Università degli Studi della Tuscia

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Tesi di dottorato in Ecologia Forestale XXIV ciclo

Assessment of microbial functional and genetic diversity of forest soils in Central Italy

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Chapter 1. Introduction

1.1 Policies to safeguard forest ecosystem and biodiversity

ONU declared the 2011 as the International Year of the Forest following 2010, dedicated to biodiversity, with the aim to stimulate more and more people to protect the environment and guarantee the future for human life and for earth. The main topics being highlighted are the conservation of forest areas and long-term forest management. Governments are availing themselves of this opportunity to raise public awareness of important forest policy issues: forest management combined with wood harvesting should contribute to the reduction of CO_2 and hence to the stabilization of the climate. Forest ecosystems, in fact, constitute a major terrestrial C reservoir containing over 60% of all C stored in the terrestrial biosphere, more than 85% of the total plant C on the earth and between 60-70% of the total soil C (FAO, 2008). In a scenario of changing global carbon balance, there is a large potential for forests to act as sinks for atmospheric CO_2 either through reforestation or through the enhanced tree growth rates (Ceulemans et al., 1999). It has been argued that conservation of forests by using good selvicultural practice and through tree planting can strongly enhance the C sink provided by terrestrial ecosystems (Baral, 2004). Since 2000 up to now, every year more than 5,2 million of hectares of forests have been lost, for a total of 161 million hectares (FAO, 2008).

Agenda 21 is a document adopted by more than 170 countries during a UN Conference about Environment and Development (UNCED) in Rio de Janeiro in 1992. The protocol of Rio de Janeiro intended to promote the scientific and international cooperation to have a better understanding of the importance of biodiversity and its ecosystems. Since its signature several laws and agreements have been enacted with the aim to defend the ecosystems and the natural habitat, maintaining and rebuilding of the plants and living species in their natural environment (Table 1).

The important innovation of Convention of Biological Diversity (CBD) and the Millennium Ecosystem Assessment (MAE) is the relationship of productivity of a natural environment with its biodiversity, in particular MAE defines biodiversity as :"...the diversity among living organisms in terrestrial, marine, and other aquatic ecosystems and the ecological complexes of which they are part. It includes diversity within and between species and the diversity of ecosystems".

Table 1: Laws and agreement to defend the ecosystems and the natural habitat

Year	Law/Agreement
1979	Convention of Barcelona
1979	Convention of Berna
1991	Protected areas act (394/1991)
1992	Convention on biological diversity (CBD)
1992	Rio Protocol
2000	Strategy of Lisboa
2001	Strategy of Goteborg
2005	Millennium Ecosystems Assessment
2006	Agreement in environmental matter (Dlgs. 152)
2006	Thematic strategy for soil conservation

The maintenance of diversity in a defined ecosystem is vital both for the productivity of the same ecosystems and for their ability to offer services important for the human species, at the same time the anthropical activities are part of the ecosystem influencing all the ecosystem processes. In particular the creation of protected areas, as National Parks or Natural Reserves with the main aim to safeguard the ecosystem or the damaged area, in a long period will not guarantee the conservation of the diversity, it will be preserved only with an holistic and sustainable approach of protected areas and the "non" protected ones.
Two different programs were conducted in Italy in the last decades with the aim to monitor forest ecosystems. The first one, ICP Forest, started in 1986 according to UNECE program (United Nations Economic Commission for Europe); it dealt with the health of forest soil, the nutrient diseases, the acidification and the rate of defoliation. An extended level studied intensively not only soil chemistry but also air quality and soil vegetation, with the aim to understand the various interactions of development among the different parameters. The second program defined "Con.Eco.For" is a national program with the same aims of the ICP Forest. The program started during the 90's by the organization of a network of 260 sample areas and 30 permanent plot areas, representative of the different national forest ecosystems. The monitoring of biotic and abiotic components, part of forest ecosystem, gives results about the prevention strategy of environmental risk and the creation of a role for the forest management. Despite forest ecosystem, the first protection plan for soil biodiversity came in 2006 with the Sixth action program for the environment. The thematic strategy for soil protection faces several soil problems in particular among them the loss of

biodiversity. If the damage deriving from soil degradation is estimated to be around 38 billion €/year (European Soil Bureau Network, 2005), the damage deriving from the loss of biodiversity is still not possible to estimate, but in the last decades thanks to an increasing knowledge, new estimation of its importance are coming up.

1.2 Ecosystem services controlled by agroforestry systems

The products and services provided by agroforestry systems are several in particular the multifunctional role of agroecosystems has also been emphasized by both the Millennium Ecosystem Assessment (2005) and the International Assessment of Agricultural Science and Technology for Development (2008). They could be defined as those properties of ecosystems that either directly or indirectly benefit human endeavors, such as maintaining hydrologic cycles, regulating climate, cleansing air and water, maintaining atmospheric composition, pollination, soil genesis, storing and cycling of nutrients (Christensen et al. 1996, Daily 1997). According with the Millennium Ecosystem Assessment and Blum (1998) the benefits that people received from ecosystem services are divided in ecological and economic functions. In particular the ecological functions carried out are resumed as follows:

1. carbon sequestration;
2. biodiversity conservation;
3. soil enrichment;
4. air and water quality.

1. The general goal of sequestration activities is to maintain ecosystems in the sink phase, in fact, if the system is disturbed (a forest burns or is harvested, or land is cultivated), a large fraction of previously accumulated C may be released into the atmosphere through combustion or decomposition. When the system recovers from the disturbance, it re-enters a phase of active carbon accumulation. The incorporation of trees or shrubs in agroforestry systems can increase the amount of carbon sequestered, in addition to the significant amount of carbon stored in above-ground biomass, agroforestry systems can also store carbon belowground. The amounts of carbon stored increase with the rotation age of trees and/or shrubs and by manufacturing durable products from them upon harvesting but the sequestration potential of the vegetation component (above and belowground) is variable according to the latitude. In general, agroforestry on arid, semiarid, and degraded sites have a lower carbon sequestration potential than those on fertile humid sites; and temperate agroforestry systems have

relatively lower rates compared to tropical systems. Gupta et al. (2009) examined soil organic carbon and aggregation under a poplar-based agroforestry system in northwest India and found that soil organic carbon concentration and pools were higher in soils under agroforestry and increased with tree age.

2. The literature on the role of agroforestry in conserving biodiversity is growing rapidly. It helps to preserve biodiversity providing an habitat for species that can tolerate a certain level of disturbance, preserving germoplasm of sensitive species reducing the rates of conversion of natural habitat by providing a more productive, sustainable alternative to traditional agricultural systems that may involve clearing natural habitats. Probably, the most important ability is to conserve the biological diversity by providing other ecosystem services such as erosion control and water recharge, thereby preventing the degradation and loss of surrounding habitat. According to Parker (2010) degradation in soil integrity may have both direct and indirect consequences for the earth's total biodiversity, in fact degradation of soil integrity may harm biodiversity directly by causing local extirpation or global extinction of certain soil organisms.

3. Soil structure and fertility provide essential ecosystem services to agro-ecosystem (Zhang et al., 2007). Well aerated soils with abundant organic matter are fundamental to nutrient acquisition by crops, as well as water retention. Soil pore structure, soil aggregation and decomposition of organic matter are influenced by the activities of bacteria, fungi and macrofauna (earthworms, termites and other invertebrates). Through decomposition of detritus and plant residues and through nitrogen fixation micro-organisms mediate nutrient availability. The incorporation of trees and crops that are able to biologically fix nitrogen is fairly common in tropical agroforestry systems. Non N-fixing trees can also enhance soil physical, chemical and biological properties by adding significant amount of above and belowground organic matter and releasing and recycling nutrients in agroforestry systems. Several studies showed the improvement of soil quality in different management or using different crops, Udawatta et al. (2008) further showed improved soil aggregates stability, soil carbon, soil nitrogen, and soil enzyme activity in soils under agroforestry buffers compared to row crops.

4. The delivery of ecosystem services to agriculture is highly dependent on the structure of the landscape in which the agro-ecosystem is embedded. Agroforestry practices such as windbreaks and shelterbelts are touted as having numerous benefits.

These benefits include effectively protecting buildings and roadways from drifting snow, savings in livestock production by reducing wind chills, protecting crops, providing wildlife habitat, removing atmospheric carbon dioxide and producing oxygen, reducing wind velocity and thereby limiting wind erosion and particulate matter in the air, reducing noise pollution, and mitigating smells from concentrated livestock operations, among others. When natural forested landscapes are denuded, rain can compact the surface and turn soil to mud; mud clogs surface cavities in the soil, reduces infiltration of water, increases runoff, and further enhances clogging (Hillel, 1991) and reduces water quality. Water delivery to agro-ecosystems depends on flow patterns across the landscape and can be influenced by a variety of biophysical factors. Stream flow is influenced by withdrawals for irrigation, as well as landscape simplification. Water provisioning is also affected by diversion to other uses in the landscape or watershed, such as domestic, industrial or energy consumption (Power, 2010). Both natural biological control services and pollination services depend crucially on the movement of organisms across the agricultural landscape, and hence the spatial structure of the landscape strongly influences the magnitude of these ecological services to agricultural ecosystems (Tscharntke et al. 2005; Kremen et al., 2007).

1.3 Mediterranean forest ecosystem

A forest is an area with a high density of trees and it can be imagined as a complex, self-organizing system with multiple natural processes that respond autonomously to internal and external drivers (FAO, 2008). The plant communities cover approximately 9.4% of the Earth's surface (or 30% of total land area) and even if a number of global forest classification systems have been proposed, none has gained universal acceptance (FAO, 2008). The Italian forest is an ecosystem with a high biodiversity counting a magnitude of woodland, habitat spread in all the national landscape. It is enriched by morphology and location of the peninsula, bringing together the alpine ecosystem characterized mainly by conifer forest and the Mediterranean ecosystem with a mixed forests; anyway even if the Italian territory is mostly mountainous, the forest covers less than one quarter of the territory.

In this work Mediterranean forests ecosystem is the centre of our attention, in fact although it represents only a little part of all forest ecosystems, it hosts 10% of plant kingdom, about 50.000 plant species are classified (Cowling et al., 1996), moreover they are not only located in the basin of the Mediterranean sea but also in California, in some part of Chile, in South Africa and in the West Coast of Australia, where it is called "bushland" (Figure 1.1).

Figure1.1: Mediterranean areas in the world (FAO 2008)

The favorable climate of this ecosystem determined the evolution of a unique vegetation constituted by evergreen trees and shrubs with accentuated sclerophyllous (hard leaf), resistant to drought and decomposition. The herbaceous layer usually survives to hot season with two different strategies, for the perennial species the aboveground layer dies and the belowground portion survives, while the annual species breed during the spring dying in summer leaving only the seed (Venturelli e Virli, 1995).

The high diversity of plant species in this area is mainly linked to the coexistence of areas with different successional stages called:

1. **Evergreen forest**. The evergreen forest is the mature stage of the ecological succession characterized by a complex structure of different layers. In the tree layer oak (*Quercus ilex*) is the main species even if it is possible to find oak (*Quercus pubescens Willd.*), maple (*Acer monspessolanum L.*), ash tree (*Fraxinus ornus L.*) and chestnut (*Castanea Sativa Mill.*). The shrub layer is constituted by arbutus (*Arbutus unedo L.*), Phillyrea (*Phyllirea latifolia L.*), buckthorn (*Rhamnus alaternus L.*) and viburnum (*Viburnus tinus L.*). The brushwood it is composed by herbaceous (*Smilax aspera L., Lonicera implexa Aiton, Rubia peregrina L.*) and shurb species (*Ruscus aculeatus L., Rosa sempervirens L.*).

2. **Maquis**. The plant species covering this area are composed by species high usually 4-6 m. Oak (*Quercus ilex L.*) carob (*Ceratonia siliqua L.*), olive (*Olea europea L.*) and sometimes pine (*Pinus pinea L.*) deriving from reforestation. The shrub layer is similar to

the evergreen stages in addition to (*Pistacia lentiscus L.*), (*Cistus sp.pl.*) and (*Myrtus communis L.*)

3. **Garrigue**. It is the last stage where it is possible to find an alternation of shrubs and herbaceous layer (Bullini et al.,1998). This kind of vegetation is located mainly in soils with a scarce differentiation in horizon, an alkaline pH and very low in phosphorus and nitrogen (Giovagnotti, 1989; Padula, 1985).

This work was focused on natural reservations located in Mediterranean areas covered by the following species:

- white pine (*Pinus strobus*),
- chestnut (*Castanea sativa*),
- oak (*Quercus cerris*).

The Mediterranean environment is characterized by different kind of soils which developed thanks to different lithological, climatic and biotic conditions. Soil Taxonomy identifies eleven order of soils, four (Entisols, Inceptisols, Vertisols and Alfisols) represent the 90% of parental material of Mediterranean basin (Torrent, 1995). Mollisols, Aridosols and Ultisols are less representative, while the remaining four orders (Andisols, Histosols and Spodosols) are rare or absents. This broad variety of soils is mainly due to the position of the Mediterranean basin which is in a transition area between temperate and subtropical regions.

1.4 The disturbance factors in Mediterranean ecosystem

The environmental characteristics of Mediterranean ecosystem are due to an extended anthropical presence (agriculture and fire management, pasture, deforestation) which provokes a reduction of the health and quality of soil.

This disturbance is started at least 10.000 years ago with the development of agriculture, even if the greater part of these changes happened in the last four decades.

The rising of mechanization and intensification of production processes have a negative impact on the quality and ecological functions. In fact, the deep workings, designed to increase the plant growth and improve water storage capacity, while having contributed to the increase of production and industrialization of agriculture, have resulted in adverse effects on the soil, as: the increased rate of mineralization of organic matter present in the already low profile, with production of nitric oxide, useful for plant nutrition but easily

leached, with the risk of pollution of surface water or groundwater. These negative effects were accentuated by the simultaneous reduction of inputs of organic matter to soil, which were originated from the production cycle such as manure to the organic matter from the use of green manure and forage programs (Bazzoffi, 2007) .

The actual causes of vegetation fires are mainly due to anthropogenic activity (Susmel, 1993). In the Mediterranean environment fire favors the presence of shrubs and herbaceous plants at the expense of the trees. In fact, a high frequency of fires does not allow vegetation to reach the mature stage and can be responsible for pronounced erosion, resulting in loss of organic matter and nutrients, to the point of not allowing the development of tree vegetation (Pignatti, 1995).

Grazing also removes organic matter that, in part, is compensated by the return of nutrients through manure and despite this, some environments are altered by an imbalance in nutrient redistribution (McIntosh, 1997). In addition, poaching of grazing animals results in increased soil compaction, thereby reducing macro-porosity and therefore the availability of air in the soil. The reduction of oxygen availability may in turn inhibit the activity of decomposers, thereby reducing the availability of nutrients useful to plants.

Forest ecosystems were exploited for centuries for the wood and coal production, anyway compared to agricultural soil ecosystem, the forest soils generally show a greater structural stability, thanks to two separate mechanisms that act with different degrees of effectiveness. The first is determined by the production of polysaccharides that contribute to aggregate stability as cement stabilization, through the formation of a network that covers a large number of micelles clay and sand, sealing the pores. Since the polysaccharides are formed and decompose rapidly, they are among the most responsible for the strong variability of the microstructure due to different soil management and climatic conditions. The second cause of stabilization is determined by the dense network of roots that surround and permeate the aggregates. The roots are responsible for the structural stability of macro-aggregates and their effectiveness develops in about three years from the conversion to a use that includes plants with extensive root system. Agroforestry systems, as well as many natural tree based ecosystems, are perceived to improve or maintain soil fertility, productivity and stability, to promote soil conservation, reduce soil degradation and achieve sustainable production.

1.5 Soil microbial diversity and its influencing factors

1.5.1 Soil factors that influence biodiversity

Biodiversity represents the broad variety of the living organisms and it can be expressed in terms of species, genetic, landscape and ecosystem diversity (Ferrari, 2001) as reported in figure XX. Anyway the ecological theories have been developed in terms of ecosystems considering only the aboveground species and leaving the belowground one.

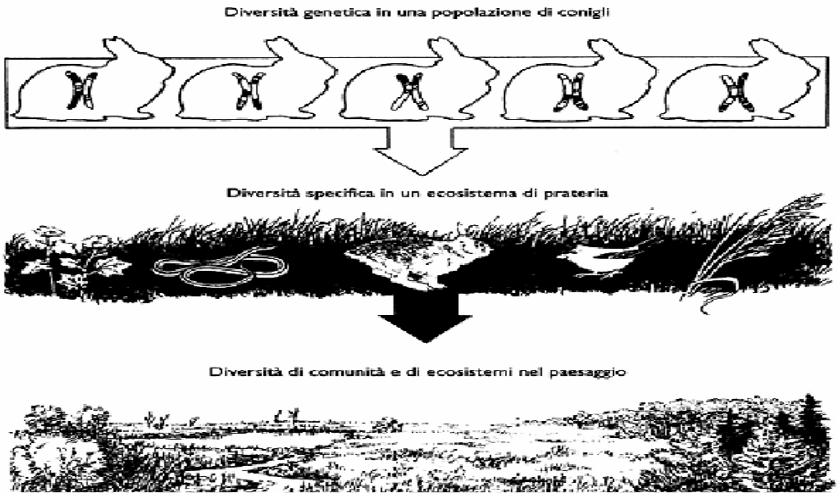

Fig. 1.2: Main levels of biological diversity (Ferrari, 2001)

As suggested by Insam (2001) and Paul et al. (1989), biodiversity may divided in four different kind of diversity as reported in Figure 1.2:

- Ecological diversity represents the number and the abundance of the habitat of biotic communities and ecosystems, where several organisms live;
- Species diversity is the number of the individues of several groups (*richness*) and their distribution inside the same groups (*evenness*);
- Functional diversity is the sum of the ecological processes developed by the organisms of a community and it can be expressed through species or important groups to maintain several functions in the soil;
- Genetic diversity represents gene and genotype variations inside the species and it is the total genetic information in the genes of all the animals, plants and microrganisms that live the earth.

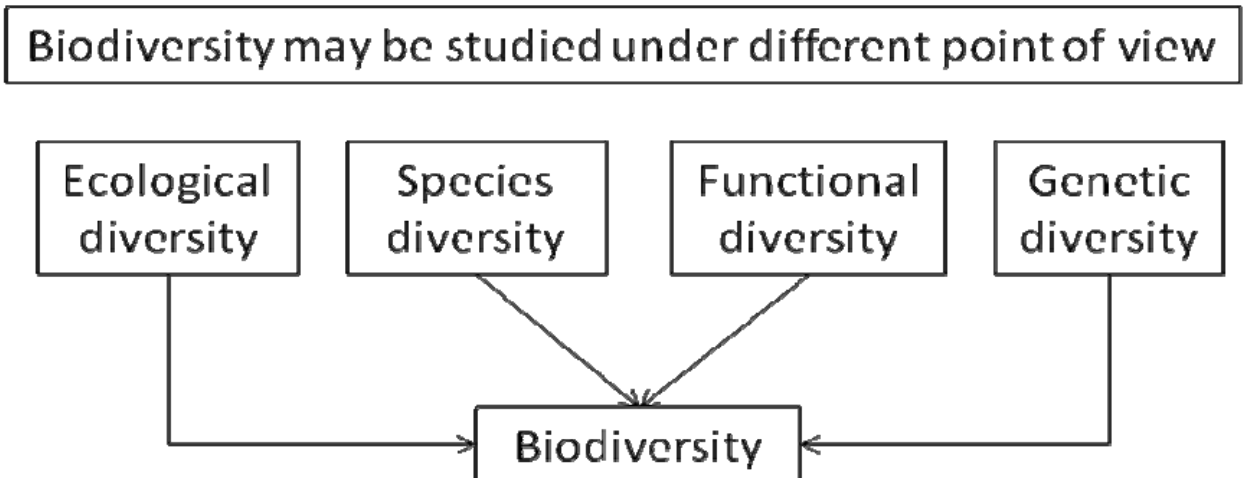

Fig. 1.3: Different levels to study soil biodiversity

Microflora is the most representative part of the soil and is also the one that most affects the biological properties regulating all the biochemical processes (Bloem et al., 2003). Despite the growing interest of scientific communities on the edaphic diversity, it has been estimated that less of 5% of soil microrganisms has been estimated and described. The number of microrganisms in soil and their biomass vary greatly both within the soil and in relation with the plant species and other organisms. The general definition of biodiversity can be applied also to microrganisms except the species diversity because it is not applicable to asexual organisms (bacteria and virus). So, microbial diversity is defined in terms of richness, as the number of individues owning at the same group (taxa), and evenness, as their distribution inside the taxa (Atlas,2005; Bartha, 1998).

Microorganisms living in the soil can be divided in bacteria, archaea, fungi, yeasts, algae and protozoa (Metting, 1993). Communities composition is not the same but it changes by disturbance in the micro-environment or by environmental and anthropical variations.

Microbial communities in soil are strongly affected by water content in soil. Water with its nutrients inside, in fact, is very important for the nutrition of microorganisms and its scarcity or abundance reflect the composition and metabolism of microbial communities (Waldrop and Firestone, 2006). Microbial cells are killed by desiccation and only the most resistant species can survive for long period of water scarcity; commonly the microbial communities, in a dried soil ten years old, reduce 90% of its population (Florenzano, 1983). The remaining living community is due to resistance mechanisms and to the presence of residual water (osmotic and hygroscopic), but desiccation killed some microorganisms that became nutrients for the survivors. Soil temperature influences the metabolic activity of microorganisms, because the microbial activities increase with the increase of temperature with an optimum of 25°/37°C, too high temperatures may have a depressive effect on

microbial metabolism (Florenzano, 1983). Usually the microbes of upper layers are more sensible to changes of temperature than the deeper ones. The pH greatly influences the edaphic microflora. A bacterial cell contains about 1000 enzymes which are very sensitive to pH conditions, the optimum pH for the greater part of bacteria is comprised between 4 and 9 while fungi are moderately acidophilic and their optimum is between 4 and 6. Several studies showed the influences of soil texture and structure on diversity and structure of microbial communities (Nannipieri et al., 2003). The network of pores regulates the movement and defines the relative location of the organisms and the substrates. The relationship between the distribution of microbial communities and the classes of the particle size, has been highlighted by Renjard and Richaume (2001) who showed that the distribution of the greater part of bacteria is located in those areas where a good availability of water, substrates, gas diffusion and protection from predators is present. Texture may strongly influence the growth and the metabolism of the microbial biomass and in particular great relevance is due to the presence of clay particles that have an important role in the formation and stabilization of aggregates. Moreover clays are able to retain a sufficient amount of water to sustain the growth of microrganisms and adsorb cations considering the surface negative charges.

Several organisms maintain the same composition inside a community, but the factors discussed above may modify some metabolic processes with functional and ecological variations. "Functional diversity" of soil microorganisms reflects the characteristics of microflora, the great variability of environmental and nutritional conditions that characterize the soil (usually with a low amount of nutrients and energy), in which microrganisms live in a microhabitat. Even if the available surface in soil is high, the "biological surface" (the area occupied by microrganisms) is only a little portion of the effective available area (less than 5%) (Nannipieri et al., 2003).

1.5.2 Influences of plant covering on soil microbial diversity

The effect of tree covering has a big relevance on soil biodiversity, probably because it represents one of the most important factors which influences edaphic communities. Covering and litter, in particular, influence the quality, the quantity and the spatial distribution of the resources for the decompositors; moreover covering influences also the temperature and moisture of the upper layer of the soil, where the largest part of the biological activity is located. The water-soluble compounds of plant residues (organic acids, sugars, amino acids), as well as substrates for microbial activities, act as compounds

with a strong ability to complex cations, contribute to the migration of these in the form of complexes and chelates, modifying also the thermic and water regime of soil.

Soil is influenced in different ways by plant species. A high plant diversity provides a litter very different with respect to a litter with just one or two species, the high diversification influences also the diversity and number of detritivores and decomposers (Hansen, 2000) and a major diversity of fungi (Widden, 1986). Bargett (2002) noted that the simultaneous presence of different litter involves a greater variety of available resources and greater complexity of habitats, resulting in a higher diversity of microbial communities.

The plant species diversity may increase the net primary production (NPP) (Schmid et al., 2002), which can increase the carbon storage in soil, or by accelerating the turnover of plant biomass or by increasing roots exudation; in this way it is possible to influence the edaphic communities which are limited by carbon resources (Zak et al., 2003).

The presence of plants, as well as providing an additional input of organic matter to soil, leads to formation of new habitat: the rhizosphere and rhizoplano. The increased complexity of the root system and metabolic activity (Foster, 1988) affects the characteristics of the microflora of the surrounding soil. Rhizosphere hosts a variety of bacterial species, with a prevalence of Gram-negative bacteria (Pseudomonas, Achromobacter) and denitrifying. Moreover, different plant species can regulate the development of rhizobacteria through the release of specific sugars and amino acids in the root zone (Burr and Caesar, 1984; Kowalchuck et al., 2002). So a higher plant diversity may produce a greater diversity of biochemical roots exudate and therefore select for more diverse microbial communities (Lavelle et al., 1995).

