, 2008; Luo et al., 2008). Some bacteria have the ability to grow by utilizing PCB congeners of one or two chlorines as sole sources of carbon and energy. Examples of growth on 4-chlorobiphenyl (4-CBp) are common (Furukawa et al., 1978; Masse et al.,1984; Shields et al., 1985; Furukawa and Miyazaki, 1986;Barton and Crawford, 1988; Ahmad et al., 1990); whereas examples on 2-CBp and 3-CBp are less frequent (Bedardet al., 1987; Parsons et al., 1988; Hickey et al., 1992). *Burkholderia cepacia* P166 (formerly Pseudomonas cepacia) utilizes4-CBp, 2-CBp and 3-CBp as growth substrates (Arensdorf and Focht, 1994).

The overall results show how compost and plant occurrence promoted not only lower-, but also higher-chlorinated PCB transformation in different times and ways and at the end of the experiment in their presence most of all congeners analyzed decreased and this is was particular evident in the Compost and Compost+Plant microcosms, showing the synergic effect of these treatments in biostimulating natural bacterial community in PCB degradation.

The comparison of PCB congeners transformation between Apirolio and HCS microcosms does not show the same effect of plant and/or compost presence at the different sampling times. The Apirolio addition was performed in order to simulate an accidental leak of this oil in the environment as it can frequently occur from devices improperly disposed. Apirolio is a mix of several PCB congeners which contains a variable chloride percentage and other organic solvents (including hydrocarbons) and when it is just added to soil its behaviour can be different from that in

an aged apirolio contaminated soil. The PCBs added with the Apirolio treatment were presumably more available to microbial attack than those present in the HCS for a long time (Malik and Elisabeth, 2011; You et al., 2007) and we cannot exclude that other organics present might be a source of carbon for microorganisms. The microcosm results confirm this hypothesis, in fact Apirolio addition to the HCS not only it was not toxic to the natural bacterial community (Figure), but it also activated bacterial degradation pathways with an increase between 0 and day 133 of PCBs 101, 138, 180, 153, 118, 105, 156 and a decrease in 28 and 52 higher than that observed in HCS microcosms (Figure). As already above mentioned, the increase in PCBs 138, 153, 180, 118 and 156 is ascribable to the dechlorination of higher-chlorinated congeners such as PCBs 189 and 194 (Wu et al., 2012; Furukawa and Fujihara, 2008). Then their subsequent significantly decrease at day 224 show their transformation which presumably occurred through anaerobic and aerobic pathways, resulting in a very low residual concentration of 28 and 52 congeners at the end of the experiment.

In the Apirolio+compost +Plant it is evident a synergic effect of the two treatments, in increasing in PCBs 52, 101, 138, 153, 118, 105 and 156, which reached about two folds the amounts observed in the Apirolio condition, however at day 224 the residual concentrations were comparable to the other conditions.

Overall comparison of HCS vs Apirolio-treated microcosm: in order to rank the congeners on their degradation percentage and gathered together on the basis of their chloride numbers, the following formula was used:

$$\% \text{ degradation PCB} = (C0 - Ct) / C0$$

C0= initial concentration (24h)

Ct= final concentration (224 d)

Negative values means an increase in concentration

In Tables 4.5 and 4.6 the degradation percentages in HCS and Apirolio-treated microcosms, respectively are reported.

Comparing lower-chlorinated congeners, a lower decrease (t test, $p<0.01$) of PCBs 28 and 52 was observed in HCS than in Apirolio microcosms.

Comparing higher-chlorinated congeners in HCS a positive effect in decreasing their final concentrations in soil due to compost alone and compost+Plant presence was observed (t test, $p<0,01$). In Apirolio, the plant and compost treatments affected differently the final concentrations of the different congeners analyzed, but their concentrations were comparable or in some cases higher than those found in Apirolio microcosms.

Table 4.5: Degradation percentages of the sum of PCB congeners grouped for Cl number in historically contaminated soils.