1.6 Soil genetic diversity

Soil comprises mineral particles of different sizes, shapes and chemical characteristics, together with the soil biota and organic compounds in various stages of decomposition. One gram of forest soil contains an estimated $4x10^7$ prokaryotic cells (Richter and Markewitz, 1995), whereas one gram of cultivated soils and grasslands contains an estimated $2x10^9$ prokaryotic cells (Torsvik et al., 2002). Based on the reassociation kinetics of DNA isolated from various soil samples, the number of distinct prokaryotic genomes has been estimated to range from 2,000 to 18,000 genomes per gram of soil. The formation of clay–organic matter complexes and the stabilization of clay, sand and silt particles through the formation of aggregates are the dominant structural characteristics of the soil matrix, soil micro-organisms often strongly adhere or adsorb onto soil particles, such as

the surfaces of the soil aggregates and the spaces between and inside the aggregates, even though some pore spaces are inaccessible for micro-organisms owing to size restrictions (Daniel, 2005).

Cultivation and isolation of micro-organisms is the traditional method but, as only 0.1% to 1.0% of the soil bacteria are culturable using standard cultivation methods (Torsvik et al., 1990; Amman and Zetterberg, 1995), to circumvent some of the limitations of cultivation approaches, indirect molecular methods based on the isolation and analysis of nucleic acids (mainly DNA) from soil samples without cultivation of microorganisms have been developed. Molecular techniques employed to study the soil microbial communities initially were applied in the medical research and only some years later have been applied to soil. Microbial diversity describes complexity and variability at different levels of biological organization.

Most molecular methods involve the separation of PCR amplicons based on differences in DNA sequence of genes of functional or phylogenetic interest, often the 16S rRNA gene.

Valid results can be obtained if the amount of sampled soil is representative of the whole soil (Nannipieri et al., 2003; Amendola et al., 2002), moreover care must be observed in handling the sample as not to affect the composition of the microflora. During the DNA extraction it is possible to extract also organic and inorganic substances, as humic substances, fulvic acids, proteins, polysaccharides, heavy metals and clay minerals, which may pollute and damage the sample. During the extraction of DNA it may occurr the extraction of organic matter (humic acids, fulvic acids, cellular debris, polysaccharides, proteins, organic solvents) and inorganic (xenobiotics, heavy metals, clay minerals), which can contaminate the sample, compromising the subsequent analytical steps. Humic substances can inhibit the polymerase enzyme, fundamental to obtain a right PCR (Chandler et al., 2000). The DNA polymerase is inhibited by an average of 1µl of humic acids, and this inhibition is independent of the amount of DNA (Tsai and Olson, 1992). Vahjen and Tebbe (1993) have found that 0.08mg/ml of humic acids inhibit the Taq polymerase, and 0.5 to 1.17g/ml inhibit restriction enzymes. The humic substances co-extracted with DNA are difficult to remove without laborious treatments (Romanowski and Burley, 1992). Even the clay minerals inhibit the Taq polymerase (Vettori et al., 1999). Hybridization efficiency and specificity of primers sometimes cause preferential amplification of certain templates, which affects the quantitative assessment of microbial diversity. Formations of PCR artifacts (e.g., chimeric molecules, deletion

mutants, and point mutants) could also lead to misleading results (von Wintzingerode et al. 1997).

Rapid profiling procedures, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993), temperature gradient gel electrophoresis (TGGE) (Felske et al., 1997; Heuer et al., 1997) and single-strand conformation polymorphism (SSCP) analysis (Lee et al., 1996; Schwieger and Tebbe, 1998), all allow the analysis of multiple samples, but the community fingerprints they generate do not directly translate into taxonomic information. There should therefore be of considerable interest in developing rapid community proling methods, which provide such information. Theoretically, use of the resolution of procedures employed in DNA sequencing to create 16S rRNA gene fragment profiles could provide data of sufficient quality for use in database interrogation to obtain taxonomic information directly (Osborn et al., 2000). The T-RFLP has been considered as a powerful tool for assessing species richness and the population sizes of various species in the complex bacterial communities based on variation in the 16S rRNA gene. It can be used to examine microbial community structure and community dynamics in response to changes in different environmental parameters or to study bacterial populations in natural habitats. T-RFLP technique is a culture-independent, rapid, sensitive and reproducible method of assessing diversity of complex communities without the need for any genomic sequence information. This method is rapid and highly automated, but does not allow the identification of bacteria in the samples.

T-RFLP data can only be regarded as “semi-quantitative” since one peak may represent many species of bacteria that share the same cutting sites for the restriction enzyme of experimenters’ choice (Zhang et al., 2008). The same as all other molecular tools, minority bacterial populations may not be detected by T-RFLP analysis since template DNAs from these populations represent a small fraction of the total extracted DNA and may not be amplified by PCR due to kinetic bias (Liu et al., 1997).

1.7 Soil functional diversity

To assess the role of soil microbial communities, it is essential to know also their functional diversity. The diversity of a system includes the diversity of functions that is characterized by the richness and consistency of expression of these functions (Kennedy and Smith, 1995). The functions that occur in an ecosystem are several but not always, all functions are carried out with the same efficiency. In addition not always a broad taxonomic variety corresponds to an high functionality of the edaphic microflora (Van

Veen and Heijnen, 1994). A minimum number of species, according to Loreau and collaborators (2001), is essential for the functioning of an ecosystem in equilibrium, while a greater number of species is probably necessary for the maintenance of stable processes in changing ecosystems. The importance of studying the relationship between functionality and soil microbial diversity is due to the fact that 80-90% of the metabolic processes are mediated by soil microorganisms (Nannipieri et al. 2003).

Microbial functional diversity is defined, operationally, as the numbers, types, activities, and rates at which a suite of substrates are utilized by the bacterial community (Zak et al., 1994), or it can be described as the composition of microbial communities needed to perform and maintain ecosystem processes in the soil such as decomposition and mineralization. The functional diversity results from genetic variability within a taxon, environmental effects on gene expression and ecological interactions among taxa. Zak et al. (1994) point out that there is a general lack of information relating taxonomic diversity to ecosystem functions, so "functional rather than taxonomic diversity may provide greater insight to microbial roles in ecosystems".

The microbial community plays a very large number of functions such as decomposition of organic matter, transformation of nutrients, atmospheric nitrogen fixation, humification etc (Emmerling et al., 2002, Nsabimana et al. 2004); it seems quite difficult to measure and estimate all activities performed by soil microrganisms, so in order to assess the capacity of the soil microbial pool functions several chemically different substrates may be used (Degens et al.2000). A microbial community characterised by a great functional diversity will be able to use a large number of compounds of the most varied chemical composition and more efficiently if compared to a reduced functional diversity level. In the last years various methods based on this principle have been developed; among these the use of enzyme activities (Bending et al., 2004), CLPP-BIOLOG (Garland and Mills, 1998) and CLPP-MicroResp (Campbell et al., 2003).

1.8 How to define soil biodiversity: hyerarchy of indicators

At the moment we dispose of different parameters to test, that can be integrated with the aim to define soil biodiversity.

Information on microbial diversity in soil can thus be obtained through different hyerarchical levels.

1. The first level will be based on the basic characterization of soil in terms of physical, chemical and biological properties. In this case the measurement of the

index of biological diversity (IBF), will be useful as a fast, synthetic, routinely determination. Parameters such as: texture, pH, TOC, microbial biomass, microbial respiration and relative microbial indexes will be determined in order to describe soil biological fertility and to calculate the IBF.

2. The second level will comprise the knowledge of genetic diversity that will be acquired by the extraction of DNA from soil and subsequent techniques of fingerprinting such as ARDRA (Amplified Ribosomal DNA Restriction Analysis) , DGGE (Denaturating gradient gel electrophoresis), TRFLP etc…
3. The third level will try to define the specific microbial functions with the aim to isolate single groups and to relate a corresponding process (phenotypic microarray, Biolog …)
4. The fourth level will try to encompass the *actual diversity* obtained with the three levels cited, aiming to define the *absolute diversity,* which is obtained monitoring the functions of a certain site through time. A long period of spatio-temporal monitoring of soil microbial functions through the tools described in the three levels will return the knowledge of microbial diversity characterizing a certain soil.

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Chapter 2. Aims of the research

2.1 Aims of the research

Biological diversity or biodiversity is the sum of genes, species and ecosystems living on earth. It is the final product of an evolutionary period and it is fundamental for the right functioning of all the ecosystems present on the earth. Antrophical activities are reducing the soil biodiversity and this trend will grow with the increasing of population and the economic development (Tolba, 1992).

The study of the microbial communities found several methodological difficulties to study the relationship between the microbial population and the specific organisms that unroll a specific function. A broad range of methods has been employed to evaluate the quality, the activity and diversity of soil microorganisms, but nothing enclose all the requirements needed to define simultaneously the structural and functional characteristics of microbial communities.

As reported in the previous paragraphs, microbial communities is affected by environmental factors such as pH, texture, soil pollution (Liesack et al., 1997), while soil microorganisms and their abundance change in relationship with soil characteristics (Ceccherini et al., 2000), in particular tree cover is an important factor to regulate the microbial communities. Plant cover influences the quality, quantity, temporal and spatial distribution of resources for decomposers. Moreover moisture and temperature of upper layer, where is located the greater part of biological activities, are influenced by covering.

Several researches study soil biodiversity, but few of them focus on the effect of changes of soil microbial community in Mediterranean areas and often they regard only the agricultural ecosystems. Few studies focused their attention on forest biodiversity and fewer of these studied the natural ecosystems. Microbial communities and forest ecosystems, in fact, are mainly studied in modified environments and usually only one or two techniques are applied to study their relationships. Lagomarsino et al. (2007) assessed microbial functional diversity by means of CLPP-MicroResp in a poplar plantation and found that microbial catabolic activity was mainly influenced by poplar species and N addition. In their research on the microbial communities structure diversity of forest ecosystems after a disturbance such as thinning, harvesting, burning, Staddon et al. (1996) showed that aggregates modifications brought a reduction of soil microbial diversity. Chaer et al. (2008) in tropical ecosystem and an agricultural soil after a deforestation showed the differences in resilience and resistance of soil microbial communities. Epelde et al. (2008) employed enzymes and Biolog™ to assess the interactions between plant and rhizosphere microbial communities in polluted soils, obtaining contrasting results with the

two techniques; Alarcon-Gutierrez (2009), by the employment of Biolog™, indicated that microbial activity and functional diversity change with litter depth on a very small scale and vary with chemical organic matter composition; Giai and Boerner (2007), employing enzyme activities and Biolog™, in a forest managed ecosystem found the same results using both approaches with a general increase of the activity for thinned plots; with the MicroResp™ technique Saul and Steinberger (2009) determined the level of microbial activity and the metabolic diversity of soil microbial communities in relation to wet and dry periods respectively and shrub eco-physiological adaptation in a desert ecosystem.

This work focused its attention on natural forest ecosystem under different tree cover, different parent material and management.
Moreover to study the differences and to have a complete information on soil functional diversity different techniques, discussed in detail in the next chapter, have been employed.
The aims of work were:

1) To assess soil microbial functional diversity in a coppiced forest system;
2) To assess soil microbial functional and genetic diversity in relation to the lithological susbstrate;
3) To assess soil microbial functional diversity in relation to different tree species;
4) To assess soil microbial functional diversity in relation to tree canopy structure;
5) Calculation of microbial diversity indexes.

2.2 References

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Chapter 3. Materials and methods

3.1 Materials and methods

The sites employed in this research are located in Central Italy and comprise three natural reservations: Monte Rufeno and Lago di Vico, Viterbo province, and Monte Peglia in Terni province (Table 3.1 and Picture 3.1).

Table 3.1: Natural Reservations interested by research

Site	Species	Parent material	Age
Monte Peglia	Oak (*Quercus cerris spp.*)	Haplic Litosol	40
Monte Peglia	Oak (*Quercus cerris spp.*)	Haplic Litosol	5
Monte Rufeno	Chestnut (*Castanea sativa Mill.*)	Dystric Cambisol	40
Monte Rufeno	E.W. Pine (*Pinus strobus*)	Dystric Cambisol	40
Monte Rufeno	Oak (*Quercus cerris spp.*)	Dystric Cambisol	40
Lago di Vico	Chestnut (*Castanea sativa Mill.*)	Melanic Andosol	40
Lago di Vico	Oak (*Quercus cerris spp.*)	Melanic Andosol	40

Picture 3.1: Natural Reservations interested by research

3.2 Physical analyses

3.2.1 Soil water content

5 g field-moist soil samples were weighed in ceramic pots. Soils were placed in a stove at 100°C. After 24 hours soils were removed from the stove and weighed again. Soil water content was referred to the fresh weight.

3.2.2 Water holding capacity (WHC)

Soil samples were saturated with water in a cylinder. The cylinder was placed on an absorbent membrane until the excess water was drawn away by gravity. When equilibrium was reached, the water holding capacity was calculated based on the weight of the water held in the sample vs. the sample dry weight.

3.3 Chemical analyses

3.3.1 Soil pH

Soil pH or soil reaction is an indication of the acidity or alkalinity of soil and is measured in pH units. Soil pH is defined as the negative logarithm of the hydrogen ion concentration. Sieved soil was suspended in a solution of deionised water (to determine active acidity) or in KCl 1N (to determine exchangeable acidity) in 1:2.5 ratio.

The pH was measured in the surnatant with a pH meter (pH 211, Hanna Instruments).

3.3.2 Cation exchange capacity (Gillman, 1979)

The cation exchange capacity (CEC) of a soil is simply a measure of the quantity of sites on soil surfaces that can retain positively charged ions (cations) by electrostatic forces.

Cations retained electrostatically are easily exchangeable with other cations in the soil solution and are thus readily available for plant uptake. Soil CEC is normally expressed in units of charge per weight of soil ($cmol^+$ kg of soil).

2 gr of soil were saturated with a 10% $BaCl_2$ solution pH 8.1. After 2 hours shaking, samples were centrifuged and solution was decanted. 0.1N MgSO4 was then added to replace Ba with Mg. After shaking, samples were centrifuged and solution was decanted. 10ml of supernatant were mixed with NH_4Cl pH 10 and then titrated with EDTA 0.05N.

3.4 Biochemical analyses

3.4.1 Microbial Biomass Carbon (Vance et al., 1987)

Several methods have been used to estimate microbial biomass in soil. In this work was used the Fumigation Extraction method was used, with some modifications, on air-dried soils conditioned by an incubation for 10 days in open glass jars at -33kPa water tension and 30°C. This kind of incubation was employed for restoring, within limits (Stotzky et al., 1962), the microbial activity of air dried soils to that of the soils in the field. Three replicates of each soil sample were used. Two portions of moist soil (20g oven-dry soil) were weighed, the first one (not fumigated) was immediately extracted with 80ml of 0.5M

K_2SO_4 for 30min by oscillating shaking at 200rpm and filtered (Whatman no. 42); the second one was fumigated for 24h with ethanol-free $CHCl_3$ and then extracted as described above. Organic C in the extracts was determined after oxidation with 0.4N $K_2Cr_2O_7$ at 100°C for 30min. To calculate the microbial C the formula is the follow:

$$\mu gMBC = ((\mu gC_F) - (\mu gC_{NF})) * 2.64$$

The conversion factor of 2.64 was calculated by relating the results of fumigation incubation and fumigation extraction method.

3.4.2 Soil respiration

Soil respiration was measured in a closed system (1000 ml jar with a rubber ring and pegs) by the method of Isermeyer (1952) applying modifications: (1) concentration and volume of NaOH and of HCl solutions were chosen to ensure that <20% of the NaOH was neutralized by CO_2 are obtained when large amounts of NaOH are neutralized (Gupta and Singh, 1977); (2) two beakers in each jar were placed, containing the soil sample and the NaOH solution; (3) on the bottom of each jar were placed 4ml of CO_2-free water to avoid the soil moisture depletion by the NaOH (Stotzky, 1965). Three replicates of each soil sample were used. CO_2 evolution was measured after days 1, 2, 4, 7, 10, 14, 17, 21, 28, 35, 42. Average values are given in mg CO_2-C/kg of soil oven dry-weight equivalent.

The organic carbon mineralization has been calculated by cumulative values of respiration through an exponential model of organic matter decomposition $C_m = C_0 (1- e^{-kt})$ (Riffaldi et al., 1996). In this model C_m corresponds to the cumulative carbon mineralized over time t of observation (days), while C_0 is the potentially mineralizable C, k is the rate constant and C_0k is the initial potential rate of C mineralization.

3.4.3 Enzyme activities

According to Marx et al. (2001) and Vepsalainen et al. (2001) enzyme activities were measured using fluorimetric method, with this technique it is possible to test several enzyme activities employing a specific substrate for each enzyme derived by methylumbelliferil (MUF or MUB)-substrates.

The eight enzymes studied are involved to the main biogeochemical cycles: Carbon (β-glucosidase, β-xylosidase, β-cellobiopyranoside), Nitrogen (leucine aminopeptidase, N-acetyl-β-glucosaminidase), Sulphur (arylsulfatase) and Phosphorus (acid phosphatase).

Furthermore acetate esterase was included as a proxy of endocellular activity (Wittman et al., 2004).

1 g of soil was weighed into a sterile jar and 50 ml of water. Soil suspension was obtained by homogenising with Ultra Turrax at 9600rev*min^{-1} for three minutes, then 100µl were withdrawn and dispensed into a 96 well microplate. Substrates were prepared with acetate buffer 0,5M pH5,5. 100µl of 1mM substrate solution were added reaching a final concentration of 500µM. Fluorescence (excitation 360nm, emission 450nm) was measured with an automatic fluorimetric plate-reader (Fluoroskan Ascent) and readings were performed after 0, 30, 60, 120, 180 minutes of incubation at 30°C.

3.4.4 Microbial indexes

Microbial indexes were calculated as follows:

Metabolic quotient (qCO_2) is the ratio of basal respiration to microbial biomass carbon:

qCO_2 = (µg C-CO_2 basal h^{-1} x µg Biomass C-1)10^3 (Dilly and Munch, 1998);

The ratio between the microbial cumulative respiration and the total organic carbon content represents the efficiency of microflora to metabolize the soil organic matter:

qM = µg C-CO_2 cumulative µg total organic carbon^{-1} (Pinzari et al., 1999).

3.4.5 Biological Fertility index (IBF)

Soil Biological fertility can be defined as an expression of microbial life and soil organic matter and depends mainly on the environment. The environment considered as a climate not only affects the development of microorganisms but also their activities and consequently the evolution of organic matter, which is the source of energy necessary for the performance of microbial life. Several discussions have been put forward proposals on the procedures to be adopted internationally for the assessment of soil quality, and were given the physical, chemical and biological indicators as a basis for soil quality. Starting by the index of biological fertility (IBF), proposed by Benedetti and Mocali (2003), based on the sum of the scores given to different chemical and biochemical parameters that are reported below:

$$IBF = Cbas + Ccum + Cmic + S.O. + qCO_2 + qM \text{ (Atlas, 2003).}$$

Cbas is the basal respiration, Ccum is the cumulative respiration, Cmic is the microbial biomass carbon, S.O. is the organic matter, qCO_2 is the metabolic quotient and qM represents the mineralization quotient.

The index ranges are enclosed between 1 and 30, and helps to classify soils in the following classes of biological fertility: 1) stress conditions, 2) pre-stress conditions, 3) average, 4) good and 5) high.

3.4.6 Community Level Physiological Profiling (CLPP)

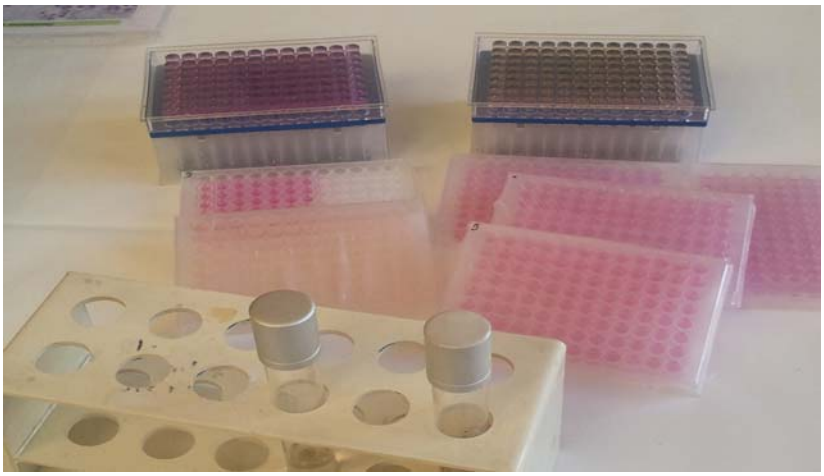
The catabolic response profile (CLPP) of soil microbial biomass was performed using the MicroResp soil respiration system (MicroResp, Macaulay Scientific Consulting Ltd, Aberdeen, UK) according to Campbell (2003) and the Biolog® (Biolog Inc., Haywood, California, USA) according to Garland and Mills (1996).

3.4.6.1 Microresp

Analysis of CLPP was performed using the MicroResp method as described in Campbell et al. (2003). Carbon Substrates were selected depending on their ecological relevance to soil and their solubility in water. In particular rhizospheric C sources (carboxylic acids and carbohydrates) were chosen considering the importance of root inputs for microbial metabolism. Each C source was dissolved in deionised water and added to soils to deliver 30 mg C g soil water^{-1}. N-acetyl-glucosamine was delivered at 7.5 mg C g soil water^{-1} because of its poor solubility. The C substrates were dispensed before the soil was added to ensure that all C sources were available at the same time for all wells. Soil was placed in 96 deep-well plates volumetrically by using another 300µl well plate from which the bottom had been removed. Approximately 0.4g of soil was added to each well. Three lab replicates were used for each field replicate. The deep-well plate was then sealed with the detection plate.

To detect the evolved CO_2, a colorimetric method relying on the change in the pH of a gel based solution of bicarbonate was used. The absorbance at 595nm was read with a Zenyth multimode reader (Anthos Labtech, Wals, Austria) immediately before and after 6h of incubation at 22°C. The absorbance after 6 h was normalized for any differences recorded at time zero and then converted to the headspace CO_2 concentration by using a calibration curve obtained with a infrared based respiration measurement device (Heinemeyer et al., 1999) using 5 different soils with and without glucose addition (1% C). The best fit for the calibration curve, using the difference of absorbance between t0 and t1 (after 6 h) was:

$y = 0.1229 \ln(x) + 0.2091$, $R^2 = 0.835$.



Picture 3.2: CLPP-MicroResp

3.4.6.2 BIOLOG

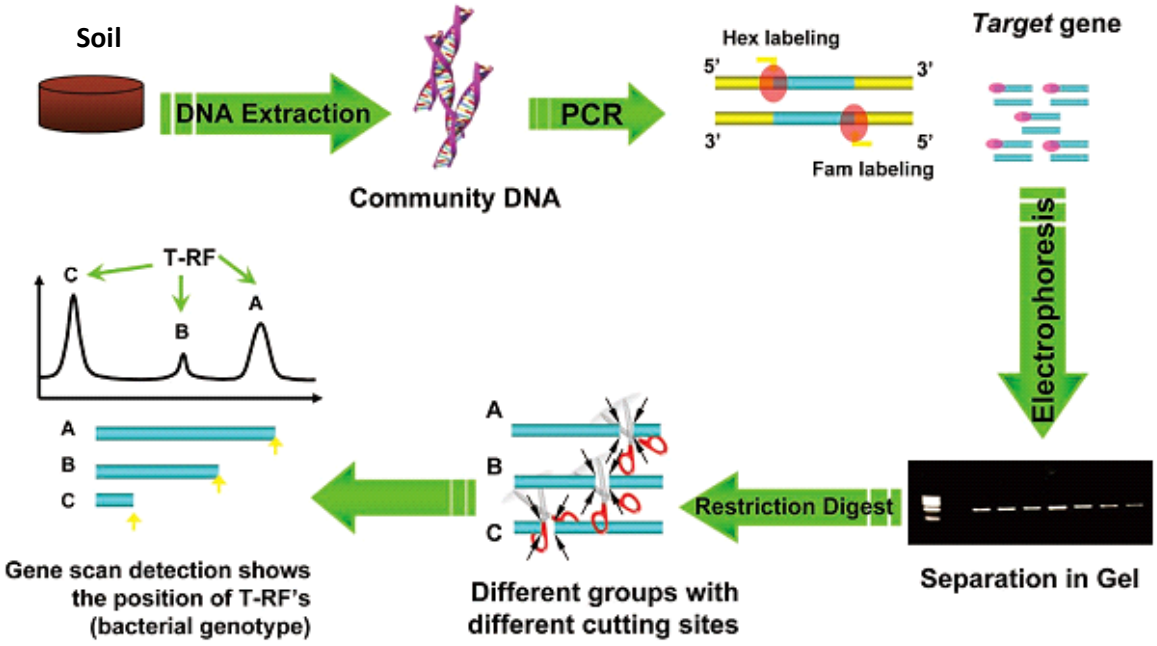
Biolog® (Haywood, California, USA) is nowadays much in favour to measure microbial functional diversity in soil because the utilisation of available carbon is the key factor governing microbial growth in soil (Insam and Goberna, 2004).

This technique is based on tetrazolium dye reduction as an indicator of sole-carbon-source utilization community-level method. ECOplates have been used to apply this technique to ecological studies to estimate metabolic potential of microbial communities (Stefanowicz, 2006), in each plate there are 96 well with 95 different carbon substrates and 1 substrate employed as control (Mills, 1991). Three grams of re-wetted soil was suspended in 30ml of physiological solution (1:10) and shaken for 30min. Sample suspensions were centrifuged for 2min. Each well of ECOplates was inoculated with 150µl of sample supernatant. Two analytical replicates were performed for each layer. All samples were incubated at 30°C and the optical density (OD) was read for each plate at 3h intervals over a period of 150h. The average well colour developments (AWCD) of the different replicates were calculated according to Garland and Mills (1996) where AWCD equals the sum of the difference between the OD of control (no substrate) and substrate wells divided by 31.

The adbsorbance measured give the reduction of measurement of the tetrazolium salt, due to catabolic activities of micro-organisms, measuring anyway only the catabolic activities of organisms able to growth in incubation condition; so it is possible that the obtained results could not reflect the real functional structure of the edaphic communities.

3.5 Molecular techniques:T-RLFP

The technique provides information on a collection of microorganisms that may be present in a given sample. Figure **XX** shows a quick overview of the T-RFLP technique, while the detailed steps follows:



Picture 3.3: Flowchart of steps required for a T-RFLP assay

1. Soil DNA extraction with "MoBio PowerSoil® DNA Isolation Kit": the technique foresees the employment of a property technology with patent right. The composition of specific reagent is not specified;
2. Dosage at Nanodrop with the extracted DNA;
3. Sample purification pre-PCR with Genereleaser (EurGentec): Genereleaser® is a proprietary reagent which releases DNA from bacterial colonies. 5µl of Genereleaser® are added to 2 µl of DNA. Lysis is accomplished directly in the amplification tubes on a thermocycler. Genereleaser® sequesters cell lysis products which might inhibit polymerase and improves amplification yield and specificity. Genereleaser® greatly simplifies the amplification of genomic DNA by avoiding the requirement to purify DNA. The thermocycler program is resumed below:

Step	Temperature	Heating rate
1.	65°C	30''
2.	8°C	30''
3.	65°C	1'30''
4.	97°C	3'
5.	8°C	1'
6.	65°C	3'
7.	97°C	1'
8.	80°C	1''
9.	50°C	∞

4. DNA amplification with primers labeled for the 16s, the employed primers are fluorescent. The selected primers in our research are:
   - ❖ FAM : emission at 517 nm (Blu)
   - ❖ VIC: emission at 552 nm (Green)

Primer	Fluorescent label	Sequence from 5' to 3'	Target gene	Specificity
P0	5' FAM/VIC	GAGAGTTTGATCCTGGCTCAG	16S rRNA	Bacteria
P6	None	CTACGGCTACCTTGTTACGA	16S rRNA	Bacteria

For a mix of 25µl we employed the following products:

Reagent	Starting concentration	Final concentration	Amount x 1
genereleaser	-	-	5
H_2O	-	-	-
Buffer	5x	1x	5
$MgCl_2$	25mM	7,5mM	7,5
Fw	10 µM	1,25µM	1,25
Rev	10 µM	1,25µM	1,25
dNTPs	2,5mM	0,25mM	2,5
Taq	5U/µl	0,025U/µl	0,125
DNA	ca 25ng/µl		5

The PCR loop is reported in the table below, at the ending take slow the amplified sample and store it without genereleaser:

Step	Temperature	Heating rate	
1.	95°C	1’30’’	
2.	95°C	30’’	5x
3.	60°C	30’’	
4.	72°C	4’	
5.	95°C	30’’	
6.	55°C	30’’	5x
7.	72°C	4’	
8.	92°C	30’’	
9.	50°C	30’’	5x
10.	72°C	4’	
11.	72°C	10’	
12.	60°C	10’	
13.	4°C	∞	

5. Analysis of the amplified DNA on agarose gel (1%); The identification and separation of nucleic acids has been achieved by electrophoresis on agarose gel (1% w/v) in TAE buffer (Tris-acetate 40 mM, EDTA 2 mM, pH 8,3).
6. Dosage of amplified sample with nanodrop;
7. Purification of amplified sample with QIAGEN Mini Elute kit®: the technique foresees the employment of a property technology with patent right. The composition of specific reagent is not specified;
8. Dosage of amplified sample with nanodrop;
9. Enzymatic digestion: This step can be resumed in the following processes:
    - Digestion of 6 ng of amplified DNA, considering for the reaction a total volume of 10/20μl;
10. Preparation of the sample for sequencing;
11. Analysis of the T-RFLP profiles chromatogram.

Picture 3.4: T-RFLP analyzer

3.6 Diversity indexes

Shannon's diversity index ($H' = \sum p_i \log_2 p_i$) was computed from enzymatic rates as a measure of functional diversity (Bending et al., 2004), where p_i is the ratio of the activity of a particular enzyme to the sum of all enzymatic activities.

The Simpson–Yule index (Magurran, 1988) calculates evenness for each treatment using the formula: $CE = 1/\sum pi^2$, where *pi* is calculated as the respiration response to a substrate as a proportion of respiration responses summed across all substrates for a soil.

Catabolic versatility (CV) is another index very that gives an estimation of the potential degradation of microbial communities from environmental matrices (Sharma et al., 1998). CV is defined by the following equation: CV= M/SD, where M represents the mean absorbance value of all wells and SD the standard deviation of absorbance values.

3.7 Statistical analysis

The effects of different variables determined with the chemical, biochemical, and microbiological analyses were examined through analysis of variance (ANOVA), followed by the Duncan test. Moreover where the variables were more than two (e.g chapter 3), CLPP and enzymatic discrimination capabilities among treatments were computed using the discriminant function analysis (DFA). DFA is a multivariate statistic that uses an *a priori* classification to maximize the power of separation of those variables that generate the greatest difference between the experimental groups.

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Chapter 4. Assessment of soil microbial functional diversity in a coppiced forest system

Submitted to “Applied soil Ecology”

Abstract

Among the bioindicators used to determine soil quality a vast interest is devoted to those related to soil biodiversity, in fact the ability of a system to withstand stress and abiotic and biotic disturbances depends on its level of biodiversity which is the basis of the functionality of ecosystems. Functional diversity is widely used to gain insight into microbial performances particularly in presence of a factor of disturbance. In this study we present the changes of microbial functional diversity and other soil chemical and biochemical properties following forest coppicing. The study was conducted in central Italy in a natural reservation under *Quercus cerris* spp plant cover; soils were sampled after three years from coppicing and in an aged coppice taken as control. Trees cutting provoked a decrease of soil TOC and an increase of pH suggesting a priming effect on native organic matter and qualitative changes in soil solution. Microbial indexes (microbial, metabolic and mineralization quotients) were significantly affected by forest management. Enzyme activities and microbial catabolic activity measured by means of community level physiological profile (CLPP) techniques (MicroResp and Biolog) increased in coppiced plots indicating higher decomposition processes promoted by plant debris and rhizodepositions released after cutting

The calculation of the diversity index using both techniques (enzyme activities and CLPP) suggested interesting speculations and perspectives on possible interpretations of these results.

Keywords: forest soil, management, functional diversity, enzyme activities, CLPP

4.1 Introduction

In the last two decades the interest on soil biodiversity and ecosystem functioning has become more and more important in the study of ecological sciences. Soil hosts several microbial species even if most of them are still unknown (Miller, et al., 1997); belowground biodiversity, in fact, represents the 95% of total diversity on Earth but only 5% of it has been classified (Gentile, 2006; Menta et al., 2008). The key role of soil microbial communities for the functioning of terrestrial ecosystems is guaranteed by their diversification in terms of structure and/or activity. Soil microbial processes include degradation of organic residues, transformations of soil organic matter, mineralization and immobilization of nutrients and formation and stabilization of soil aggregates (Haynes and Beare, 1996; Gregorich et al., 1997; Nannipieri et al., 2003).

In forest ecosystems most of the studies on soil microbial community regard the response to anthropic disturbances such as prescribed burning, erosion, harvesting, forest management, changes in plant diversity and soil pollution (Epelde et al., 2010; Giai and Boerner, 2007; Lalor et al., 2007; Leckie, 2005). The clearing of trees, in particular, causing the restructuring of vegetation, modification in quality and quantity of litter, alteration of root exudates, leaching of some plant nutrients, and changes in the microclimate can create conditions that could affect soil biota and influence the diversity of microbial communities (Marshall, 2000).

To have a better understanding of the role of the soil microbial communities it is essential to know their functional and genetic diversity. According to Insam et al. (1989) the microbial functional diversity represents the sum of the ecological processes developed by the organisms of a community and it can be expressed through species or important groups to maintain several functions in the soil, while the genetic one represents gene and genotype variations.

The functional diversity of soil microbial communities results from genetic variability within a taxon, environmental effects on gene expression and ecological interactions among taxa. Distinct from the genetic diversity of the soil microbial biomass (Emmerling et al., 2002; Wellington et al., 2003; Zak et al., 1994) which assess potential diversity, functional diversity is thus related to the actual activities resulting from that potential so that "functional rather than taxonomic diversity may provide greater insight to microbial roles in ecosystems" (Zak et al., 1994).

To assess the functional diversity of soil microbial communities both enzyme activities and the metabolic activity of soil samples can be measured. In particular the metabolic activity

of microbial communities can be determined by using two community level physiological profile (CLPP) techniques, such as Biolog™ (Garland and Mills, 1991) and MicroResp™ (Campbell et al., 2003).

To date few studies focused on the changes of soil microbial diversity associated with cutting and burning of vegetation, afforestation or reforestation of bare areas in Mediterranean soils (Rutigliano et al., 2004), especially in Italy. Furthermore few studies focused their attention on forest soil biodiversity using both enzyme activities and CLPPs methods.

Giai and Boerner (2007), in the evaluation of the effects of four alternative forest ecosystem restoration strategies on soil microbial diversity in two mixed-oak (*Quercus* spp) forests in southern Ohio (USA), employing both enzyme activities and CLPP, found similar results using both approaches with a general increase of the activity for thinned plots. Alarcon-Gutierrez et al. (2009), by the employment of CLPP and enzymes, on forest litter spatial diversity found similar results using both techniques. Dalmonech et al. (2010) in a Mediterranean forest subjected to N fertilization found negative effects on microbial functional diversity at high N doses using both enzymes and MicroResp approaches.

The present work was carried out in a forest ecosystem (*Quercus cerris* spp) in Central Italy managed as a coppice with standards. The aim of this work was to evaluate the effect of coppice on the size, metabolic activities and functional diversity of soil microbial communities. Microbial functional diversity was determined by means of enzyme activities and CLPP techniques (Biolog and MicroResp).

4.2 Material and methods

4.2.1 Site description and soil sampling

The Natural Reservation "Selva di Meana – Monte Peglia" is located in Umbria region (Italy), Terni and Perugia district (N42°43'36'', E12°11'78''), exposition North-West, mean altitude 550m a.s.l., annual rainfall 900mm. The total area is 4.535ha. Turkey oak (*Quercus cerris* spp) is the main species. The forest has been managed as a 'coppice with standards' with residues left on soil surface.

In June 2009 six plots were selected for soil sampling: the first three, for a total of 17,5ha, were coppiced in the year 2006 (coppice with standards, CS), while the last three, for a total of 24ha, were coppiced 50 years before and can thus be considered as an aged coppice (AC). For soil sampling particular attention was paid to the uniformity of the lithological substrate which was classified as *Haplic litosol* (FAO classification). Three soil samples

were collected in each plot in the A horizon after removal of litter layer. Soil samples were collected at least 10m from the nearest tree in order to be uniformly influenced by the root system and 25m distant from each other. All soil samples (for a total of 12 cores) were air-dried and sieved at 2mm before being analyzed. The moisture content was adjusted to 60% of their water holding capacity (WHC) and soil samples where then left to equilibrate at room temperature in the dark for 10 days before biochemical and microbiological analyses (Pinzari et al., 1999).

4.2.2 Chemical and biochemical analyses

The techniques applied in this work are reported in detail in the chapter two (materials and methods) and here they are resumed as follows:

(1) Chemical analysis (pH, TOC, TN and C/N);
(2) Microbial biomass carbon, basal and cumulative soil respiration;
(3) Enzyme activities (β-glucosidase, β-xylosidase, β-cellobiopyranoside, Leucine aminopeptidase, N-acetyl-β-glucosaminidase, arylsulfatase, acid phosphatise and acetate esterase);
(4) CLPP-MicroResp™ and BIOLOG;
(5) Calculation of diversity indexes (Index of biological fertility (IBF), Simpson-Yule index, Shannon index and Catabolic Versatility);
(6) Statistical analysis.

4.2.3 Statistical analysis

The analysis of variance (ANOVA) was performed on six replicates for each management, to evaluate the significant differences between the tested treatments. All statistical analyses were performed using SPSS 16 Linux edition. Statistica 7 (StatSoft, Tulsa, USA) for Windows has been employed. Statistical significance was determined at $p \leq 0.05$.

The average well colour development (AWCD) of the different replicates was calculated according to Garland and Mills (1991) where AWCD equals the sum of the difference between the OD of control (no substrate) and substrate wells divided by 31 (n. of substrates). Moreover about CLPP-Biolog further parameters derived from the growth curves were r and s: where r is the flex slope and s the number of hours to reach the flex. They were obtained by fitting the curve of OD_{592} vs time to a density-dependent logistic growth equation (Lindstrom et al., 1998):

$$Y = \mathrm{OD}_{592} = \frac{K}{1 + e^{-r(t-s)}}$$

where K denotes the asymptote or maximum degree of color development (OD_{592}), r the exponential rate of OD_{592} change (h^{-1}), t the time following inoculation of the microplates (h), and s the time at the midpoint of the exponential portion of the curve (i.e., when y =K /2) (h).

The diversity indexes for both CLPPs were computed similarly to those for enzymatic activities, except substituting the term p_i with the respiration rate of each single C-substrate as SIR (Degens et al., 2000).

4.4 Results

4.4.1Chemical and biochemical analyses

Soil pH (active and exchangeable acidity) was significantly increased after coppice (CS) while total organic carbon content was reduced by 13% ($p<0.05$) (Table 4.1). No significant variations were observed for soil total nitrogen nor for C/N ratio (Table 4.1). Soil microbial biomass (+46%, $p<0.05$), the microbial quotient (q_{mic}) (+36%, $p<0.05$), the metabolic quotient (qCO_2) (+ 42%, $p<0.05$), basal (+52%, $p<0.01$) and cumulative respiration (+58%, $p<0.01$), the potentially mineralizable C (C_0) (+63%, $p<0.05$) were all significantly increased in the managed forest ecosystem while the rate constant (k) decreased by 29% ($p<0.01$) (Table 4.2 and Figure 4.1).

4.4.2 Enzyme activities

CS plots showed a generally higher activity (+47.8%, averaged across all the enzymes) than the AC (Table 4.3). Figure 2 reports the percentage variation of each enzyme activity due to coppice with respect to AC plots. The highest effect was recorded for β-glucosidase which increased by almost 90% ($p<0.05$). All three diversity indexes calculated with the enzymatic activities showed a higher diversity for the managed ecosystem, in particular both Simpson-Yule and Catabolic Versatility showed the highest values in the managed ecosystem (Table 4.4).

4.4.3 MicroResp™ and Biolog™

The community level physiological profile (CLPP), measured by means of MicroResp™, showed a general higher consumption of the substrates for the coppiced plots (Table 4.5). The highest rates were observed in the following ranking for the different groups of

substrates: aminoacids > phenolic acids > carboxylic acids > carbohydrates > amide (Fig. 4.3). MicroResp data were highly correlated with enzyme activities ($r=0.709$, $p<0.05$, Fig. 4.4) both averaged across managements. Although a general increase of all diversity indexes in the AC plots was observed, only Simpson-Yule (CE) could significantly discriminate between the treatments (Table 4.6).

Figure 5 shows Biolog-AWCD values for soil microbial communities both in CS and AC plots calculated during a 190.5h incubation experiment. Microbial activity, as measured by AWCD, continued to increase with time and was higher for coppiced plots. Table 4.7 reports AWCD, r, s and area calculated from AWCD values obtained with CS and AC plots. Only s was significantly higher in CS plots than on AC.

Despite the MicroResp™ results, Biolog™ carbon-sources utilization was extremely heterogeneous. Some chemical groups as aminoacids and carboxylic acid had a major activity in the AC than in the coppiced plots (data not shown).

The diversity indexes calculated with Biolog™ data gave higher values for the AC plots and were significant only for CE and CV (Table 4.8).

4.5 Discussion

4.5.1 Chemical and biochemical properties

In our study significant effects were observed for soil pH and total organic carbon which were modified after coppice. Grady and Hart (2006) did not find any difference in chemical parameters between a clear cut system and a 30 years old unmanaged plot. Soil pH is known as one of the most sensible parameters to ecosystem variations (Pizzeghello et al. 2001; Berger et al. 2004). In this work the increase of pH_{H2O} and pH_{KCl} suggested qualitative changes, arisen during the 3 years after coppice, in the soil solution and in the cationic composition of the organic component of soil colloidal fraction (Oorts et al., 2003). A consequence of trees cutting is the cessation of annual litter fall and root exudation, in fact the fine roots of cut trees become subject to degradation. In particular the intense extrusion of H^+ occurring in a living root when acquiring nutrients was interrupted after coppice. Consequently the reduced H^+ concentration in the soil solution combined to an increase of other cations released from litter decomposition could have qualitatively changed the cationic composition of ions adsorbed on soil solid phase**.** The increase of soil pH was also reported after similar forest management practices by Pietikainen et al. (2001), Brais et al. (2003) after clear cut and Chauvat et al. (2003) after thinning**.**

In this study trees coppice significantly decreased soil total organic carbon content as a likely consequence of both the intense microbial decomposition activity observed (enhanced enzyme activities and respiration) and, at the same time, a strongly reduced input of plant litter in the years following trees cutting. In soils of natural forests a general condition of homeostasis leads to a long-term substrate constraint which controls microbial functioning. Conversely new inputs of fresh C due to coppicing as root exudates, plant residues and low molecular organic substances can activate microbial groups that were dormant or inactive, with synthesis of a broad variety of enzymes and possible SOM decomposition leading to a "priming effect" (Kuzyakov, 2010). Giai and Boerner (2007) found no significant differences in soil organic C content among different kind of forest management (prescribed fire, thinning, the combination of fire and thinning, and an untreated control), though they observed a significant increase in C/N ratio in the thin-only treatment. They supposed this increase in C/N ratio to be related to accumulations on the forest floor of woody remains from the trees cut during the thinning treatment. Several authors (Brais et al., 2003; Lundgren 1982; Pietikainen et al. 1995) observed that the greater part of changes in nutrient cycling occurred during the first years after management and included an increase in forest floor organic C, total N, base cations availability and a decrease in microbial C/N ratio. These changes may have occurred in response to reduced vegetation uptake and woody debris abundance.

It is well known that microbial biomass plays a key role in the processes of soil organic matter dynamics and nutrient availability in soil ecosystem. Soil management practices strongly affect the microbial biomass, particularly the inputs of C substrates (Brookes et al. 1990), thus clear cut or coppice may have either positive or negative effects on soil microbial biomass.

The root system of felled trees and other harvest residues represent decomposable source of carbon and nitrogen for the soil microorganisms (Thibodeau et al., 2000); this carbon source is long-lasting and mycorrhizal root tips are able to live long after the associated tree has been cut (Hagerman et al., 1999).

In this study the microbial biomass increased in coppiced plots probably due to enhanced availability of carbon substrates deriving from litter and rhizodepositions (Merila et al., 2002; Saetre and Bååth, 2000). Chauvat et al. (2003) in a chronosequence of four spruce forest stands found no differences in soil microbial biomass content. In boreal forest soils Bååth et al. (1995), using PLFA, demonstrated that, few years after a strong management (prescribed burning, clear-cut, harvesting) all managed sites were structurally similar

among them, but that they were significantly different from unharvested sites. In our study also the new herbaceous and shrubs plant cover, colonizing the coppiced plot, may have enhanced microbial biomass through changes in root exudates and litter. In fact Hannam et al. (2005) found that the chemical composition of forest floor was different and that pre-harvest stand type, rather than disturbance by harvesting, had the strongest influence on microbial community.

In our study microbial respiration increased significantly both as basal and cumulative respiration after coppicing. Lytle and Cronan (1998) found that basal respiration in coniferous forest floors increases in the months following clearcutting, and they ascribed this effect to a flush of fine root-root death and decay in the immediate aftermath of harvesting. However, this initial flush is ephemeral and subsequent years should show a marked reduction in available C for heterotrophic metabolism due to the loss of rhizodeposition, throughfall, and litterfall (Bradley et al., 2001).

In this work we can formulate two hypotheses: a) fine root mortality and decomposition of the leaf litter detritus contributed to enhance soil respiration. In fact, in a study performed in a turkey oak forest of the same geographical area as this study, the fine root inventory showed that 59% of roots, in terms of biomass, were recognized as dead in the recently coppiced plot (De Parri, 2001); b) the improved solar radiation availability on the forest floor after coppicing promoted the development of a grass/herb layer, a rapid root turnover and a diverse kind of rhizodeposition could have contributed to fuel soil microbes and thus their metabolic activity (Raich and Tufekcioglu, 2000; Sylver and Miya, 2001). Higher C_0, C_0k and lower k, derived from the kinetic model used, confirm qualitative changes of carbon substrates and increase of microbial mineralizing activity in CS plots (Riffaldi et al., 1996).

The metabolic quotient is considered as an index of microbial efficiency in utilizing the available resources; when high values of qCO_2 are recorded soil microbes are operating inefficiently and are diverting a high proportion of C to maintenance requirements than biosynthesis (Anderson and Domsch, 1993). This may occur under stresses ranging from climate changes, fertilization, pollution, human impact and management practices (Dilly et al., 1998). Furthermore in Odum's theory of ecosystem succession (Odum, 1969) when ecosystems approach a steady state or “climax” stage of development, the ratio of respiration to biomass declines as biomass becomes more energy efficient. In our study the metabolic quotient increased significantly in CS plots indicating a disturbance of microbial metabolism probably induced by changes in quantity and quality of available substrates.

4.5.2 Enzymatic activities

The actual rate of enzyme production and the fate of produced enzymes are modified by environmental effects as well as by ecological interactions (Moscatelli et al. 2005; Kandeler et al., 1996). Management can significantly impact soil enzyme activities, soil physico-chemical properties and substrate quality (Frankenberger and Dick, 1983; Mungai et al., 2005). Giai and Boerner (2007) found that tree canopy thinning resulted in increased acid phosphatase activity with respect to the untreated control, while no significant differences among restoration treatments in chitinase activity were observed. For Hassett and Zak (2005) all harvest treatments tended to reduce the extracellular enzyme activity from 10 to 30%, confirming the observations of Waldrop et al. (2003), who found that post-harvest treatments reduced the activities of extracellular enzymes in the forest floor of mixed forest in California. In this study an opposite trend was found, with a clear increase of almost all enzyme activities probably because the study was performed after three years from coppice while, in the cited works, the elapsed time was 8-10 years. Busse et al. (2006) report, in fact, a resilience response of microbial communities in the surface soil (size, activity, and composition) in a 5- to 10-yrs period after harvest. In particular C-cycle enzymes as cellulose, β-glucosidase and xylosidase were particularly enhanced (+41, 87 and 51% respectively) in CS plots due to the accumulation of lignocellulosic material released after coppicing. Furthermore the colonization of the coppiced site by fast-growing pioneer plants stimulates the microbial biomass to increase the activity of nutrients acquisition which also depends on the quality of new substrates available (eg. rhizodeposition). In particular Kuzyakov (2010) reports that addition of easily available organic substrates to the soil enhances microbial activity and acceleration of SOM mineralization by means of co-metabolism (i.e. enzyme production). Root exudates, in fact, can stimulate the mineralization of existing C pools (Dalenberg and Jager 1989; Mary et al. 1993; Subke et al. 2004), resulting in the ‘‘priming’’ effect, already mentioned for microbial respiration and total organic carbon.