	PCB Congeners	Cl No.	% at day 133	% at day 224
HCS	28	3 (tri-CB)	**23,48**	**68,95**
	52	4 (tetra-CB)	**57,51**	**46,30**
	101+105+118+126	5(penta-CB)	**42,85**	-10,98
	138+153+156+157+167	6(esa-CB)	**45,62**	-13,37
	180	7(epta-CB)	**1,83**	-49,85
HCS +Plant	28	3 (tri-CB)	**14,39**	**63,31**
	52	4 (tetra-CB)	**49,69**	**51,73**
	101+105+118+126	5(penta-CB)	**8,98**	-8,56
	138+153+156+157+167	6(esa-CB)	-31,43	**7,11**
	180	7(epta-CB)	-89,06	-0,72
HCS +compost	28	3 (tri-CB)	-11,11	**60,34**
	52	4 (tetra-CB)	**62,80**	**68,45**
	101+105+118+126	5(penta-CB)	**55,72**	**51,45**
	138+153+156+157+167	6(esa-CB)	**44,57**	**24,57**
	180	7(epta-CB)	**61,01**	**45,18**
HCS +compost +Plant	28	3 (tri-CB)	-12,18	**54,43**
	52	4 (tetra-CB)	**59,50**	**70,74**
	101+105+118+126	5(penta-CB)	**45,32**	**52,49**
	138+153+156+157+167	6(esa-CB)	**39,03**	**16,22**
	180	7(epta-CB)	**61,16**	**42,92**

Table 4.6: Degradation percentages of the sum of PCB congeners grouped for Cl number in Apirolio treated soils.

	PCB Congeners	Cl No.	% at day 133	% at day 224
Apirolio	28	3 (tri-CB)	**85,51**	**94,86**
	52	4 (tetra-CB)	**51,46**	**86,48**
	101+105+118+126	5(penta-CB)	*-161,03*	**51,52**
	138+153+156+157+167	6(esa-CB)	*-72,10*	**26,65**
	180	7(epta-CB)	*-22,63*	*-43,46*
Apirolio +Plant	28	3 (tri-CB)	**84,15**	**93,87**
	52	4 (tetra-CB)	**72,91**	**82,37**
	101+105+118+126	5(penta-CB)	**13,68**	**47,16**
	138+153+156+157+167	6(esa-CB)	**21,14**	**12,50**
	180	7(epta-CB)	**1,42**	-52,55
Apirolio+compost	28	3 (tri-CB)	**73,94**	**93,45**
	52	4 (tetra-CB)	**64,04**	**75,33**
	101+105+118+126	5 (penta-CB)	**19,49**	**32,70**
	138+153+156+157+167	6 (esa-CB)	**18,25**	**7,50**
	180	7 (epta-CB)	**15,98**	-21,22
Apirolio+compost +Plant	28	3 (tri-CB)	**69,74**	**91,00**
	52	4 (tetra-CB)	-148,75	**79,46**
	101+105+118+126	5 (penta-CB)	-423,72	**38,85**
	138+153+156+157+167	6(esa-CB)	-112,31	-5,50
	180	7(epta-CB)	**2,57**	-23,74

4.5 Conclusions

The co-presence of the compost and plant promoted in the historically contaminated soil the transformation of higher chlorinated congeners (not detected in microcosm experiments, but present in soil) activating both co-metabolic and metabolic degradation pathways in different ways. The compost can stimulate plant growth and at the same time to be a carbon source for anaerobic dechlorination (PCB with Cl>4), the rhizosphere can stimulate directly PCB degradation through its exudates and indirectly providing carbon source or microhabitat suitable for microbial activity; it is also possible that depending on the organic carbon occurrence in the soil anaerobic and aerobic micro-niches can coexist and favour at the same time aerobic (metabolic) and anaerobic (co-metabolic) degradation.

The overall results of microcosms experiments show how the co-presence of *Medicago sativa* and compost can be the best biostimulation strategy for improving PCB biodegradation in the historically contaminated soil. In fact, if we consider just the decrease in PCB contamination the compost alone had the most effective effect on the decrease of almost all congeners analyzed, however if we consider the structure and activity of the natural microbial community we observed a significant improvement in the quality state when also the plant was present.

The fact that PCBs (28, 52 and 167 and 157) added with Apirolio were promptly transformed, independently from the addition of the compost and/or the plant, show that soil autochthons bacterial populations were adapted to these toxic compounds and able to transform some PCB congeners contained in it which were presumably more available than the same congeners occurred in the HCS. The time of contact of contaminants like PCBs in soil can significantly affect their bioavailability for degradation because different kinds of bonds can establish and this aspect needs to be considered in planning a bioremediation strategy.

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