4.5.3 Biolog and MicroResp

The use of Ecoplates™ to study the community level physiological profile (CLPP-Biolog) is widely performed and more common than MicroResp™ (Chapman et al., 2007). The Biolog™ method essentially targets the fraction of the microbial community that can grow within the microtitre plate wells; moreover it is subjected to changes in the microbial community during the incubation and the contribution of fungi is not measured for their

slow growth (Nannipieri et al., 2003; Chapman et al., 2007). Some authors showed that CLPP-Biolog do not necessarily reflect the functional potential of the dominant community members (Smalla et al., 1998) and that it is a culture-based assay which reflects the functional abilities of a very limited fraction of the entire soil microbial community (Ros et al., 2008). Nevertheless it has been also found that even non-culturable cells may respond to substrate supply (Garland and Lehman, 1999). Furthermore it has been successfully used as a fast and highly reproducible tool to study changes in soil microbial functional diversity in natural environments (Gomez et al, 2004; Ros et al., 2008; Zak et al., 1994).

MicroResp™ was designed to be a 'whole soil' technique while still maintaining the convenience of the 96-well microtitre plate format as Biolog™. Moreover substrate concentrations can be optimized in order to reflect the concentrations which likely occur in soils (Lalor et al, 2007).

The utilisation of the different carbon substrates in Biolog™ was significantly increased in coppiced soils only for a few carbohydrates (data not shown), moreover the growth curves were almost overlapping in CS and AC soils. Among the derived parameters the s value (the time to reach the flex) differed significantly between managements suggesting a longer period of adaptation to a higher variety of different organic substrates. The lack of other significant differences could be probably ascribable to some limitations of Biolog™ approach which have been discussed by Chapman et al., (2007), Chodak et Niklinska, (2010), Nannipieri et al. (2003).

MicroResp™ was more effective than Biolog™ to point the effects of forest management in terms of changes of microbial metabolism as also shown by the significant correlation with enzyme activities. MicroResp showed significant increased utilization of the different carbon sources, in CS soils, with highest rates for aminoacids and phenolic acids. Trees coppice provided a greater availability of C compounds whose assumption by microbes requires additional acquisition of nitrogen compounds; furthermore increased wood debris on forest floor may have selected those microorganisms more efficient in the use of phenolic compounds.

The two CLPP approaches, here investigated, differed substantially in their ability to distinguish between soil treatments; we can hypothesize that, beyond the methodological differences previously described (i.e. the employment of soil for Microresp and a cell suspension for Biolog), additional variation can be due to the different C concentration of the substrates used. In fact the availability of organic substrates in soil has been shown to

be a major regulator of the dynamics and composition of heterotrophic microbial communities; varying substrate concentrations can alter microbial community composition as a result of the affinity of different microorganisms for particular substrate concentrations (Griffiths et al., 1999; Wardle, 1992).

4.6 Conclusions

The oak forest coppicing significantly increased soil microbial pool and metabolism probably due to quali-quantitative changes of root products and litter that provided enhanced availability of C substrates.

Microbial functional diversity, assessed by means of CLPP (MicroResp™ and Biolog™) and enzyme activities was affected by forest management; however the different results obtained suggest that the two analytical approaches used may probably target diverse ecological processes depending on the quality of the available substrates.

4.7 - Tables

Table 4.1 – Main chemical parameters: pH, total organic carbon (TOC), total nitrogen (TN), C/N ratio determined in coppiced (CS) and aged coppice (AC) plots. Standard errors are reported in Italics, n =6, * p<0.05, ** p<0.01, *** p<0.001, ns not significant.

	pH (H_2O)		pH (KCl)		TOC (%)		TN (%)		C/N ratio	
	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.
CS	6.72	*0.12*	6,51	*0.14*	5.49	*0.73*	0.526	*0.15*	12.74	*0.9*
AC	5.84	*0.35*	5.74	*0.08*	6.30	*0.68*	0.472	*0.05*	11.91	*0.8*
Analysis of variance										
Management	*		*		*		ns		ns	

Table 4.2 – Microbial Biomass Carbon (MBC), microbial quotient (*q*mic), metabolic quotient (qCO_2), potentially mineralizable C (C_0), kinetic constant (K), initial potential rate of C mineralization (C_0K), microbial cumulative respiration and basal respiration determined in coppiced (CS) and aged coppice (AC) plots. Standard errors are reported in Italics, n=6, * p<0.05, ** p<0.01, *** p<0.001, ns not significant.

	MBC (µg C biomass g^{-1})		qmic (µg C biomass * µg C org^{-1})		qCO_2 (µg C-CO_2 h^{-1} µg C $biomass^{-1}$)		Cumulative respiration (µg C-CO_2 g^{-1} soil)		Basal respiration (µg C-CO_2 h^{-1} g^{-1} soil)		C_0		K		C_0K	
	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.
CS	589.7	*77*	0.90	*0.12*	0.27	*0.018*	840.97	*69.6*	1.171	*0.10*	993.9	*86.6*	0.048	*0.004*	47.23	*4.41*
AC	403.5	*23*	0.66	*0.05*	0.19	*0.016*	531.81	*46.7*	0.771	*0.06*	608.5	*98.7*	0.068	*0.009*	37.93	*0.76*
Management	*		*		*		**		**		*		**		ns	

Table 4.3 - Enzyme activities, expressed in nmol MUF $g^{-1}h^{-1}$, determined in coppiced (CS) and aged coppice (AC) plots. Standard errors are reported in Italics, n=6, * p<0.05, ** p<0.01, *** p<0.001, ns not significant.

	Cellulase		Chitinase		β-glucosidase		Phosphatase		Arilsulphatase		Xylosidase		Acetate esterase		L-Leucine aminopeptidase	
	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.
CS	107.0	*8.3*	4.03	*0.24*	445.7	*23.2*	830.4	*51.4*	346.8	*12.5*	93.25	*6.0*	3125.0	264.0	945.9	*46.8*
AC	75.7	*12.5*	2.95	*0.35*	237.8	*47.8*	603.4	*107.9*	234.8	*10.6*	61.57	*2.7*	2047.0	257.3	744.0	*74.7*
Analysis of variance																
Management	ns		*		*		*		***		*		*		ns	

Table 4.4 - Enzyme diversity indexes determined in coppiced (CS) and aged coppice (AC) plots. H' represents Shannon diversity index, CE represents Simpson-Yule and CV the Catabolic Versatility. Standard errors (reported in Italics), n=6, * p<0.05, ** p<0.01, *** p<0.001, ns not significant.

	H'		CE		CV	
	Mean	s.e.	Mean	s.e.	Mean	s.e.
CS	2.83	*0.07*	7.11	*0.38*	8.61	*1.27*
AC	2.57	*0.08*	5.48	*0.38*	3.15	*0.85*
Analysis of variance						
Management	*		*		*	

Table 4.5 – CLPP-MicroResp substrates consumption, expressed in μg C-CO_2 $g^{-1}h^{-1}$, determined in coppiced (CS) and aged coppice (AC) plots. Substrates used are Citric acid (Cit), Oxalic acid (Ox), Ascorbic acid (Asc), Arginine (Arg), Glycine (Gly), Leucine (Leu), Aspartic acid (Asp), Butirric acid (But), Galattose (Ga), Arabinose (Ara), Glucose (Glu), Fructose (Fr), Vanyllic acid (Van), Siringic acid (Sir), N-Acetyl-Glucosamine (Nag). CA is carboxylic acids, AA is amino acids, CH is carbohydrates and A is amide. Standard errors (in Italics), n=6, * p<0.05, ** p<0.01, *** p<0.001, ns not significant.

		CS		**AC**		**Analysis of variance**
Chemical groups	Substrates	μg C-CO_2 g^{-1} h^{-1}	s.e.	μg C-CO_2 g^{-1} h^{-1}	s.e.	**Management**
CA	Cit	2.910	*0.255*	1.507	*0.200*	*
CA	Ox	1.027	*0.066*	0.942	*0.057*	ns
CA	Asc	1.403	*0.098*	1.467	*0.108*	ns
AA	Arg	0.919	*0.213*	0.872	*0.052*	ns
AA	Gly	2.746	*0.093*	1.180	*0.066*	*
AA	Leu	2.618	*0.112*	1.041	*0.048*	*
AA	Asp	3.553	*0.206*	1.414	*0.065*	*
AA	But	2.262	*0.383*	1.076	*0.099*	*
CH	Ga	4.505	*0.227*	2.364	*0.191*	*
CH	Ara	3.447	*0.113*	1.916	*0.087*	*
CH	Glu	3.467	*0.173*	2.107	*0.030*	*
CH	Fr	3.018	*0.093*	2.441	*0.173*	ns
PA	Van	2.306	*0.227*	0.959	*0.078*	*
PA	Sir	2.457	*0.103*	1.225	*0.081*	*
A	Nag	0.655	*0.217*	0.433	*0.026*	ns

Table 4.6 – CLPP-MicroResp functional diversity indexes determined in coppiced (CS) and aged coppice (AC) plots. H' represents Shannon diversity index, CE represents Simpson-Yule and CV the Catabolic Versatility. Standard errors (in Italics), n=6, * p<0.05, ** p<0.01, *** p<0.001, ns not significant.

	H'		CE		CV	
	Mean	s.e.	Mean	s.e.	Mean	s.e.
CS	3.58	*0.04*	10.64	*0.27*	1.60	*1.16*
AC	3.62	*0.06*	12.50	*0.19*	2.43	*0.11*
Analysis of variance						
Management	ns		*		ns	

Table 4.7 – CLPP-Biolog: AWCD measured at curve plateau (K) and flex slope (r), number of hours to reach the flex (s), area under the curve, determined in coppiced (CS) and aged coppice (AC) plots. Standard errors (in Italics), n=6, * p<0.05, ** p<0.01, *** p<0.001, ns not significant.

	K		r		s		Area	
		s.e.		s.e.		s.e.		s.e.
CS	1.28	*0.04*	0.025	*0.002*	124.68	*8.19*	96.53	*9.17*
AC	1.18	*0.20*	0.027	*0.003*	101.48	*4.70*	93.12	*13.90*
Analysis of variance								
Management	ns		ns		*		ns	

Table 4.8 – Biolog functional diversity indexes determined in coppiced (CS) and aged coppice (AC) plots. H' represents Shannon diversity index, CE represents Simpson-Yule and CV the Catabolic Versatility. Standard errors (reported in Italics), n=6, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns not significant.

	H'		CE		CV	
	Mean	s.e.	Mean	s.e.	Mean	s.e.
CS	4.62	*0.01*	21.64	*0.20*	1.53	*0.02*
AC	4.58	*0.01*	23.22	*0.17*	2.06	*0.06*
Analysis of variance						
Management	ns		*		*	

4.8 Figures caption

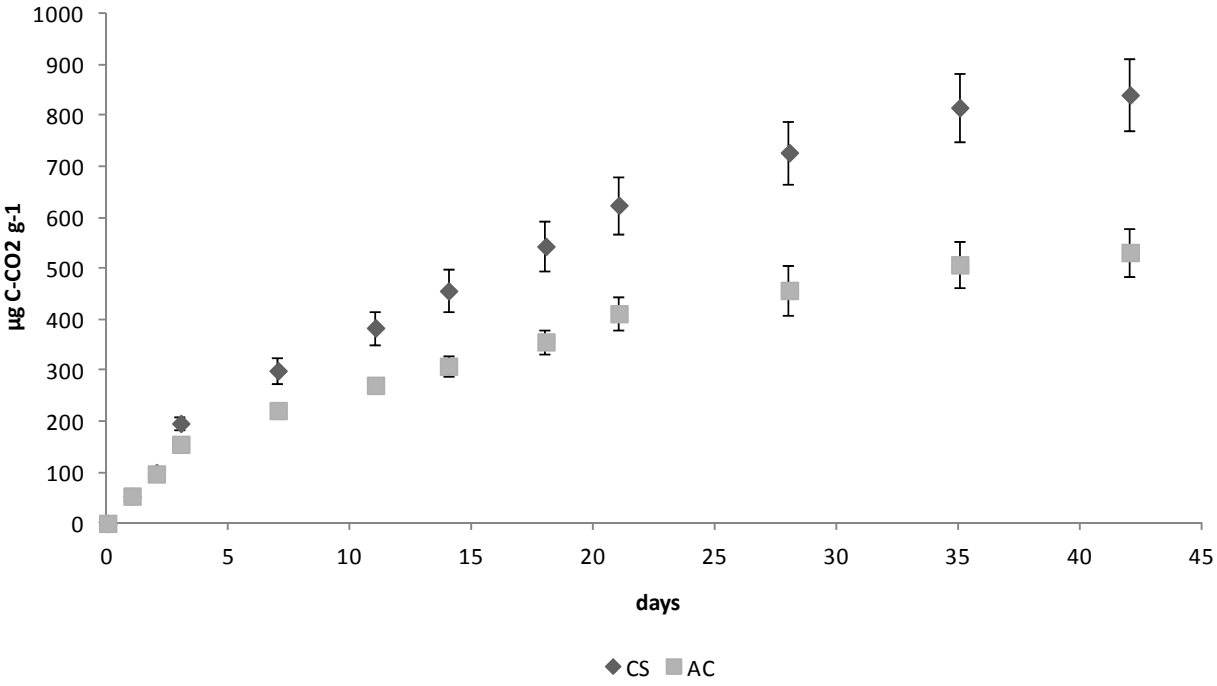
Figure 4.1 – Cumulative (a) and basal respiration (b) measured in CS (white diamond) and AC (black square) soils. Standard error bars are reported, *n*=6.

Figure 4.2 – Effect of coppice on enzyme activities expressed as percentage variation. Substrates used are: Cellulase (Cell), Chitinase (Chit), β-Glucosidase (β-Gluc), Acid Phosphatase (A. Phos), Arylsulfatase (Aryl), Xylosidase (Xylo), Acetate Esterase (Ace), L-Leucine-Aminopeptidase (L-Leu). *n*=6, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns not significant.

Figure 4.3 – Effect of coppice on CLPP-MicroResp expressed as percentage variation. Substrates used are: Citric acid (Cit), Oxalic acid (Ox), Ascorbic acid (Asc), Arginine (Arg), Glycine (Gly), Leucine (Leu), Aspartic acid (Asp), Butirric acid (But), Galattose (Ga), Arabinose (Ara), Glucose (Glu), Fructose (Fr), Vanyllic acid (Van), Siringic acid (Sir), N-Acetyl-Glucosamine (Nag). *n*=6, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns not significant.

Figure 4.4 – Correlation between MicroResp and enzyme activities both averaged across managements ($r=0.709$, $p<0.05$).

Figure 4.5 – Biolog average well colour development (AWCD) curves at the end of the incubation experiment measured in CS (straight line) and AC (dotted line).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700
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days
CS
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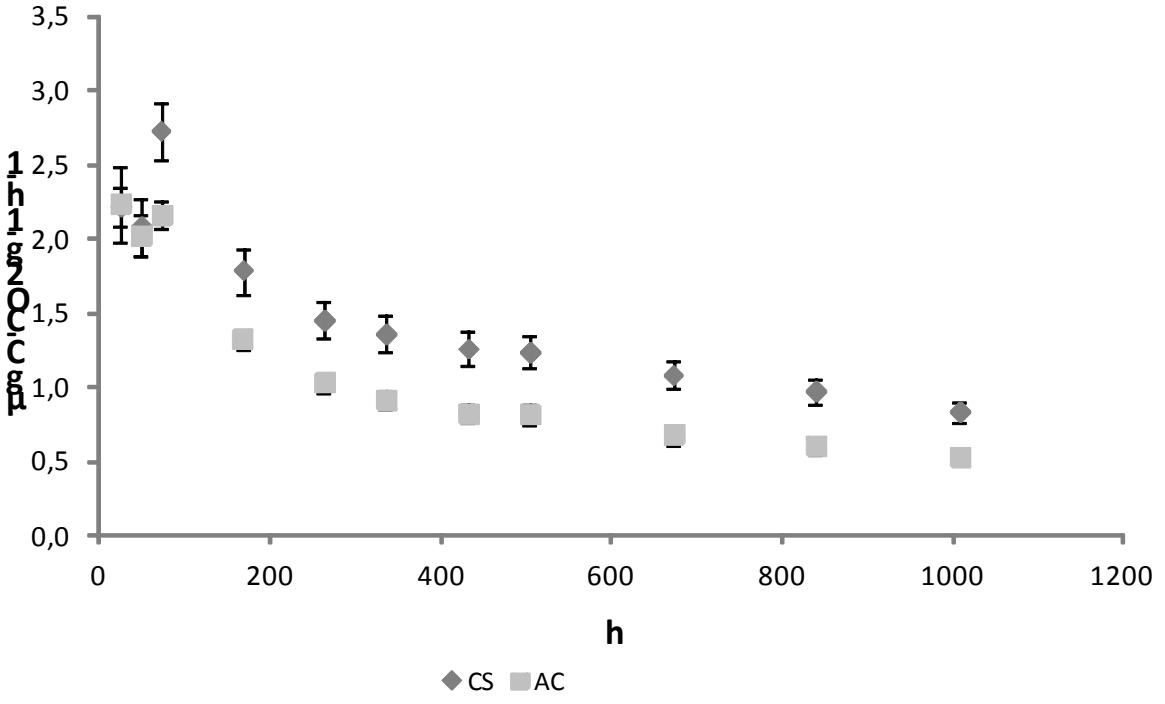
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Figure 4.1

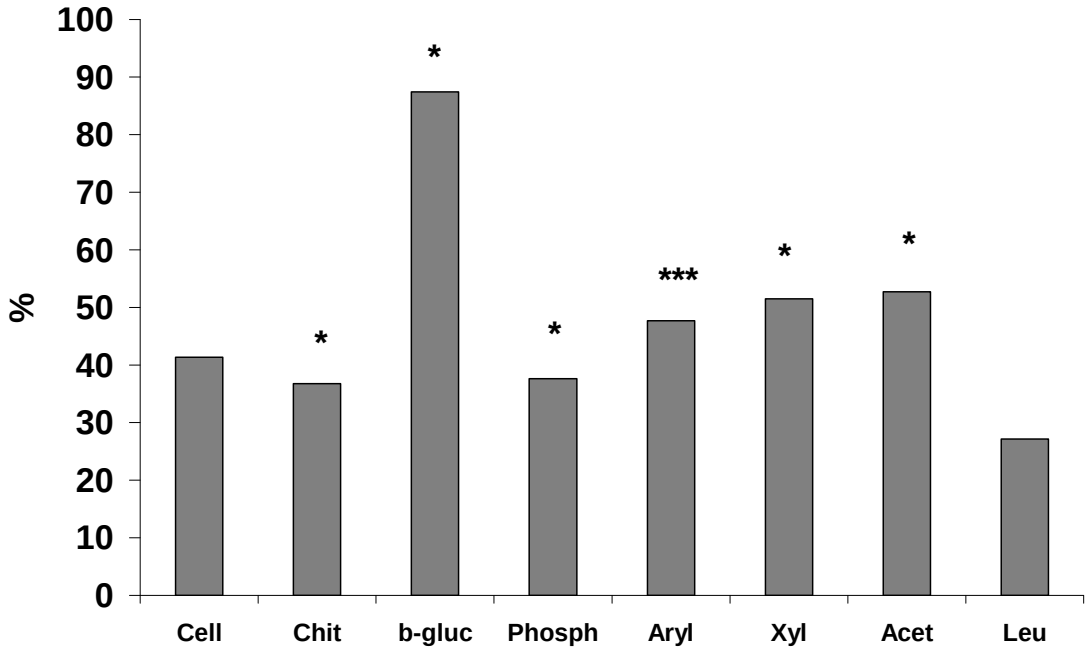

Figure 4.2

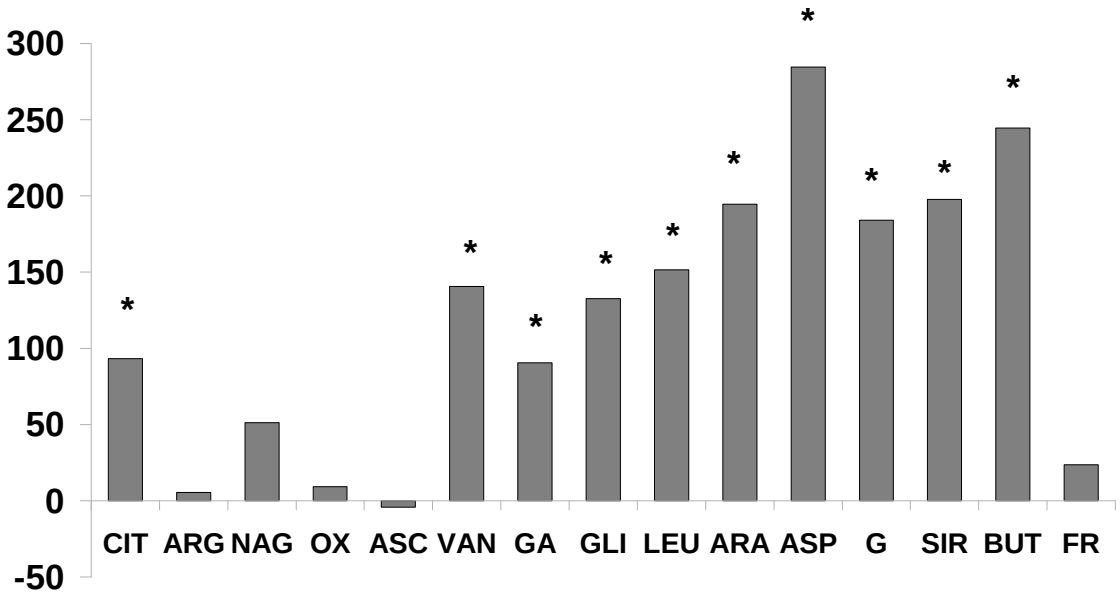

Figure 4.3

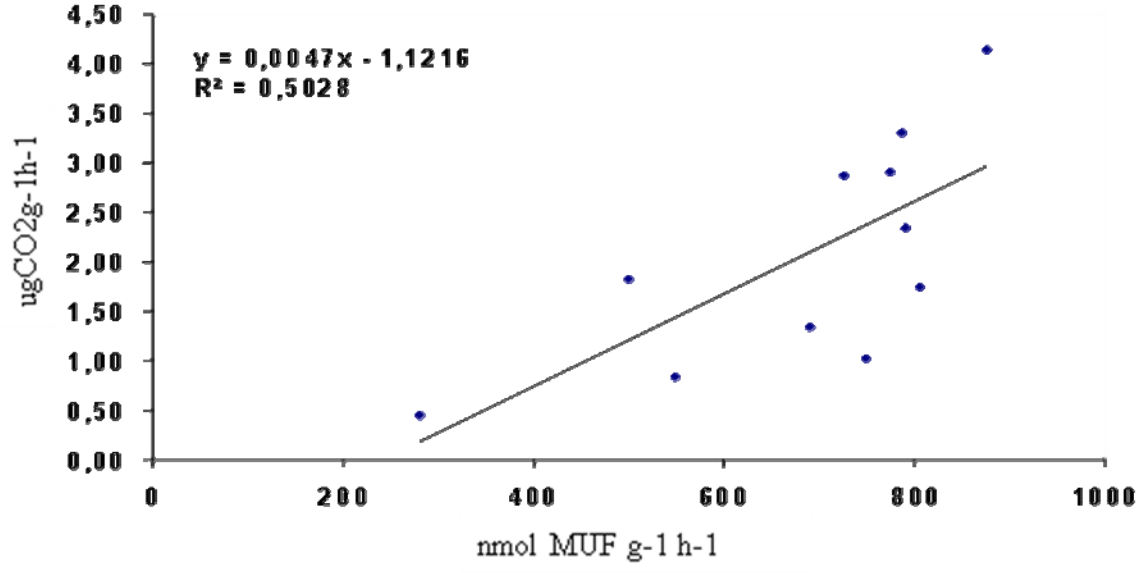

Figure 4.4

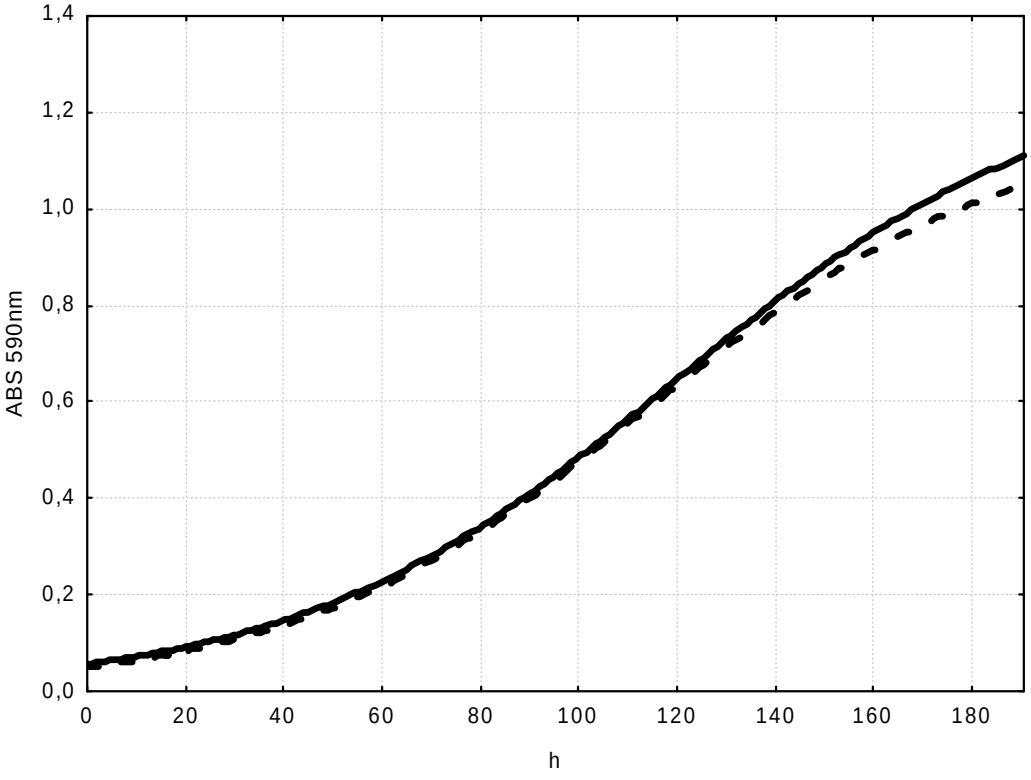

Figure 4.5

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Chapter 5. Assessment of soil microbial functional and genetic diversity in relation to the lithological susbstrate

Published on “Environmental Quality” 7 (2011) 49-57

Abstract

Soil biological functions, in particular linked to the activities of microbial communities, are influenced by the interaction between the species (canopy, quantity and quality of litter, roots and rhizodepositions) and the type of soil.

The present study focused on the influence of different pedogenic substrates on the composition and the activities of microbial soil communities.

Three systems with the same plant cover (*Quercus cerris* spp.) and same topographic conditions but with different pedogenic material (Andosol, Entisol, Inceptisol) were chosen. The soils were sampled in June 2009 in three Natural Reserves in the Centre of Italy (Selva di Meana/Monte Peglia, Monte Rufeno, Lago di Vico) at 0-20cm in horizon A.

Functional diversity was calculated by estimating eight enzyme activities and the Community Level Physiological Profile (CLPP), together with soil chemical characterization.

Key words: *soil, forest ecosystem, microbial diversity, enzymes, CLPP (MicroResp, Biolog)*

5.1 Introduction

Italian soils are extremely various as genesis, features, properties and distribution. Their lithological variety and different morphological processes occurred in the centuries and clustered the national territory in morphological areas with different characteristics. They can be employed both for anthropical and natural use: natural forest ecosystems represent the place where biodiversity, both aboveground and belowground, is preserved. Soils host a big variety of microbial species, most of them still unknown. The main role carried out by soil microbial component for ecosystem functioning is guaranteed by its diversification (Giller et al., 1997).

Soil microorganisms, in fact, have a main role in biotic natural systems where they are key players in nutrient turnover.

Microorganisms respond differently to prevailing environmental conditions and forest stand characteristics (i.e. soil and vegetation properties) influence the composition of the soil microbial community in a specific way (Hackl et al., 2005).

The main aim of this paper is to evaluate the differences in soil microbial communities induced by specific pedogenic processes of soils and functional characteristics (soil temperature and moisture) under the same tree species (*Quercus cerris spp.*) but different parental material. The information on the microbial community composition in undisturbed forest soils could then be used to facilitate interpretation of microbial community data derived from measurements in managed or even damaged or degraded soils.

5.2 Materials and methods

5.2.1 Site description and soil sampling

Soils have been sampled at 0-20cm in three different natural reserves: The Natural Reserve "Selva di Meana – Monte Peglia" is located in Umbria region (Italy), Terni and Perugia district, N42°43'36'' E12°11'78'', exposition North-West, mean altitude 550m a.s.l., annual rainfall 900mm. The Natural Reserve of "Monte Rufeno" is located in Lazio region (Italy), Viterbo district, 32T 0736390 UTM 4742149, exposition North-West, mean altitude 600m a.s.l., annual rainfall 800mm. The Natural Reserve "Lago di Vico" is located in Lazio region (Italy), Viterbo district, N42°19'47'' E12°08'08'', exposition North, mean altitude 550m a.s.l. , Six soil samples were collected in each area. Soil cores were collected, in June 2009, at least 10m from the nearest tree and 25m distant from each other. All soil samples were dried and sieved at 2mm. The moisture content was adjusted to 60% of their water holding capacity (WHC) and soil samples where then left to equilibrate at room

temperature in the dark for 10 days prior to biochemical and microbiological analyses (Pinzari et al., 1999). Turkey Oak *(Quercus cerris* spp.*)* is the main tree species. The parent material has been classified, with the International Classification World Reference Base (2006), as follows: Monte Peglia is on a Haplic Litosol (Entisol very thin), Monte Rufeno is on a Dystric Cambisol (acid soil with a cambic horizon) and Lago di Vico is on a Melanic Andosol (Andosol with Melanic epipedon). Soil physical data regarding textural classes of the three selected soils are coming from previous studies: for the Haplic Litosol of Monte Peglia from the CRA National Soil Database (Napoli R., personal communication), for the Dystric Cambisol of Monte Rufeno from the CRA-RPS soil data of the forestry monitoring CONECOFOR Project (Alianiello et al., 2002) and for the Melanic Andosol of Lago di Vico from previous pedological studies of the same site (Lorenzoni and Quantin, 1990).

5.2.2 Chemical and biochemical analyses

(1) The techniques applied in this work are reported in detail in the chapter three (materials and methods) and here they are resumed as follows:
(2) Chemical analysis (pH, TOC, TN and C/N);
(3) Microbial biomass carbon, basal and cumulative soil respiration;
(4) Enzyme activities (β-glucosidase, β-xylosidase, β-cellobiopyranoside, Leucine aminopeptidase, N-acetyl-β-glucosaminidase, arylsulfatase, acid phosphatase and acetate esterase);
(5) CLPP-MicroResp™ and BIOLOG;
(6) Calculation of diversity indexes (Index of biological fertility (IBF) and Simpson-Yule index);
(7) Statistical analysis.

5.2.3 Statistical analysis

The analysis of variance (ANOVA) was performed, on six replicates for each management, to evaluate the main differences among the tested treatments. Duncan's Post hoc test has been applied. Statistical analyses were performed using SPSS 16.0 Linux edition. Statistical significance was determined at $p \leq 0.05$.

5.3 Results

The main soil physical and chemical properties are reported in table 5.1.

Significant differences among sites were observed in the C_{org} and of N_{tot} content.

Since the three areas are characterised by significant differences in organic carbon content all biochemical data were normalized for their relative amount of organic C to reduce other sources of variation.

Enzyme activities (Fig. 5.1) and CLPP-MicroResp (Fig. 5.2) ranked in the three sites as follows: Monte Rufeno > Monte Peglia > Lago di Vico. In particular in the last site microbial activity decreased by one order of magnitude than in the other ones.

The community level physiological profile performed with MicroResp (Fig. 5.2) and Biolog (Fig. 5.3) showed a high consumption of substrates in Monte Rufeno. In particular glucose substrate, used in both techniques, is one of the most employed by soil microbial communities. The parameters derived from Biolog data are reported in table 5.3, in particular it is possible to observe that the microbial growth (slope, hours and area) for the microorganisms in Monte Peglia is slower than in the other sites.

Among the different substrates, used with the BIOLOG technique, polymers (such as Tween 40 and 2-hydroxy benzoic acid) used were better employed by the microbial biomass in the Andosol (Lago di Vico) than in the Inceptisol and Entisol. The CH/AA ratio was significantly higher in Monte Rufeno (Table 5.3).

5.4 Discussion and conclusions

The studies assessing the effect of parent material on soil microbial properties are several, in particular those regarding the natural forest ecosystems (Vittori Antisari et al., 2001; Stursova, 2011; Chodak and Niklinska, 2010).

The differences in physical and chemical properties of the studied soils were reflected in their microbial activity, in fact Monte Rufeno exhibited higher values for enzyme activities and MicroResp. Lago di Vico soil potentially showed the best conditions for the growth and metabolism of microorganisms (higher CEC, sub-acidic pH and higher C_{org} and N content, lower C:N ratio); conversely this site was characterized by a “static system” showing the lowest microbial activities. These results could suggest different hypotheses that will be tested in the future: i) a likely stress condition for soil microorganisms caused by the low quality of dissolved organic matter (DOC) of soil (i.e. presence of recalcitrant chemical material) or ii) the site typical microclimate which maintains a high level of soil

moisture for a long period of the year which, combined to a high content of humic material, could (Lorenzoni et al., 1986) retard all biological processes.

The marked low microbial activity determined in andosols (Lago di Vico) may also be related to the presence of organo-metallic (Al based) compounds of high molecular weight, typically associated with the melanic epipedon on pyroclastic deposits rich in amorphous materials (Piccolo, 1990). Soil moisture, very high trough the whole year, associated with quite low average temperature could represent a cofactor in damping the microbial activity in this particular soil located in Vico Caldera's inner slopes.

Monte Peglia (Entisol) showed intermediate values of microbial activity, generally quite low if compared to Monte Rufeno. This site is characterized by a very shallow depth caused by strong surface water erosion. The topographic factor could be one of the main reason in controlling the low availability of nutrients in weathered pedogenic materials and soil moisture.

The highest values of microbial activity found at Monte Rufeno could be explained according to the relatively young profile horizons structure and the consequent presence of pedogenic weathering and alteration processes both in A and Bw horizons. Furthermore, at Monte Rufeno, the less dense canopy structure of oak stands allows the sunlight to reach the soil surface for a long period, this can promote a higher microbial decomposition and turnover of organic matter because of the increased topsoil temperature (Singh et al., 1999). Bach et al. (2010) found that soil microbial community structure in a boreal forest is spatially structured by the distribution of single trees, and that soil microbial community structure varies seasonally and is affected by tree removal, in a manner that reflects the initial influence of trees. Biolog data showed more heterogeneous results and did not allow to evidentiate a higher use of all substrates in a specific site, probably Biolog technique was sensitive to the differences in pH and in clay content among the three sites. At Lago di Vico site a higher consumption of complex polymers (Tween 40 and 2-Hydroxy Benzoic Acid) was recorded, suggesting a specific ability of microorganisms to metabolize recalcitrant compounds.

The relative use of amino acids, carboxylic acids and polymers was affected by the soil type. Sharma et al. (1998) suggested that higher CH-to-AA ratios indicate higher content of easily degradable carbon compounds in the soils. The higher values of CH/AA ratio found at Monte Rufeno confirm thus the occurrence of specific organic substrates quality that promoted the elevated levels of microbial activity determined in this site with all the techniques used.

The preliminary results suggest that even if plant cover is the same in each site and the topographic conditions are similar, parent material and the specific pedogenic processes could influence in a strong way the functional characteristics of microbial communities. In order to obtain a more comprehensive picture of parent material influence on soil biodiversity our research will be completed with the study of microbial genetic diversity.

5.5 Tables

Table 5.1 - *Physical, chemical and biochemical properties.*

	Monte Peglia	**Monte Rufeno**	**Lago di Vico**
U%	15	25	30
% Clay	19	21	9
% Silt	45	49	42
% Sand	36	30	49
pH	6,18^{b} ±0,08	5,85^{a} ±0,05	6,22^{b} ±0,05
% C_{org}	6,30^{a} ±0,67	2,85^{b} ±0,15	14,27^{c} ±0,40
% N_{tot}	0,47^{a} ±0,03	0,18^{b} ±0,01	1,15^{c} ±0,03
C/N ratio	14,69^{a} ±0,90	15,53^{a} ±0,35	12,38^{b} ±0,01
P (µg P g-1)	6,04^{b} ±0,73	8,18^{a} ±0,46	6,72^{b} ±0,12
CEC $cmol_{(+)kg}^{-1}$	10^{a} ±2,6	7,5^{a} ±2,07	23,5^{b} ±2,70
qmic (µg C bio $TOC^{-1)}$	0,66^{b} ±0,057	1,80^{a} ±0,052	0,65^{b} ±0,055

Table 5.2 - *Biolog parameters: AWCD measured at curve plateau (K) and flex slope (r), number of hours to reach the flex (s), area under the curve.*

	Monte Peglia	**Monte Rufeno**	**Lago di Vico**
K	1,183^{a} ±0,200	1,436^{a} ±0,13	1,335^{a} ±0,06
r	0,0271^{a} ±0,003	0,042^{b} ±0,003	0,052^{b} ±0,002
s	114,57^{a} ±46,85	82,46^{b} ±16,62	80,74^{b} ±5,79
Area	93,11^{a} ±13,90	163,32^{b} ±14,00	153,03^{b} ±6,25
CH-to-AA ratio	1,119^{b} ±0,61	4,282^{a} ±1,84	1,60^{b} ±0,46

5.6 Figures caption

Figure 5.1 – *Enzyme activities*

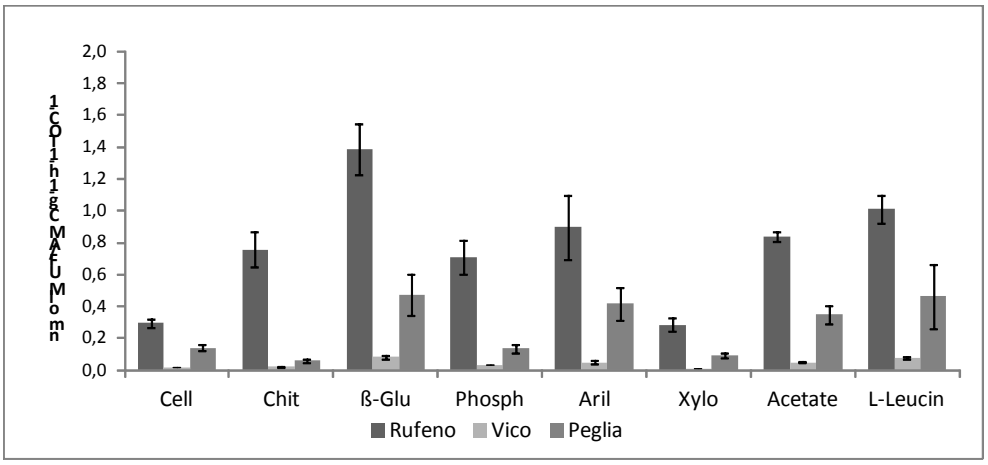


Figure 5.2 - *CLPP-MicroResp activities*

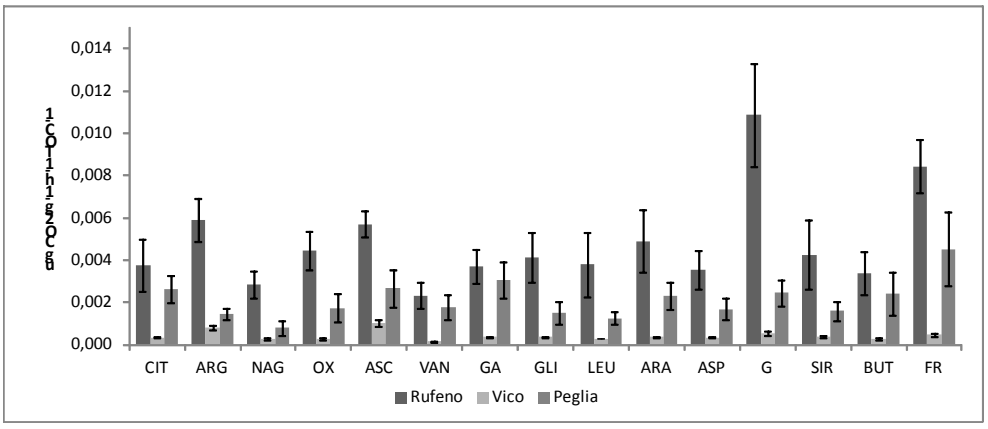
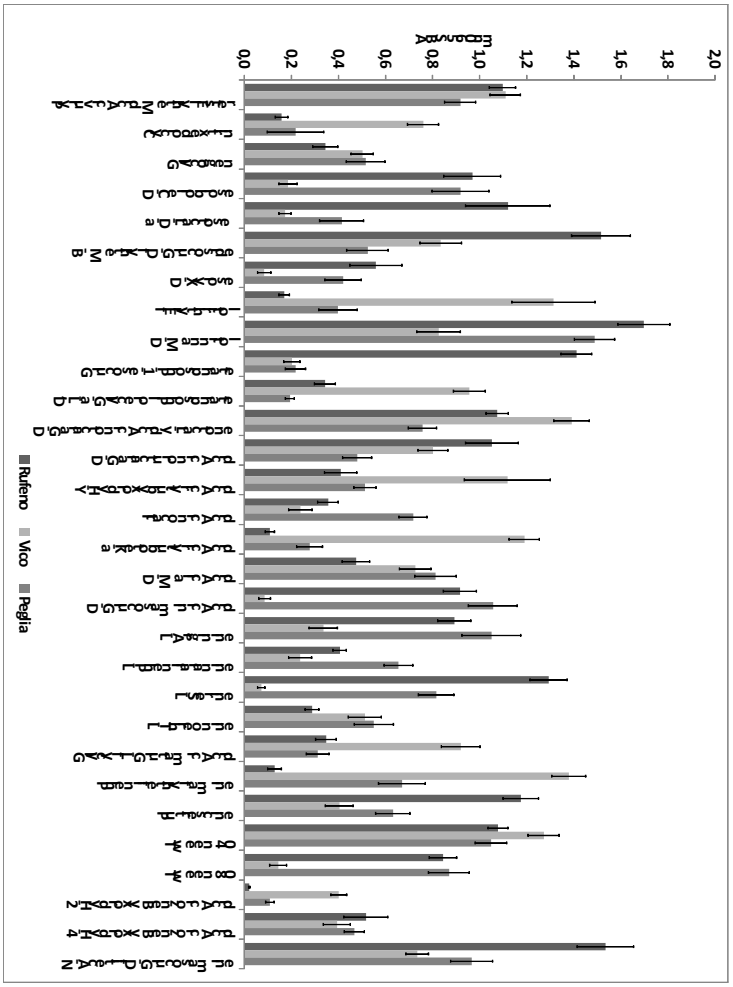


Figure 5.3 - *CLPP-Biolog activities*



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Appendix to chapter 5
Preliminary investigation on soil genetic diversity: effect of lithological substrate

A 5.1 Introduction

Soil microbes control many belowground processes critical to ecosystem functioning through their influence on decomposition of organic matter and their creation of soil structure. Shifts in soil microbial community composition and abundance can significantly influence the dynamics of these fundamental processes. Microbial processes are regulated by a variety of substrates and environmental conditions (Scharenbroch and Bockheim, 2007); it has been difficult to establish the mechanisms responsible for changes in microbial community composition because soil variables are highly correlated (Allison et al., 2007).

Soil properties are highly related to their parent material, profile depth and stone content (Leifeld et al., 2005). Bedrock type, in particular, has been correlated with Mediterranean wood-land ecosystems productivity (Kooijman et al. 2005), while less attention has been paid to the role of geology in ecosystem functioning, although on a local scale parent material can affect the soil organic carbon cycle. Variation in soil mineral assemblage resulted in the dominant control of both cumulative soil C mineralization and soil C priming in a previous study (Rasmussen et al. 2007). Geologic parent material also turned out to be the most discriminating of three predictor variables on forest floor bacterial community composition in the southern boreal forest of western Québec, Canada (Lamarche et al. 2007).

The high spatial variability that characterizes Mediterranean areas suggests that regional studies must include a morphology component; slope aspect, in fact, is an important topographic factor influencing local site microclimate mainly because it determines the amount of solar radiation received.

The effects of stand age on soil and on the microbial community stimulated increasing interest in forest ecology. Natural differences in inputs and transformations of C are likely to occur in stands of different ages due to differences in inputs of above- and below-ground litter (both from trees and from ground vegetation), through fall, light, water and nutrient availability. Changes in DOC dynamics and forest floor bacterial community composition through various stand development stages have been previously investigated (Clarke et al., 2007).

Analysis of the spatial distribution of bacteria at microhabitat levels showed that, in soils subjected to different treatments, more than 80% of the bacteria were located in micropores of stable soil micro-aggregates (2–20 μm) (Torsvik and Ovreas, 2002).

As reported in literature several researchers widely used PLFA (Phospholipid fatty acids) in microbial ecology as chemotaxonomic markers of bacteria and other organisms, in particular forest ecosystems are studied in relation of spatial variability (Weber et al., 2011), management (Leckie, 2005). Nevertheless in the last decades molecular techniques are coming up with the aim to obtain a complete scenario of soil microbial communities: this can be achieved analyzing bacterial communities by different methods based on 16S rDNA clone library (Giovannoni et al. 1990) or, more and more promising, the analysis of a metagenomic library (Rondon et al. 2000).

Such approaches include Denaturing Gradient Gel Electrophoresis, DGGE (Muyzer et al., 1993), Amplified Ribosomal DNA Restriction Analysis, ARDRA and T-RFLP (Osborn, 2000). Among them the last one can be considered potentially promising for microbial community analysis. The potential of T-RFLP to analyze variation between amplified 16S rRNA genes from different bacteria and obtain information on microbial community structure was shown by Liu et al. (1997) and Clement et al. (1998). Liu et al. (1997) discussed the possibility that T-RFLP analysis may permit at least a semi-quantitative analysis of the relative proportions of dominant members/genotypes within a microbial community, while cautioning that T-RFLP analysis is subject to all the biases inherent in any PCR amplification approach. However, due to the complexity of soil communities and the effort required for this type of analysis, clone libraries have been restricted to the analysis of a single or a few samples in an environment. To circumvent the limitations of the clone library approach, several PCR-based methods exist which allow rapid fingerprinting and monitoring of many samples. Terminal-restriction fragment length polymorphism (T-RFLP) is a PCR-based tool which has been introduced for specifically studying the genetic diversity of bacterial communities (Liu et al., 1997). This analysis is based on the detection of a single restriction fragment in each sequence amplified directly from the environmental sample of DNA and is capable of surveying dominant members comprising at least 1% of the total community (Dunbar et al., 2000). T-RFLP has been widely used in recent years for the analysis of bacterial communities in different conditions (Osborn et al., 2000; Fierer et al., 2003) and to assess spatial and temporal heterogeneity and dynamics of bacterial communities in soil (Kuske et al., 2002), sediments and water environments (Braker et al., 2001; Casamayor et al., 2002). Moreover, T-RFLP has

recently been proposed as a standard methodology for assessing soil fertility in comparison to fatty acid methyl ester (FAME) analysis (Suzuki et al., 2005).

Soil biological functions, in particular linked to the activities of microbial communities, are influenced by the interaction between the species (canopy, quantity and quality of litter, roots and rhizodepositions) and the soil type. The present study focused on the influence of different pedogenic substrates on the composition and the activities of microbial soil communities.

A 5.2 Results and discussion

The results confirm our hypothesis that soil chemical data and microbial community composition in natural forest ecosystems vary as a function of geologic parent material. Parent material has been previously found affecting the functional diversity both productivity (Kooijman et al., 2005) and bacterial community (Lamarche et al., 2007) in different ecosystems.

The three forest soils have showed a very different DNA concentration which ranged from 21 ng/µl to 93 ng/µl, suggesting that, as reported in literature (Carletti et al., 2009; Dequidet et al., 2011) and in according with this we expected this type of soil a high genetic biodiversity particularly at Lago di Vico (Figure A5.1).

The concentration of amplified products were calculated prior to purification in order to estimate the batcterial richness of forest soil sample. As we can observe in figure A5.2, 16S rDNA amplified showed very similar patterns in spite of different DNA content in the three sites also using two diverse fluorochromes (VIC and FAM). However the TRFLP results yielded very few peaks (data not shown); this could be due to the fact that this approach mainly targets bacterial population and do not inform on fungi. This is in accordance with what already discussed for CLPP-BIOLOG technique which reflects a very limited fraction of the entire soil microbial community (Ros et al., 2008), where the contribution of fungi is not measured for their slow growth (Nannipieri et al., 2003; Chapman et al., 2007).

Rembering the definition of metagenomics which is the study of metagenomes, genetic material recovered directly from environmental samples, we could hypothesize that probably in the considered natural forest ecosystems fungi biomass is more representative than the bacterial one.

A5.3 Figures caption

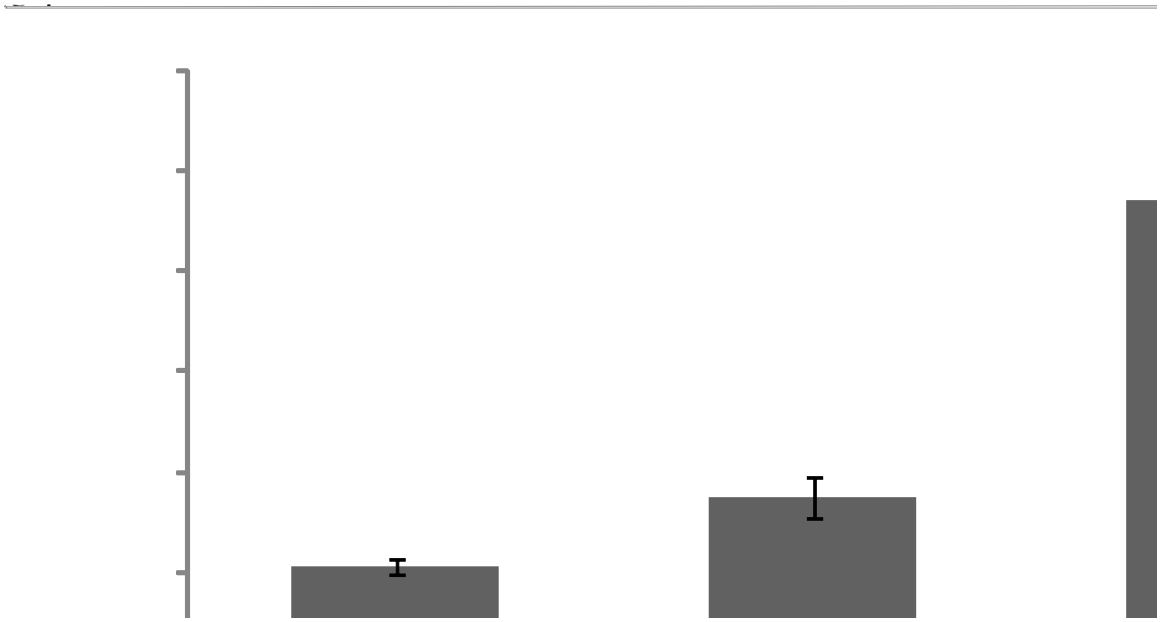

Figure A5.1

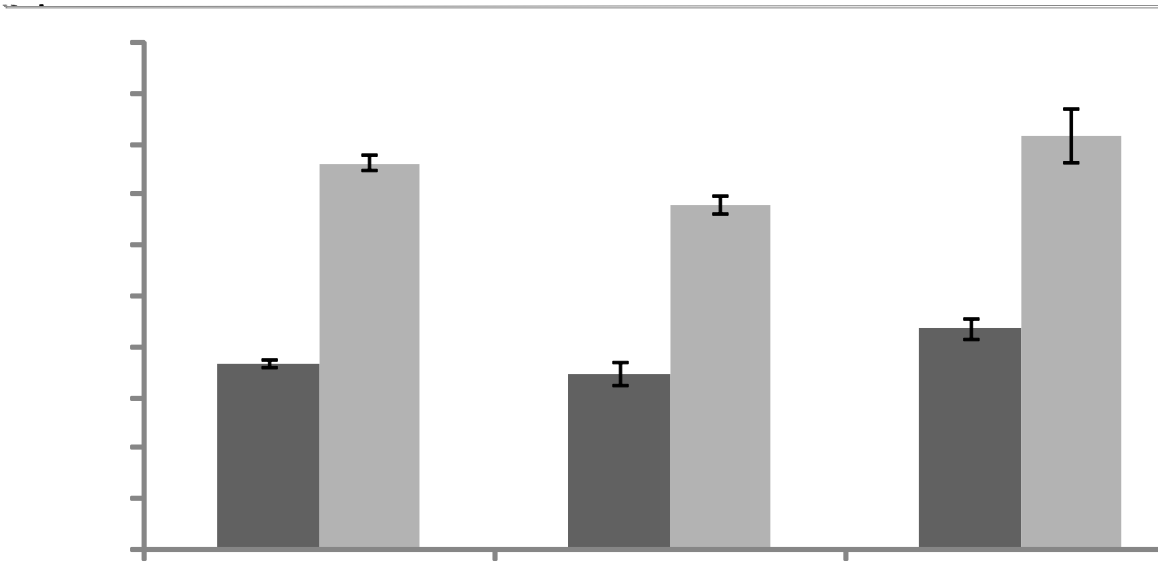

Figure A5.2

A 5.5 References

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Chapter 6. Microbial functional diversity as influenced by different tree species in forest soils under Mediterranean climate

6.1 Introduction

The effects of different plant species on soil microbial communities composition and/or activity have been demonstrated for rhizosphere (Soderberg et al., 2002), bulk soil (Carney and Matson, 2006), trees (Priha et al., 2001; Grayston and Prescott, 2005) and herbaceous plants (Zak et al., 2003). Particularly, the relationship between the soil organic matter (SOM) (quantity and quality) derived from plant remains and the microbial community is not clear in forest sites, while the effect of grassland and leguminous species has been better explored (Grayston and Prescott, 2005; Lucas-Borja et al., 2011). In forest ecosystems, little is known about the link between aboveground tree species diversity and soil microbial diversity. Most studies focused their attention on the interactions between aboveground and belowground communities in microcosms/manipulated field plots under controlled conditions (Porazinska et al., 2003; Van der Putten, 2005; Gleixner et al., 2005). Nevertheless, these have failed to show clear trends between productivity, bacterial diversity and stability. Plant diversity exerts a control on soil microbial community by increasing the diversity of litter and rhizodeposition products (Bartelt-Ryser et al., 2005), as well as the number of mycorrhizal hosts (De Bellis et al., 2006).

Additionally the size and longevity of trees make them important ecosystem engineers that affect both above- and belowground ecosystem components (Bach et al., 2010), in fact the effects of trees on belowground properties are linked with litter and belowground deposition of matter through root exudation and root death. Tree species secrete different types of root exudates, and as the roots grow older, qualitative and quantitative changes occur (Grayston and Campbell, 1996). Root exudates of higher plants include sugars, amino acids and amines, aliphatic and aromatic acids, fatty acids, sterols, enzymes, vitamins and miscellaneous phenolics (Grayston et al., 1996). Trees will also, through their extended root system, take up nutrients over large areas and deposit them under or close to their canopy (Gibson, 1988), and thereby redistribute soil resources (Pärtel and Wilson, 2002).

The quality and quantity of inputs, in the form of litter and rhizodepositions, and character of soil organic matter, are likely important factors for structuring the microbial communities in different ecosystems. Conifers litter in particular undergoes a slower decomposition rate than broadleaves one because of its high concentration in lignin,low presence of degradable compounds and the increase of the recalcitrant ones which inhibit the degradation processes. Pine litter contains large amounts of tannins and lignins leading

thus to a likely shift in microbial community composition being fungi the main degraders of lignin (Dix and Webster, 1995).

The concentrations of micro-macronutrients, the presence of complex molecules (such as chitin, cellulose, hemicelluloses and lignin) very resistant to enzymatic degradation may affect in a strong way the degradation processes of plant litter, moreover influencing the nutrients availability (Rutigliano et al., 2004).

The presence of plant cover however is not the only the factor which most influences the composition and activity of the soil microbial biomass (Klose et al., 2004), in fact plant diversity is also regarded as a driver for ecosystem productivity (Tilman et al., 1996), which may also improve microbial stability. Soil microbial communities benefit of the carbon compounds and nutrients released by roots, *viceversa* the soil biota decompose the soil organic matter, stabilize soil structure producing other nutrients for plants growth.

According to Insam (2001), functional diversity represents the sum of the ecological processes developed by the organisms of a community and it can be expressed through species or important groups to maintain several functions in the soil, while genetic diversity represents gene and genotype variations inside the species and it is the total genetic information in the genes of all the animals, plants and microrganisms that live the earth. The loss of plant species, as it may occur after coppicing or thinning, in certain ecosystems can lead to changes in the community of soil decomposers which, in turn, affect the mineralization of organic matter with consequences for other ecosystem processes (Spehn et al., 2000). Saetre and Baath (2000) suggested that microbial community composition is related to the tree species influence on soil organic matter turnover more than the effects of tree species on soil moisture, light, and ground vegetation. Under field conditions, two obvious factors, plant diversity and geologic parent material, come to mind as probable determinants of soil microbial stability. Lithological substrate, in fact, controls site nutrient capital, including base cations which control soil pH, as well as CEC (cation exchange capacity) and drainage (Carletti et al., 2009). Thus when studying the effect of different tree species on soil microbial communities it is important to consider also soil properties as a likely additional variation factor.

Soil enzyme activities and CLPP-MicroResp are very useful techniques for the assessment of the status or the condition of the soil environment and are both widely used as a measure of functional diversity of the whole microbial community (Lalor et al., 2007; Naseby and Lynch, 2002).

Loynaz et al. (2008) in a study of five different vegetation types and applying different enzyme activities found several correlations between plant diversity, soil physicochemical properties, and soil enzyme activities. Marinari and Antisari (2010) in an investigation of the microbial activity, along forest brown soil profiles sequence developed on different lithological substrates, found that the biochemical activity along profiles was mainly regulated by different soil organic matter content and quality derived from different plant species litter.

The aim of this study was thus to evaluate the effect of different tree species on the size, metabolic activities and functional diversity of soil microbial communities in naturally developed plant communities in Central Italy of native oak, chestnut and white pine forest without imposing any disturbances to already existing plant–soil relationships. In particular we focused our attention on soil and not on litter because regarding the first one few information are available in particular in monospecies stands. We chosen the same lithological substrate to compare the functional diversity linked to different species in the same environment.

6.2 Materials and methods

6.2.1 Site description

The present work was carried out in a natural forest located in Central Italy: Monte Rufeno Natural Reservation (32T 0736390 UTM 4742149). It has been established in 1983 and it is enclosed in the regional systems of protected areas. The Reservation is located at 780m a.s.l. and in an area of 2.892 hectares with a greater part enclosed in the municipality of Acquapendente, district of Viterbo. The forested areas cover a large part of the Natural Reservation and the main species are chestnut (*Castanea sativa*), oak (*Quercus cerris spp.*) with the presence also of the other trees and shrubs such as willow (*Salix Alba L.*), poplar (*Populus Alba*), maple (*Acer monspessolanum L.*) and ash tree (*Fraxinus ornus L.*), arbutus (*Arbutus unedo L.*), phillyrea (*Phyllirea latifolia L.*), buckthorn (*Rhamnus alaternus L.*), viburnum (*Viburnus tinus L.*). Moreover in the last decades Police Forestry introduced a new tree species, eastern white pine (*Pinus strobus*), now covering a part of the Reserve.

In this research we selected three areas where the plant cover was monospecies: eastern white pine (*Pinus strobus*), chestnut (*Castanea sativa*) and oak (*Quercus cerris*), all grown on the same lithological substrate (*Dystric Cambisol*) (clay 21%, silt 49%, sand 30%) and with the same environmental characteristics: NNE exposure, around 650mt height a.s.l, 900mm annual rainfall. This aspect was considered with the aim to discriminate additional

factors of variation. The eastern white pine or white pine (*Pinus strobus*) has the distinction of being the tallest tree in eastern North America. In natural pre-colonial stands it is reported to have grown to as tall as 70 m. Like all members of the white pine group, *Pinus* subgenus *Strobus*, the leaves ('needles') are in fascicles (bundles) of five (rarely 3 or 4), with a deciduous sheath. White pines, prefer well-drained soil and cool, humid climates, but also grow in boggy areas and rocky highlands.

Oak is a tree or shrub in the genus *Quercus*, which is composed of about 600 species. Oaks are keystone species in a wide range of habitats from Mediterranean semi-desert environments to subtropical rainforest. For example, oak trees are important components of hardwood forests. It can grow under optimum conditions 20-30 m in height and can be found from coastal spots up to the mountains.

Chestnut, or sweet chestnut, (*Castanea Sativa Mill.*) is a genus of eight or nine species of deciduous trees and shrubs in the beech family Fagaceae. *Castanea Sativa* is the only European species of chestnut, though successfully introduced to the Himalayas and other temperate parts of Asia. Chestnut trees range from moderate growth rate to fast-growing for American and European species. Their mature heights vary from the smallest species of chestnut, often shrubby, to the giant of past American forests, Castanea dentata that could reach 60 m. Usually the height of the European chestnut (Castanea sativa) is around 30 m.

From a geological viewpoint, Monte Rufeno Natural Reservation is made up of sedimentary formations, lying in the immediate vicinity of the Vulsini volcanic complex (Burragato et al., 2010). Flysch facies are dominant (Buonasorte et al., 1988) and consist of clays and marly clays, argilloschists, marly limestones and marls, interbedded with siliceous limestones, calcarenites and sandstones. Basic magmatic rocks, which underwent metamorphic processes, are found among the flysch facies. These are ophiolitic formations of secondary deposition with respect to the flysch and at times associated with euphotites. The latter developed as result of low-grade metamorphism of serpentinous breccias with carbonate cement of marine sedimentary origin (Brotzu et al., 1973). Based on the paragenetic talc-tremolite-calcite assemblage of the ophicalcites, the intensity of the metamorphic-metasomatic event may attributed to the green schist facies. Asbestiform materials are observed within the serpentinites and ophicalcites (Burragato et al., 2001).

Soil samples were collected in September 2009. For each species twelve samples in A horizon, after removal and litter and O layer, were sampled where no shrubs were present in order to reduce bias in the study. Soil cores were collected under the canopy in a middle position between the stem and end of canopy. All soil samples were air dried and then

sieved at 2mm. Before biochemical and microbiological analyses the soil moisture content was adjusted to 60% of their water holding capacity (WHC) and then left to equilibrate at room temperature in the dark for 10 days (Pinzari et al., 1999).
All biochemical data were presented on total organic carbon unit in order to eliminate differences in site properties.

6.2.2 Chemical and biochemical analysis

The techniques applied in this work are reported in detail in the chapter two (materials and methods) and here they are resumed as follows:

(1) Chemical analysis (pH, TOC, TN and C/N);
(2) Microbial biomass carbon, basal and cumulative soil respiration;
(3) Enzyme activities (β-glucosidase, β-xylosidase, β-cellobiopyranoside, Leucine aminopeptidase, N-acetyl-β-glucosaminidase, arylsulfatase, acid phosphatase and acetate esterase);
(4) CLPP-MicroResp™;
(5) Calculation of diversity indexes (Index of biological fertility (IBF) and Simpson-Yule index);
(6) Statistical analysis.

6.1.3 Statistical analysis

The analysis of variance (ANOVA) was performed on six replicates for each species, to evaluate the significance of differences. All statistical analyses were performed using SPSS 16 Linux edition. Statistica 7 (StatSoft, Tulsa, USA) for Windows has been employed. Statistical significance was determined at $p \leq 0.05$. Discriminant Functional Analysis (DFA) for enzyme activities and MicroResp™.

6.3 Results

6.3.1 Chemical and biochemical analyses

Most of the chemical properties (pH, total organic carbon and total nitrogen, basal respiration) showed significant differences under the three species (Table 6.1).
Oak soil showed the highest values for microbial biomass, basal and cumulative respiration (table 1 and figure 1), and the microbial quotient. Also microbial indexes (qmic, IBF and qCO_2) pointed out significant different values for oak (Table 6.2). All the species reported

an IBF value higher than 20, falling into the class (5) indicating thus a high degree of biological fertility.

6.3.2 Enzyme activities

The discriminant functional analysis (DFA) showed a clear species effect due to the different tree cover, in particular there is a strong separation between conifer and broadleaves (Fig. 6.1), the X axis accounted for 88,42% of the data variability and Y axis accounted 11,56%. Cellulase, β-glucosidase and leucine-amino-peptidase were significantly higher under Eastern White Pine.

In general oak soil showed the lowest activity (Fig. 6.1).

The diversity index calculated with the enzyme activities pointed out a higher functional diversity for the eastern white pine (Fig. 6.3).

6.3.3 CLPP-MicroResp

The DFA, reported in figure 6.2, showed a strong species effect. The community level physiological profile (CLPP), measured by means of MicroResp™, showed a clear consumption of the different substrates employed, the X axis accounted for 86,97% of the data variability and Y axis accounted 13,03%. In particular, grouping the results for guilds, we observed a high consumption of carboxylic acids for the white pine, a general high activity for amino acids and carbohydrates in the oak, the phenolic acids pointed a major consumption for the white pine and oak, while amide had the higher consumption in broadleaves. The diversity index Simpson-Yule indicated the oak soil as the one with higher values of microbial functional diversity (Fig. 6.2)

6.4 Discussion

6.4.1 Chemical and biochemical properties

Before its establishment Monte Rufeno Natural Reserve was affected by different forms of anthropic exploitation. The area with deciduous covering was exploited for coal production, while conifer species were introduced only in the last decades by Police Forestry. Forest floor microbial communities play a major role in the retention and cycling of nutrients in old-growth coniferous forests. The activity of these communities is partially controlled by climatic and biochemical conditions imparted by the dominant tree cover. For example, a closed forest canopy may affect forest floor temperature and moisture (Vesterdal et al.,

2012), which may in turn affect microbial processes such as decomposition and nutrient mineralization (Martin et al. 1999).

Changes in soil chemical and physical environment (Beck et al., 1995) may influence the quantity and composition of microbial biomass. Decomposition is an important process for cycling of nutrients in forest ecosystems. Decomposition processes are influenced by macro- and micro-climate, litter quality, activity of decomposing organisms and soil nutrient status (Vesterdal et al., 1999). Kara et al. (2008) reported that soil pH is considered as one of the most important factors which influence the composition of soil microbial communities. Several studies pointed out, with different methods such as bacteria isolation (Kytöviita et al., 1990) or PLFA (Bååth et al., 1995), the variation of soil communities to metabolize different carbon substrates after a pH variation. Significant differences in our work were observed in chemical properties (pH, TOC, TN) among the species and between the species belonging to the same family (*Fagales*); previous studies conducted in boreal area indicated that different tree species planted within the same sites have different amounts of SOC (Vesterdal et al., 2008). In this study lower values of soil pH were observed under white pine; as known, pine litter produces a soil acidification (Pennanen, 2001; Iovieno et al., 2010). Most of the chemical (pH, C, N and C/N ratio) and biochemical properties (Cbas, Cmic and qmic) agree to prove for white pine soil a delay in the decomposition processes and a hard ability to grow for microorganisms (Balvanera et al., 2006; Stephan et al., 2000; Zak et al., 2003). Raich and Tufekcioglu (2000) reviewed vegetation influence on soil respiration and found an indication that broadleaf stands have higher respiration rates than conifer stands at the same site. Despite oak and chestnut a higher level of soil moisture combined to a diverse quality of litter and rhizodepositions under pine cover may present sub-optimal environmental conditions for soil microbes.

Odum (1985) argued that, during the evolution of ecosystems, organisms improve their efficiency in the use of energy by reducing respiration and increasing the production of biomass, in agreement with this theory, numerous studies have reported decreases in metabolic quotient during primary succession (Schipper et al., 2001; Ohtonen et al., 1999). According to these considerations the site dominated by oak and characterized by high q_{mic} and low qCO_2 could suggest a major efficiency in the carbon utilization in the soil of a mature system. A low qCO_2 agrees with a higher catabolic evenness index (measured with MicroResp) suggesting, in a mature stand, a major efficiency to exploit the different carbon sources and a higher diversification of its metabolic activities, representing at the same time a low stress for microbial communities in that plot. Conversely in conifer plot,

the results indicate that high values of qCO_2 reflect difficulties in the use of organic substrates by the microbial biomass (low values of soil qmic) (Pinzari et al., 1999). Jiang et al. (2011) in a study on soil restoration demonstrated that the metabolic quotients were much higher under the coniferous forest ecosystem than those under broadleaf forest ecosystem, with the lowest under broadleaf forest ecosystems. High metabolic quotient values and low catabolic diversity, found in this study are in accordance with Jiang et al., (2011). For Nsamibana et al., (2004), the difference between the microbial pool size and qCO_2 could indicate the predominance of non-metabolically active microorganisms within biomass pool, and/or an increase in fungi:bacteria ratio induced by the diverse nature and quality of available organic substrates.

6.4.2 Enzyme activities

The potential effects of tree species on soil properties have been studied for a long time, and the associated plant–soil interactions provide important feedbacks that regulate ecosystem processes (Porazinska et al., 2003). Studies regarding plant–soil interactions revealed that plant species may have significant impacts on soil physicochemical properties (e.g., soil water content and pH) and on the quality of substrate for soil microbes (e.g., total carbon, nitrogen, C/N ratio, and the phenolic concentrations in soil) under the plant species through their litter quality and quantity (Binkley and Giardina, 1998). Soil physicochemical properties and substrate quality have been used to explain differences in decomposition processes (i.e., mineralization of soil organic matter) under different plant species. Soil enzyme activities have been related and are deeply modified by soil physico-chemical characters (Amador and Rones, 1997), microbial community structure (Waldrop et al., 2000), vegetation (Waldrop et al., 2000), lithological substrate (Pignataro et al., 2011), disturbance (Boerner et al., 2000), management practices (Mosca et al., 2007).

In this study significant fluctuations of almost all biochemical properties were found, revealing a high degree of sensitivity in relation to tree diversity. Soil physico-chemical properties (pH, OM content, total N) have been reported to affect soil enzyme activities (Ushio et al., 2008), affecting the diversity and composition of the soil microbial community (Bardgett et al., 2005). Enzyme production in a soil reflects the availability and chemical forms of nutrients but other environmental conditions, such as soil pH, may additionally modify the enzyme activity (Protovska and Mazurer, 2009). Loynaz et al. (2008) found that soil pH affect the activity of soil enzymes through different mechanisms such as the ionization or protonation of the acidic or basic groups in the enzyme active

centre, this is thought to account for most of the decrease in enzyme activity observed when pH deviates from optimum (Wang et al., 2006). Moreover soil pH can alter the concentration of inhibitors, activators, and substrates (Wang et al., 2006). According with Loynaz et al. (2008) we found positive correlations with pH for cellulase ($r=0,48$; $p<0,001$), β-glucosidase ($r=0,47$; $p<0,05$) and leucine amino-peptidase ($r=0,83$; $p<0,001$). According with Ushio et al., (2008) organic matter accumulated on the forest floor suggests species-specific differences primarily due to the its quality. Litter decomposition results from the activity of microbial extracellular enzymes that degrade the cellulose (β-glucosidase and cellobiohydrolase), hemicellulose (xylosidase) (Waldrop et al., 2004). Enzyme activities in litter and wood are good predictors of litter decomposition and may be a limiting step in litter decomposition in some systems (Sinsabaugh et al., 1994). In hardwood forest with litterfall season limited, the changes in the chemical composition of litter during its decomposition are another important cause of differences in enzyme activities (Wittmann et al., 2004; Fioretto et al., 2000). The needles have been found to degrade in a long period (several years) (Geoffroy et al., 1987; Molfetas, 1981) than deciduous leaf litter due to their lignin content (Yu and Peng, 1996), while in deciduous around 70% of the total leaf litter is easily decomposed.

White pine soil shows low biomass, low activities, low use of carbon substrates but a general higher enzymatic activity. This could indicate high recalcitrance of litter and carbon substrates that need to be degraded and hydrolysed before being utilised. Syringic acid is highly consumed under White Pine where litter is characterized by high content of phenolics (Nsabimana, 2004; Grayston et al., 1996). Though litter chemistry was not measured in this study, it is reported that pine litter is characterized by a lower quality (high C/N and lignin/N ratio) that broadleaves one (Perez-Suarez et al., 2009). The significant difference in leucine amino-peptidase among the three different species could suggest a diverse microbial N demand, higher under pine. Several authors (Guckland et al., 2009; Jacob et al., 2009) found that different tree litter, affected the nutrient status of the soil surface layers, providing different initial nutrient concentrations, which influence the soil nutrient status of the topsoil layer. In our study microbial biomass was positively correlated to leucine amino-peptidase ($r=0,70$; $p<0,001$) in soils under white pine indicating that a greater microbial demand for N is required to balance the elevated C availability from litter (Kuzyakov, 2002). Although total soil N is higher under pine it is possible that it is immobilized in forms not available for microbes.

In conclusion, soil mineralization activities were significantly different among tree species. The difference could result from the combined effects of the soil substrate quality, physicochemical properties. This localized mineralization activity and the soil properties could subsequently influence soil nutrient availability in this forest.

6.4.3 CLPP-MicroResp

Our analysis pointed out that the CLPP-MicroResp™ system can detect considerable variation in the ability of soil microbial communities under different tree species to metabolize different C compounds (Fig. 6.3). The microbial catabolic activity was directly influenced by the soil nutrient availability and plant inputs (litter and roots), since the plant species significantly affected C utilization rates, and at the same time the higher metabolic rates were observed by the soil communities under the broadleaved forest ecosystems than under the conifer forest ecosystems (table 3.1). As reported by Paul and Clark (1989) this confirms the role of the C substrate quality in determining C utilization. For (Jiang et al. 2010) this is explained that, at the same experimental site, organic carbon was the greatest under the broadleaf forest ecosystems, followed by the conifer forest ecosystems.

Under natural conditions, the largest source of labile carbon (C) inputs to the soil is root exudates (Bertin et al. 2003; Hutsch et al. 2002; Kuzyakov 2002). The quantity and quality of root exudates released by trees show considerable variation between species (Grayston et al., 1996), exuding a broad variety of low-molecular weight organic compounds (sugars, amino acids, organic acids and phenolics) which change with plant age (Hale et al., 1978; Hamlen et al., 1972). The availability of organic substrates in soil has been shown to be a major regulator of the dynamics and composition of heterotrophic microbial communities (Wardle et al., 1992; Griffiths et al., 1999). In this study a positive significant correlation ($r=0,64$; $p<0,05$) was observed between Cmic and CLPP, as also reported by Hackl et al. (2005), this relationship under different forest ecosystem could only have been attributable to the presence of different microbial species, which was optimal to the typical micro-environment developed under different tree species. CLPP approach can distinguish among different plant covering in fact Fu and Cheng (2002) found that soil microbial catabolic response was dependent on plant species. In our study we found that soils under oak canopy utilized most of the C sources (with an average of 0,036 $\mu g\ C\text{-}CO_2 g^{-1} h^{-1} TOC^{-1}$) despite the conifer ones (with an average of 0,020 $\mu g\ C\text{-}CO_2 g^{-1} h^{-1} TOC^{-1}$); nevertheless soil microorganisms under white pine canopy metabolized better than oak three different substrates (syringic acid, oxalic acid and ascorbic acid), in agreement with Jiang et al.

(2011). On the other hand the higher use of carbohydrates under oak is also reported by Burton et al. (2010).

For Vivanco and Austin (2008) long-term effects of plant species create specific conditions that enhanced decomposition of their own litter, establishing affinity effects between single-species litter and their own microenvironment. In agreement with these authors as reported in our DFA results it is possible to observe a clear species effect on microbial communities. Previous studies have shown that coniferous and deciduous tree species differentially affect the chemical and microbial properties of soil (Priha and Smolander, 1997; Saetre, 1999), and species have various soil fertility requirements (Sims, 1990). Deciduous litter may be more favourable for microbial decomposers than coniferous litter (Mikola, 1985), and the greater quality of deciduous species litter may have a positive effect on the functional diversity of soil (Sharma et al., 1998), as reported in picture XXX it is possible demonstrate that deciduous litter exploited better than coniferous one all the substrates available. White pine litter, in fact, is more resistant to decomposition, due to the high content of recalcitrant polymeric phenolic compounds (lignin and tannin) contained in the needles (Niemi et al., 2007).

We could hypothesize moreover another factor that could have influenced exudation patterns and hence the microbial communities, in fact with different tree species different mycorrhizal symbionts may be associated (Molina et al., 1992). Ectomycorrhizal fungi have been reported to be dominant colonisers of species within the conifer (Smith and Read, 1997), while deciduous have both endomycorrhizal and ectomycorrhizal associates (Janos 1980). Anyway actually few studies found relationships between mycorrhizae and enzyme activities (Mosca et al., 2007) while we did not find any work that confirmed our hypothesis with CLLP, however further analysis are going to confirm this observation.

Litter, root and soil data from this study and previous studies at this site tend to suggest that the change in tree species/stand composition has reduced the quality and quantity of organic matter input, which may have contributed to the shift in soil microbial community composition and diversity (Burton et al. 2010).

6.5 Conclusions

Microbial functional diversity was significantly different under the three plant species (*Q. cerris, C. sativa* and *P. strobus*) pointing thus to three different systems:Microbial biomass under *Quercus* seemed to be particularly active showing high respiration rates, high carbon substrates utilisation, high efficiency in the use of carbon substrates (low qCO_2). *Castanea*

soil showed intermediate values of biomass, activities, diversity and a homogeneous use of carbon substrates. *Pinus* soil showed low biomass, low activities, low use of carbon substrates but a general higher enzymatic activity. This could indicate high recalcitrance of litter and carbon compounds that need to be degraded and hydrolysed before being utilised.

6.6 Tables

Tables 6.1: Soil chemical and biochemical properties, different letters represent significant differences among the species ($p<0.05$).

	U%	pH H_20		N%		C%		C/N		Cbas		Ccum		Cmic	
EW Pine	9,73 [a]	5,3 [a]	0,03	0,21 [a]	0,006	2,19 [a]	0,080	10,48 [a]	0,213	0,718 [a]	0,014	1021,52 [a]	32,015	104,97 [a]	25,964
Chestnut	6,26 [b]	5,5 [b]	0,04	0,12 [b]	0,007	1,86 [b]	0,140	15,85 [b]	0,540	0,873 [b]	0,041	857,23 [b]	65,991	186,39 [b]	40,402
Oak	6,99 [b]	5,9 [c]	0,05	0,19 [c]	0,011	2,86 [c]	0,141	15,53 [b]	0,336	1,056 [c]	0,056	1328,43 [c]	38,920	530,77 [c]	12,675

Tables 6.2: Metabolic quotient (qCO_2), microbial quotient (qmic), index of biological fertility (IBF). Different letters represent significant differences among the species ($p<0.05$).

	qCO2		IBF		qmic	
EW Pine	0,035 [a]	0,009	22 [a]	0,365	0,49 [a]	0,107
Chestnut	0,025 [a]	0,005	23 [a]	0,601	0,96 [b]	0,134
Oak	0,008 [b]	0,000	25 [b]	0,833	1,96 [c]	0,168

Tables 6.3: Soil enzyme activities expressed in MUF/AMC$g^{-1}TOC^{-1}$.Cellulase (CELL), Chitinase (CHIT), ß-Glucosidase (ß-GLU), Acid phosphatase (PHO), Arylsulfatase (ARY), Xylosidase (XYL), Acetate (ACE), L-Leucine (L-LEU), different letters represent significant differences among the species ($p<0.05$).

	CELL		CHIT		ß- GLU		PHO		ARY		XYL		ACE		L- LEU	
EW Pine	0,83 ^{a}	0,05	0,17 ^{a}	0,03	3,01 ^{a}	0,20	9,58 ^{a}	0,35	3,16 ^{a}	0,17	0,54 ^{a}	0,04	21,5 ^{a}	0,11	6,07 ^{a}	0,12
Chestnut	0,54 ^{b}	0,07	0,14 ^{a}	0,02	1,99 ^{b}	0,46	10,36 ^{b}	0,69	3,84 ^{a}	0,36	0,49 ^{a}	0,07	21,0 ^{a}	1,88	2,44 ^{b}	0,29
Oak	0,52 ^{b}	0,06	0,13 ^{a}	0,03	2,39 ^{b}	0,29	12,18 ^{b}	0,49	1,63 ^{b}	0,41	0,49 ^{a}	0,06	14,5 ^{b}	0,92	1,88 ^{b}	0,12

Tables 6.4: CLPP-MicroResp substrates consumption, expressed in µg C-$CO_2g^{-1}h^{-1}TOC^{-1}$. Substrates used are Citric acid (Cit), Oxalic acid (Ox), Ascorbic acid (Asc), Arginine (Arg), Glycine (Gly), Leucine (Leu), Aspartic acid (Asp), Butirric acid (But), Galattose (Ga), Arabinose (Ara), Glucose (Glu), Fructose (Fr), Vanyllic acid (Van), Siringic acid (Sir), N-Acetyl-Glucosamine (Nag). CA is carboxylic acids, AA is amino acids, CH is carbohydrates and A is amide. Different letters represent significant differences among the species ($p<0.05$).

	CIT (CA)		OX (CA)		ASC (CA)		ASP (AA)		BUT (AA)		ARG (AA)		GLI (AA)		LEU (AA)	
EW Pine	0,027 ^{a}	0,006	0,058 ^{a}	0,004	0,064 ^{a}	0,006	0,011 ^{a}	0,007	0,014 ^{a}	0,006	0,057 ^{a}	0,006	0,017 ^{a}	0,001	0,009 ^{a}	0,004
Chestnut	0,060 ^{b}	0,008	0,036 ^{b}	0,004	0,031 ^{b}	0,004	0,027 ^{b}	0,006	0,015 ^{a}	0,002	0,075 ^{b}	0,007	0,021 ^{a}	0,003	0,009 ^{a}	0,003
Oak	0,029 ^{a}	0,003	0,026 ^{c}	0,002	0,039 ^{c}	0,003	0,034 ^{b}	0,007	0,025 ^{b}	0,002	0,055 ^{a}	0,007	0,032 ^{b}	0,006	0,030 ^{b}	0,010

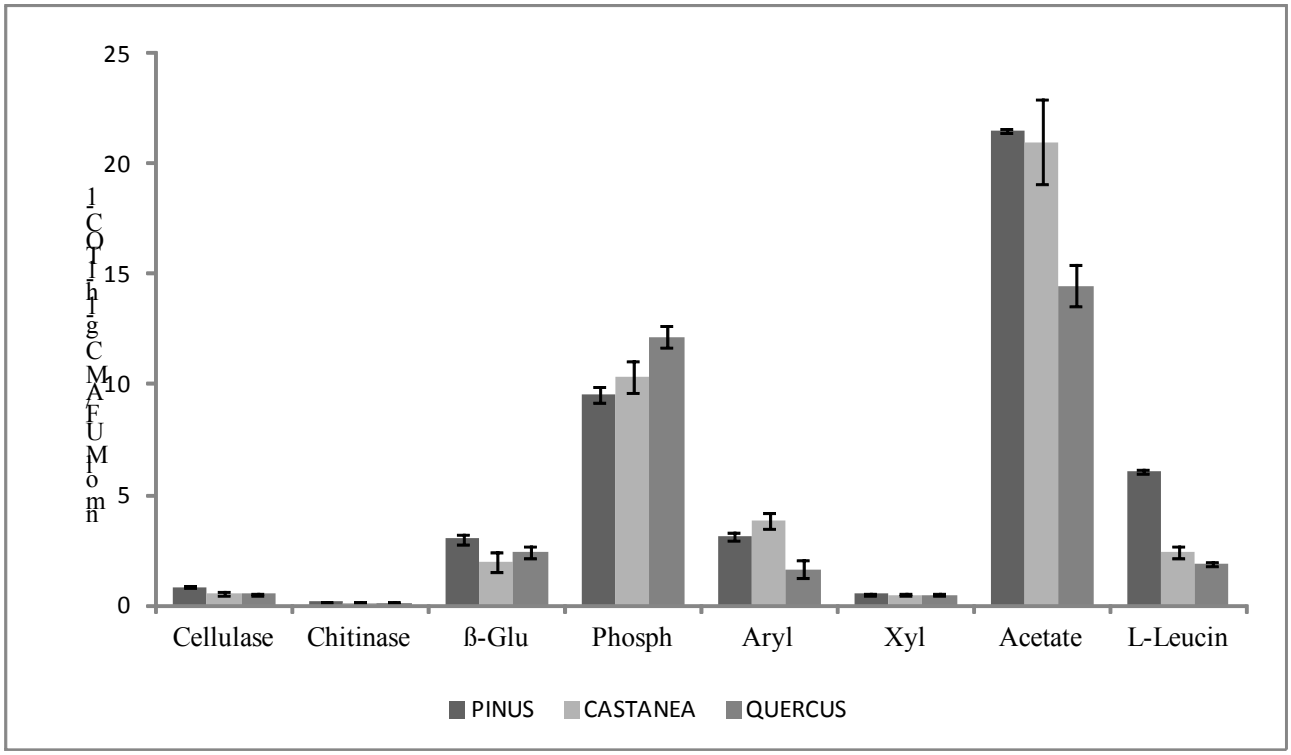
	FR (CH)		ARA (CH)		GA (CH)		G (CH)		SIR (PA)		VAN (PA)		NAG (A)	
EW Pine	0,019 ^{a}	0,008	0,020 ^{a}	0,007	0,013 ^{a}	0,004	0,041 ^{a}	0,014	0,053 ^{a}	0,013	0,008 ^{a}	0,002	0,009 ^{a}	0,003
Chestnut	0,032 ^{a}	0,005	0,022 ^{a}	0,005	0,021 ^{a}	0,005	0,034 ^{ca}	0,003	0,023 ^{b}	0,004	0,011 ^{a}	0,004	0,024 ^{b}	0,003
Oak	0,052 ^{b}	0,007	0,049 ^{b}	0,009	0,035 ^{b}	0,004	0,056 ^{ba}	0,004	0,034 ^{ca}	0,003	0,024 ^{b}	0,006	0,025 ^{b}	0,005

6.7 Figures caption

Figure 6.1 – Effect of tree species on enzyme activities and Discriminant Functional Analysis. Enzymes used are: Cellulase (Cell), Chitinase (Chit), β-Glucosidase (β-Gluc), Acid Phosphatase (Phosh), Arylsulfatase (Aryl), Xylosidase (Xyl), Acetate Esterase (Acetate), L-Leucine-Aminopeptidase (L-Leucin). Standard error bars are reported, *n*=6.

Figure 6.2 – Effect of tree species on CLPP-MicroResp and Discriminant Functional Analysis. Substrates used are: Citric acid (Cit), Oxalic acid (Ox), Ascorbic acid (Asc), Arginine (Arg), Glycine (Gly), Leucine (Leu), Aspartic acid (Asp), Butirric acid (But), Galattose (Ga), Arabinose (Ara), Glucose (Glu), Fructose (Fr), Vanyllic acid (Van), Siringic acid (Sir), N-Acetyl-Glucosamine (Nag). Standard error bars are reported, *n*=6.

Figure 6.3 – Simpson-Yule diversity index applied to enzyme activities and CLPP-MicroResp. Standard error bars are reported, *n*=6.


25
20
15
10
5
0
Cellulase
Chitinase
ß-Glu
Phosph
Aryl
Xyl
Acetate
L-Leucin
PINUS
CASTANEA
QUERCUS

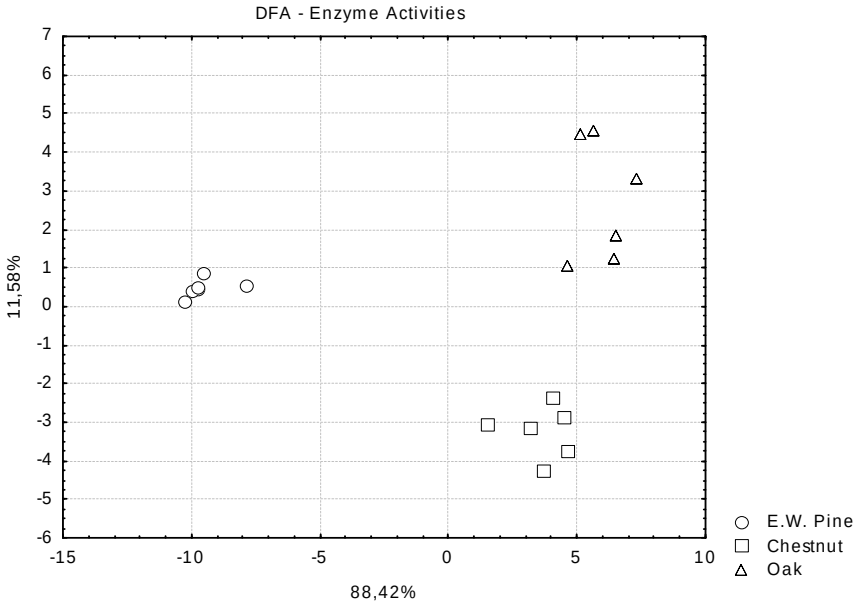
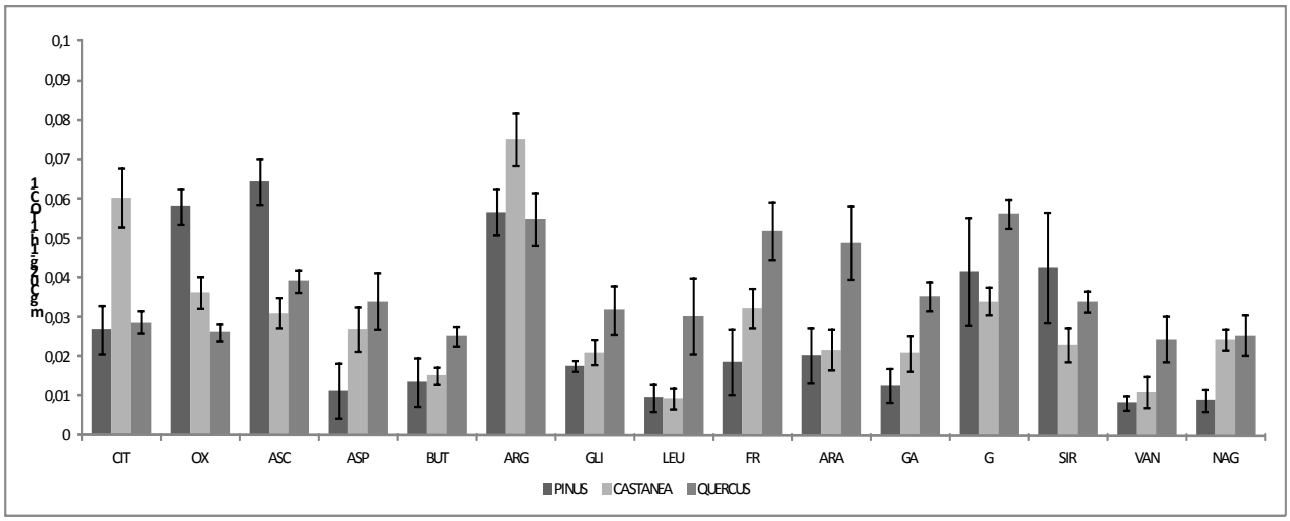

DFA - Enzyme Activities
11,58%
88,42%
E.W. Pine
Chestnut
Oak

Figure 6.1



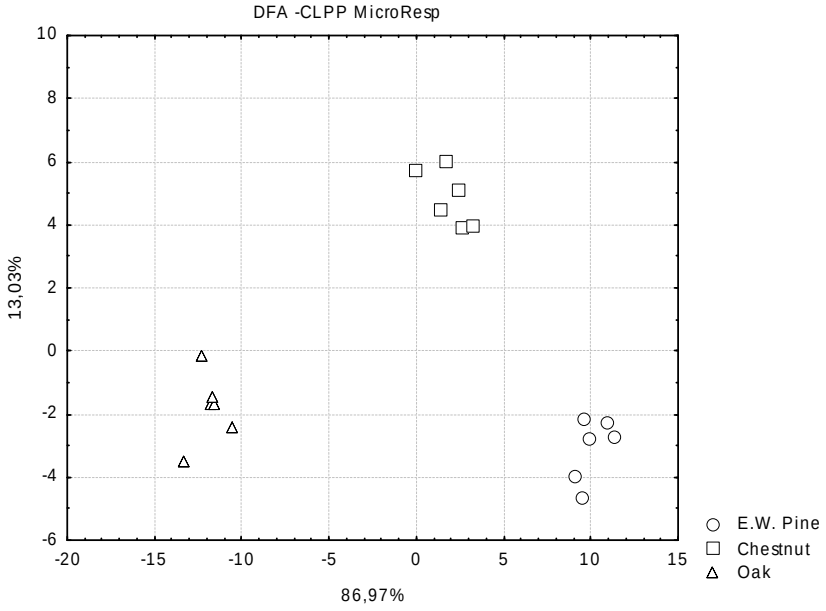


Figure 6.2

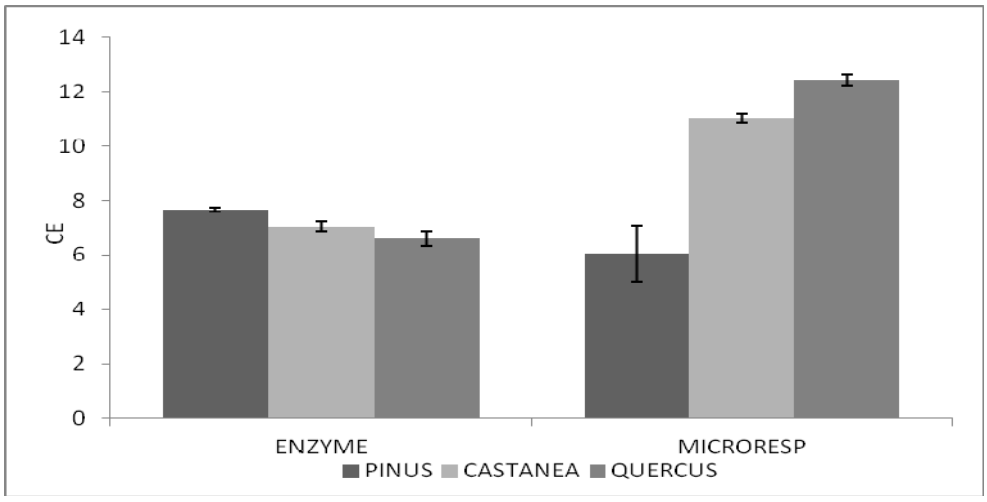


Figure 6.3

6.8 References

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Chapter 7. Microbial functional diversity in forest soils of Centre of Italy in relation to tree canopy structure

7.1 Introduction

In the last decades the studies on forest dynamics are mainly linked to variation in ecosystem processes such as nutrient cycling (Dalmonech et al., 2010), elevated atmospheric CO_2 (Lagomarsino et al., 2009), N fertilization (Moscatelli et al., 2008), management (Pignataro et al., 2011), burning (Kissling et al., 2009) and diverse lithological substrate (Pignataro et al., 2011). Particular attention is devoted to the relationship between aboveground and belowground diversity in natural forest ecosystems.

Plant cover and land-use history are two major factors conditioning the amount, composition, and distribution of soil organic carbon inputs in Mediterranean areas (Goberna et al., 2005), influencing the composition and activity of soil microbial biomass (Klose et al., 2003).

Trees strongly influence the soil biological activity under the canopy, and their presence for long period leads to the creation of a micro-environment different from out of the crown; in a recent paper, Weber et al. (2008) showed that large old trees change patterns of soil δ^{13} C and δ^{15} N in their zone of influence.

Forest differ fundamentally from other vegetation types, in fact they develop superficial O horizon, which greatly modify the microclimate and all the other chemical and biochemical parameters. The scale of spatial variation as reported by Riha et al. (1986) differs for forests, every tree may affect soil at a scale of around 10 m if compared with shrubs or grasslands. The spatial variation around each tree develops from spatial patterns of stemflow/throughfall input of water (sometimes concentrated around the periphery of the canopy) and variations in litter inputs (Binkley and Giardina, 1998). With stem flow, in example, water arrives at the soil with a low energy and slowly than throughfall fluxes, remaining available for plants thanks to a low evaporation, moreover the water reaching the soil with a low kinetic energy does not affect the soil with erosion processes or breakdown of soil agglomerates. Anyway, as suggested, the total area of stemflow zones on a forest floor is probably not larger than the stand basal area (Abee and Lavender, 1972).

Some studies show the patterns of chemical and physical factors (moisture, pH, and N content), that influence C and N mineralisation, and that have been related to species-specific canopy structures, crown habitus, the quantity and chemical quality of rain throughfall, stemflow, and the litter input (Chang and Matzner, 2000; Staelens et al., 2003; Tan et al., 2004). Other studies focused their interest on the effect of microbial activity in soil profiles (Marinari and Antisari, 2010), upper layers (Snajdr et al., 2008) or effect of plant distribution and soil environment on root-associated fungal communities at fine

spatial scales (Burke et al., 2009). In semi arid environments, as reported by Grego et al. (2003), the influence of trees canopy and roots originates a declining gradient (starting from the stem) in nutrients content, microbial numbers and activity so evident to give birth to the so called "islands of fertility" (Herman et al., 1995).

Actually few studies put in relationship the spatial variability with the soil microbial diversity, employing techniques such as enzyme activities (Marinari and Antisari, 2010; Penne et al., 2010), PLFA (Weber and Bardgett, 2011) or CLPP-Biolog (Yuan et al., 2011; Alarcon-Gutierrez et al., 2009).

In this study two natural forests of Central Italy (Monte Rufeno and Lago di Vico Natural Reserves) and three different species (Oak, Chestnut and White Pine) representative of these areas were selected. Within the considered species, chestnut is particularly interesting; the Italian chestnut is in fact characterized by a high yield (3.1 tons/ha against 2.0 as world average), being the production likely to be greater in Lazio, Campania and Calabria Region.

The aim of the study was to assess any differences in soil biochemical properties and microbial diversity due to canopy structure. This work aimed to provide further information on the actual health condition and level of microbial diversity of chestnut soils in natural stands which could be used as benchmark to compare with other sites where the cultivation of chestnut is intensive or over-exploited.

7.2 Materials and methods

7.2.1 Site description and soil sampling

The research was conducted in two different natural forest reserves both located in Lazio Region, in Viterbo district.

The Monte Rufeno Natural Reserve (32T 0736390 UTM 4742149) is located on the boundary line of Toscana and Umbria Region, and it is totally enclosed in the municipality of Acquapendente. Before its establishment in 1983 greater part the area was exploited for the production of wood-coal, while another part was employed as plant farm by Police Forestry. Actually the forested areas cover a large part of the Natural Reserve and the main species have been described in the previous chapter. An area with three different species Eastern white pine (*Pinus strobus*), chestnut (*Castanea sativa*), oak (*Quercus cerris*) was selected and all with the same parent material (*Dystric Cambisol*) and with the same environmental characteristics (exposure, height above sea level, annual rainfall).

The Lago di Vico Natural Reserve (N42°19'47'' E12°08'08''), as Monte Rufeno, is enclosed in the regional systems of protected areas, and established in the same year (1983), nevertheless before its constitution Lago di Vico did not suffer any form of antrophic exploitation. The forested areas cover a large part of the Natural Reserve and the main species are characterised by a strong presence of eastern white pine (*Pinus strobus*), Chestnut (*Castanea sativa*), oak (*Quercus cerris*) and beech (*Fagus sylvatica*). Anyway in this site we decided to collect only soils with chestnut covering. The soil is classified as Melanic Andosol (Andosol with Melanic epipedon, Lorenzoni and Quantin, 1990). Soil samples were collected in the summer of 2009. For each species twelve soil cores were sampled in the A horizon (0-20cm) after removal of litter layer for a total of 24 samples.
According to a similar experiment (Weber et al., 2011) we applied a "natural experimental sampling design" defined two zones of influence around each individual tree: the inner zone (*int*), comprising the area from the stem to the middle of the crown projection; and the outer zone (*est*), including the adjacent area outside of the crown projection (Figure 7.1).

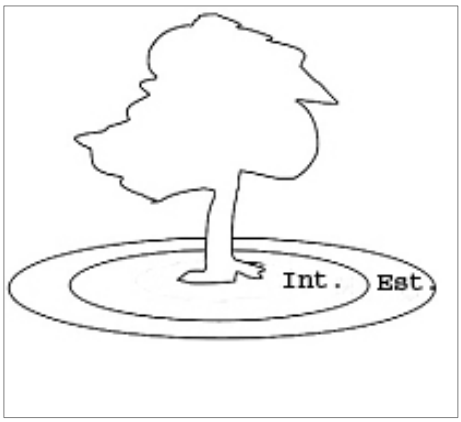

Figure 7.1: Soil sampling scheme

The soils were thus named as follows: WP (white pine), CSTV (Chestnut Lago di Vico), CSTR (Chestnut of Monte Rufeno) and QS (oak).
The outer zone of influence reached out a distance as far as half the crown projection and did not touch the outer zone of influence of any other tree. Soil cores were collected in the inner and outer zone. This sampling design allowed us to analyse differences in belowground properties along a gradient of increasing distance from the stem centre.
All soil samples were dried and sieved at 2mm. The moisture content was adjusted to 60% of their water holding capacity (WHC) and soil samples where then left to equilibrate at

room temperature in the dark for 10 days before biochemical and microbiological analysis (Pinzari et al., 1999).

7.2.2 Methods

(1) The techniques applied in this work are reported in detail in the chapter two (materials and methods) and here they are resumed as follows:
(2) Chemical analysis (pH, TOC, TN and C/N);
(3) Microbial biomass carbon, basal and cumulative soil respiration;
(4) Enzyme activities (β-glucosidase, β-xylosidase, β-cellobiopyranoside, Leucine aminopeptidase, N-acetyl-β-glucosaminidase, arylsulfatase, acid phosphatase and acetate esterase);
(5) CLPP-MicroResp™;
(6) Calculation of diversity indexes (Index of biological fertility (IBF) and Simpson-Yule index);
(7) Statistical analysis.

7.3 Results

7.3.1 Chemical and biochemical analyses

Moisture content was significantly higher under the crown in all plots accompanied by lower values of pH but only under chestnut (Table 7.1). A lower content of TOC and TN was present under the canopy of chestnut at Lago di Vico (Table 7.1).

Higher microbial respiration was observed at Monte Rufeno under the canopy of both species (Table 2). In general most parameters were higher in the soil sampled under the crown although not always significantly (table 7.2).

7.3.2 Enzyme activities

The enzyme activities showed a heterogeneous effect of spatial variability (Table 7.3 and Fig. 7.1). In fact for the CSTR we found an increase in the activity out of the canopy (+57% as average) while for the same species in the Lago di Vico the results were opposite with a strong reduction (-25%), for the other considered species a small increase (WP +2%) or decrease (QS -4%) were observed.

In detail, as reported in table 3, for CSTR acid phosphatase, acetate esterase and β-glucosidase showed the lowest values under the canopy, in CSTV with the only exception of cellulase, chitinase and β-glucosidase we observed a higher activity in the area surrounding the tree. QS despite the two chestnut species did not show a clear effect of

spatial variability in fact in some cases we observed to a high activity under the tree canopy (cellulase, chitinase, acetate esterase and β-glucosidase) while in other a high activity out of it (L-leucine), for the coniferous we observed a similar trend but with only three enzymes.
The diversity index calculated with the enzyme activities showed no significant differences in functional diversity apart from higher values in the soil under quercus canopy (Table 5).

7.3.4 CLPP-MicroResp

The study of community level physiological profile showed a homogeneous effect of spatial variability for all the tree species considered; a general significant decrease of microbial consumption of all substrates was recorded in all plots (-36%, -65%, -28%, -22% as average values) for CSTR, CSTV, WP and QS respectively (Table 7.4 and Fig. 7.2).
The diversity index showed significantly higher values of functional diversity, under the crown, only for chestnut in both sites.

7.4 Discussion

7.4.1 Chemical and biochemical properties

Similarly to other studies (Teste and Simard, 2008), we found a gradient of increasing moisture content away from the stem, which is most likely due to the trees water uptake and transpiration and due to interception losses. The influence of trees on soil moisture availability may act as a driver for the detected differences in the soil microbial activity and the rate of decomposition among the studied zones of influence (Bardgett, 2005). Kara et al. (2008) reported that pH is one of the of the most important parameters which affects the soil activities, Penne et al. (2010) did not show any significant differences in chemical properties of areas under tree crowns or in canopy gaps, even though they highlighted a significantly lower pH under the tree crowns compared with under the canopy gaps. In our research soil pH showed a significant difference between the samples inside and outside the crown of two chestnut plots. It is possible that different levels of soil moisture combined to specific characteristics of this species litter were particularly effective in modifying hydrogen ions availability under chestnut.
In general most of biochemical properties (Cbas, Ccum, and qmic) agree among them to prove under canopy an increase in the decomposition processes.
No significant differences were observed for biochemical parameters under chestnut at Lago di Vico while these were evident, with higher values under the canopy, at Monte

Rufeno. This indicates that spatial diversity is also the result of specific interactions with site properties. For instance it may be possible that the high levels of soil organic matter at Lago di Vico (6% on average), high moisture content (20%) and peculiar lithological substrate (Pignataro et al., 2011) may level any difference in biochemical characteristics.
The broadleaves at Monte Rufeno showed higher respiration rates under the canopy. Soil basal and cumulative respiration is considered to reflect the availability of C for microbial maintenance and it is a measure of basic turnover rates in soil (Insam et al., 1991); respiration activity reflects the soil microbial physiological status (Ananyeva et al., 2002) that was also studied through CLPP analytical approach and both results are in accordance. Salazar et al. (2011), in a study regarding spatial scale at level species and forest management, did not find significant differences between chestnut and oak, while in our case we found greater respiration rates under oak than under chestnut; although we do not have any data on litter quantity and quality we may hypothesize that microbial activity was strictly dependent on the specific plant residues as also the content of microbial biomass indicates. This last hypothesis may be confirmed by the higher CH/AA ratio measured by means of CLPP-MicroResp that showed higher values (about two-fold higher) under oak. According with Sharma et al. (1998), in fact, a higher CH-to-AA ratio indicates a major content of easily degradable carbon compounds in the soils.
Anderson and Domsch (1993) reported that qCO_2 is considered as a good indicator of environmental stress on microbial communities and ecosystem development, and according with Odum (1985) soil microbes need more energy for survival in a not climax environment/condition, resulting in an increase of metabolic quotient. In our study deciduous stands microbial communities had a higher efficiency in carbon utilisation than those of conifers; these results agreed with Iovieno et al. (2010), Moscatelli et al. (2007) Pinzari et al. (1999).

7.4.2 Functional diversity - enzyme activities and CLPP-MicroResp

On the landscape scale, the parent material, climate and geological history are major importance for soil organic matter distribution (Jian-Bing et al., 2006), while at stand scale, other environmental factors too (topography and log distribution) may influence organic matter variability (Spielvogel et al., 2009). In this study different tree species, in fact, had significant impacts on soil chemical and biochemical properties (Chapter 4), probably due to different substrate quality among the species; because organic matter accumulates on the forest floor and our soil samples were from the layer close to the organic one, we suggest

that these species-specific differences are primarily due to the difference in above-ground litter quality and quantity.

The techniques (enzyme activities and MicroResp) applied to study the functional diversity pointed out for broadleaves a different substrates exploitation by microorganisms under the crown and outside of it (Økland et al., 2003; Weber et al.,, 2011), while in conifer stands our results agree with Parker (1983) who reported that spatial variability was negligibly small and could not induce remarkable variation in soil properties.

To our knowledge in our study most enzyme activities showed a high degree of sensitivity in relation to tree and spatial diversity. Soil enzyme activities have been related and are deeply modified by several parameters in particular by microbial community (Waldrop et al., 2000). It is also known that "old" trees (around 50 years old) exert a strong influence on the composition of the soil microbial community in forests, soil closest to the stem will have the longest legacy of tree impact both above and belowground Bach et al. (2010), and Ruark and Zarnoch (1992) emphasised that it is important how long a site is a forest site. Our results agree with Snajdr et al. (2008) who found that the activities of extracellular enzymes in forest soils show considerable variations depending probably by species, and only the acetate esterase all the different plots was sensible to point out a variation in spatial diversity. Witmann et al. (2004) found that acetate esterase is considerate as a representative enzyme to point out the endocellular activity, consequently we could hypothesize that this enzyme is influenced by chemical properties and composition of litter despite the other enzyme activities. About the other enzymes in general it has been pointed out pointed out a major activity under the canopy particularly for chitinase activity suggesting an increasing of fungi relative to bacteria (Bach et al., 2010), while the remaining (e.g L-leucine and acid phosphatase) reported a general discrepancy between the soils under and outside the canopy; at landscape level in Monte Rufeno natural reserve a higher activity has been observed under the canopy, but in Lago di Vico we found the opposite condition. The differences regarding the two sites are probably the same reported also in the chapter regarding the lithological substrate. Penne et al. (2010) found no significant differences among tree-crown classes, even though Kandeler et al. (2001) defined enzyme activities as sensitive indicators for the small changes in the environment. Despite the several studies that confirm the positive relationship between enzymes and pH, C, N, C/N content (Salazar et al., 2011), in our work we observed significant negative correlations for the greater part of our results (data not shown), probably because in natural forest ecosystem other factors soil microorganisms are nutrient limited (Leiros et al., 2000)

or are influenced by phenolic or recalcitrant compounds. Sinsabaugh et al. (2002) showed that a decreased decomposition of recalcitrant organic matter occurs due to inhibitory effects of high nitrogen levels on extracellular enzyme activities of the N and C cycles.

About CLPP-MicroResp, this technique incorporates the advantages of Biolog and SIR, but its importance in the study of functional diversity increased only in the last years; particularly regarding forest soils. MicroResp has been able to evidence differences in soils with different land use (Lagomarsino et al., 2011), mined areas (Lalor et al., 2007) or climatic condition (Oren and Steinberger, 2008). To our knowledge, no other study measured functional diversity with MicroResp technique in soil spatial variability (horizontal), so a literature comparison is quite difficult. While in enzyme activities we had a homogeneous results in spatial diversity in almost all the considered species, MicroResp better pointed out these differences, demonstrated that catabolic abilities of microbial communities can be significantly modified by plant canopy structure (Grayston et al., 2001).

CLPP data indicated that soils under the canopy not only resulted in higher substrate utilization activity and diversity, but also resulted in different exploitation of individual substrates and substrate guilds, with the deciduous species generally showing the greatest utilization of almost all carbon sources than the communities outside the canopy. In the studies conducted by Yuan et al. (2011) and Li et al. (2007) the composition of plant species affected microbial community functioning more greatly than the structure of the plant species. On the contrary Perez-Piqueres et al. (2005) argued that variation in the microbial community were not necessarily accompanied by significant shifts in community functional diversity. Conversely conifer in a dense stand with strong competition allocate more growth to stem wood and coarse roots compared to pines in a sparse stand and with increasing tree dominance growth allocation to needles and branches increases (Vanninen and Makela, 2005). However according with Chodak and Niklinska (2010) is doubtful considering that qmic was in opposition suggesting higher share of stabile C in these soils. The higher values of CH/AA ratio found at Monte Rufeno and Lago di Vico confirm thus the occurrence of specific organic substrates quality that promoted the elevated levels of microbial activity determined in these sites.

In conclusion, different trends were observed under conifer and deciduous plants indicating that morphology and type of canopy affect the properties of the soil below it. In particular while for the broadleaves we observed a general decreasing gradient of most biochemical

and microbiological properties from the stem to the outer part of the canopy, the opposite, in some cases, or no significant differences were found under pine.

The spatial diversity of microbial communities in forest soils seems to depend also on additional factors beyond the type of plant cover. We found in fact that the specific features of Lago di Vico soil buffered any differences in microbial biomass spatial diversity or, at least, these where evidenced, by very low values if compared to the other sites, only in CLPP and enzyme approaches.

The influence of canopy structure on soil microbial diversity may thus result from the combined effects of the soil lithological properties, physicochemical characteristics, litter quantity and quality.

Understanding the ecology of forest soils will enable us to predict and assess the long-term effects of our activities on forest ecosystems and will help to determine how to manage our use of forests responsibly.

7.5 Table content

Table 7.1: Soil chemical properties, different letters represent significant differences among the species (* p<0.05, ** p<0.01, *** p<0.001, *ns* not significant).. WP is white pine, CSTR is chestnut Rufeno, CSTV is chestnut LAgo di Vico and QS is oak.

	pH H_2O	SE	C%	SE	N%	SE	C/N	SE	U%
WP int	5,48	0,10	2,073	0,10	0,218	0,02	10,16	0,35	9,73
WP est	5,50	0,17	2,314	0,13	0,215	0,01	10,73	0,25	9,50
Anova	*ns*		*ns*		*ns*		*ns*		*
CSTR int	5,45	0,00	2,203	0,17	0,126	0,01	16,08	0,63	6,26
CSTR est	5,56	0,04	1,494	0,18	0,107	0,01	14,89	0,26	5,50
Anova	*		*ns*		*ns*		*ns*		*
QS int	5,58	0,26	2,749	0,13	0,174	0,01	16,36	0,66	6,99
QS est	5,50	0,38	2,479	0,15	0,200	0,02	15,03	0,66	6,01
Anova	*ns*		*ns*		*ns*		*ns*		*
CSTV int	6,36	0,07	6,996	0,26	0,557	0,02	12,85	0,17	21,61
CSTV est	6,53	0,06	9,479	0,55	0,765	0,05	12,40	0,14	19,74
Anova	*		*		*		*ns*		*

Table 7.2: Soil biochemical properties, metabolic quotient (qCO2), microbial quotient (qmic), index of biological fertility (IBF). Different letters represent significant differences among the species (*p<0.05, **p<0.01, ***p<0.001, ns not significant).

	Cbas	es	Ccum	es	Cmic	es	qCO2	es	qM	es	IBF	es	qmic	es
WP int	1,32	0,07	1046,45	62,6	64,75	13,4	0,093	0,025	5,1	0,53	20	1,86	0,32	0,06
WP est	1,32	0,06	996,58	24,1	145,2	25,1	0,038	0,009	4,34	0,3	20	2,67	0,66	0,1
Anova	*ns*		*ns*		**		*		*ns*		*ns*		*	
CTR int	1,3	0,04	979,08	42,6	251,63	16,7	0,022	0,001	4,57	0,5	24	0,33	1,18	0,16
CTR est	1	0,08	735,37	71,5	111,8	34,4	0,042	0,008	4,93	0,56	22	0	0,69	0,06
Anova	*		*		*		*		*ns*		**		*	
QS int	1,88	0,06	1399,76	44,4	552,14	0,7	0,014	0,001	5,3	0,58	23	3,33	1,91	0,15
QS est	1,64	0,06	1257,11	22,7	516,53	16,8	0,013	0	5,18	0,46	25	0,33	2,13	0,23
Anova	*		*		*ns*		*ns*		*ns*		*ns*		*ns*	
CTV int	1,66	0,04	1140,1	115,2	302,66	58,4	0,024	0,004	1,64	0,11	21	0,33	0,34	0,04
CTV est	1,49	0,4	1059,02	210,3	426,65	64,5	0,015	0,005	1,13	0,28	21	0,33	0,44	0,04
Anova	*ns*		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	

Table 7.3: Soil enzyme activities expressed in MUF/AMC$g^{-1}TOC^{-1}$.Cellulase (CELL), Chitinase (CHIT), ß-Glucosidase (ß-GLU), Acid phosphatase (PHO), Arylsulfatase (ARY), Xylosidase (XYL), Acetate (ACE), L-Leucine (L-LEU), different letters represent significant differences among the species * p<0.05, ** p<0.01, *** p<0.001, *ns* not significant).

	CELL	es	CHIT	es	ß-GLU	es	PHO	es	ARY	es	XYL	es	ACE	es	L-LEU	es
WP int	0,829	0,049	0,218	0,040	3,059	0,073	8,866	0,220	3,124	0,189	0,536	0,057	21,722	0,030	5,976	0,117
WP est	0,822	0,106	0,117	0,010	2,970	0,441	10,291	0,255	3,204	0,320	0,537	0,074	21,238	0,047	6,172	0,214
Anova	*ns*		*		*ns*		*		*ns*		*ns*		**		*ns*	
CSTR int	0,449	0,051	0,130	0,027	1,284	0,212	9,678	0,060	3,338	0,078	0,345	0,038	16,826	0,137	1,845	0,052
CSTR est	0,634	0,118	0,156	0,028	2,704	0,716	16,577	2,396	4,338	0,627	0,633	0,068	25,196	0,414	3,035	0,236
Anova	*ns*		*ns*		*		*		*ns*		*		***		**	
QS int	0,564	0,089	0,157	0,049	2,867	0,323	11,887	0,488	1,482	0,491	0,550	0,060	15,219	0,802	1,648	0,146
QS est	0,471	0,085	0,112	0,023	1,917	0,279	12,474	0,943	1,786	0,758	0,428	0,100	13,772	1,762	2,113	0,011
Anova	*ns*		*ns*		*		*ns*		*ns*		*ns*		*		*	
CSTV int	4,255	0,725	0,383	0,104	1,582	0,063	3,775	0,197	1,300	0,028	2,212	0,141	8,658	0,403	2,024	0,387
CSTV est	3,467	0,849	0,267	0,062	1,441	0,161	3,035	0,352	0,826	0,082	1,732	0,162	6,083	0,310	1,198	0,043
Anova	*ns*		*ns*		*ns*		*		*		*		**		*	

Table 7.4: CLPP-MicroResp substrates consumption, expressed in μg C-$CO_2 g^{-1} h^{-1}$. Substrates used are Citric acid (CIT), Oxalic acid (OX), Ascorbic acid (ASC), Arginine (ARG), Glycine (GLY), Leucine (LEU), Aspartic acid (ASP), Butirric acid (BUT), Galattose (GA), Glucose (G) Arabinose (ARA), Glucose (GLU), Fructose (FR), Vanyllic acid (VAN), Siringic acid (SIR), N-Acetyl-Glucosamine (NAG). CA is carboxylic acids, AA is amino acids, CH is carbohydrates and A is amide. Standard errors (in Italics), n=6, * p<0.05, ** p<0.01, *** p<0.001, *ns* not significant.

	CIT	OX	ASC	BUT	ARG	GLI	LEU	ASP	ARA	GA	G	FR	SIR	VAN	NAG	CH/AA
WP int	0,034	0,052	0,085	0,024	0,044	0,015	0,011	0,052	0,050	0,007	0,034	0,024	0,055	0,007	0,006	0,988
	0,010	*0,005*	*0,007*	*0,005*	*0,010*	*0,004*	*0,002*	*0,009*	*0,014*	*0,003*	*0,014*	*0,009*	*0,010*	*0,002*	*0,003*	*0,006*
WP est	0,043	0,060	0,060	0,008	0,055	0,019	0,024	0,006	0,012	0,010	0,027	0,015	0,011	0,005	0,008	0,718
	0,011	*0,006*	*0,005*	*0,003*	*0,006*	*0,004*	*0,009*	*0,001*	*0,002*	*0,003*	*0,013*	*0,003*	*0,006*	*0,002*	*0,002*	*0,005*
Anova	*ns*	*ns*	*	*	*ns*	*ns*	*	*	*	*ns*	*ns*	*ns*	*	*ns*	*ns*	*
CSTR int	0,072	0,035	0,037	0,015	0,063	0,023	0,013	0,028	0,024	0,020	0,032	0,030	0,029	0,010	0,019	0,933
	0,004	*0,003*	*0,003*	*0,002*	*0,005*	*0,002*	*0,002*	*0,004*	*0,003*	*0,003*	*0,002*	*0,004*	*0,003*	*0,002*	*0,003*	*0,005*
CSTR est	0,040	0,024	0,024	0,005	0,072	0,011	0,005	0,030	0,011	0,005	0,021	0,012	0,010	0,004	0,015	0,489
	0,003	*0,005*	*0,005*	*0,002*	*0,007*	*0,005*	*0,002*	*0,009*	*0,002*	*0,002*	*0,003*	*0,002*	*0,003*	*0,002*	*0,004*	*0,007*
Anova	**	*ns*	*ns*	*ns*	*ns*	*	*	*ns*	*	*	*	*	*	*	*	ns
QS int	0,031	0,033	0,043	0,025	0,065	0,038	0,037	0,041	0,055	0,042	0,035	0,079	0,025	0,035	0,024	1,276
	0,003	*0,002*	*0,003*	*0,003*	*0,005*	*0,006*	*0,009*	*0,006*	*0,010*	*0,006*	*0,006*	*0,010*	*0,005*	*0,008*	*0,003*	*0,006*
QS est	0,017	0,023	0,036	0,023	0,048	0,025	0,020	0,031	0,036	0,030	0,057	0,051	0,036	0,019	0,023	1,486
	0,004	*0,002*	*0,002*	*0,003*	*0,005*	*0,002*	*0,003*	*0,002*	*0,004*	*0,003*	*0,005*	*0,004*	*0,004*	*0,004*	*0,004*	*0,004*
Anova	*	*	*	*ns*	*	*	*	*	*ns*	*ns*	*	*	*	*	*ns*	*
CSTV int	0,012	0,008	0,017	0,009	0,013	0,010	0,008	0,013	0,012	0,014	0,015	0,014	0,010	0,006	0,006	1,289
	0,002	*0,002*	*0,001*	*0,002*	*0,003*	*0,002*	*0,002*	*0,001*	*0,001*	*0,001*	*0,002*	*0,001*	*0,002*	*0,001*	*0,002*	*0,001*
CSTV est	0,005	0,004	0,010	0,002	0,011	0,004	0,002	0,003	0,002	0,003	0,004	0,003	0,001	0,002	0,004	0,689
	0,001	*0,001*	*0,001*	*0,001*	*0,002*	*0,001*	*0,001*	*0,001*	*0,000*	*0,001*	*0,001*	*0,001*	*0,000*	*0,000*	*0,000*	*0,001*
Anova	*ns*	*ns*	*ns*	**	*ns*	*	**	*	*	*	*	*	*	*	*	**

Table 7.5: Simpson-Yule diversity index. Standard errors (reported in Italics), n=6, * p<0.05, ** p<0.01, *** p<0.001, *ns* not significant.

	CE enzymes		CE Mresp	
WP int	7,605	0,066	8,941	0,794
WP est	7,446	0,112	8,025	0,709
Anova	*ns*		*ns*	
CSTR int	7,341	0,007	11,703	0,640
CSTR est	7,551	0,096	7,312	1,779
	ns		*	
QS int	6,988	0,213	11,321	0,809
QS est	7,657	0,126	12,748	0,512
	*		*ns*	
CSTV int	7,672	0,141	14,017	0,476
CSTV est	7,376	0,002	9,622	1,750
	ns		*	

7.6 Figure caption

Figure 7.1 – Effect of tree species on enzyme activities and Discriminant Functional Analysis. Enzymes used are: Cellulase (Cell), Chitinase (Chit), β-Glucosidase (β-Gluc), Acid Phosphatase (Phosh), Arylsulfatase (Aryl), Xylosidase (Xyl), Acetate Esterase (Acetate), L-Leucine-Aminopeptidase (L-Leucin). Standard error bars are reported, *n*=6.

Figure 7.2 – Effect of tree species on CLPP-MicroResp and Discriminant Functional Analysis. Substrates used are: Citric acid (Cit), Oxalic acid (Ox), Ascorbic acid (Asc), Arginine (Arg), Glycine (Gly), Leucine (Leu), Aspartic acid (Asp), Butirric acid (But), Galattose (Ga), Arabinose (Ara), Glucose (Glu), Fructose (Fr), Vanyllic acid (Van), Siringic acid (Sir), N-Acetyl-Glucosamine (Nag). Standard error bars are reported, *n*=6.

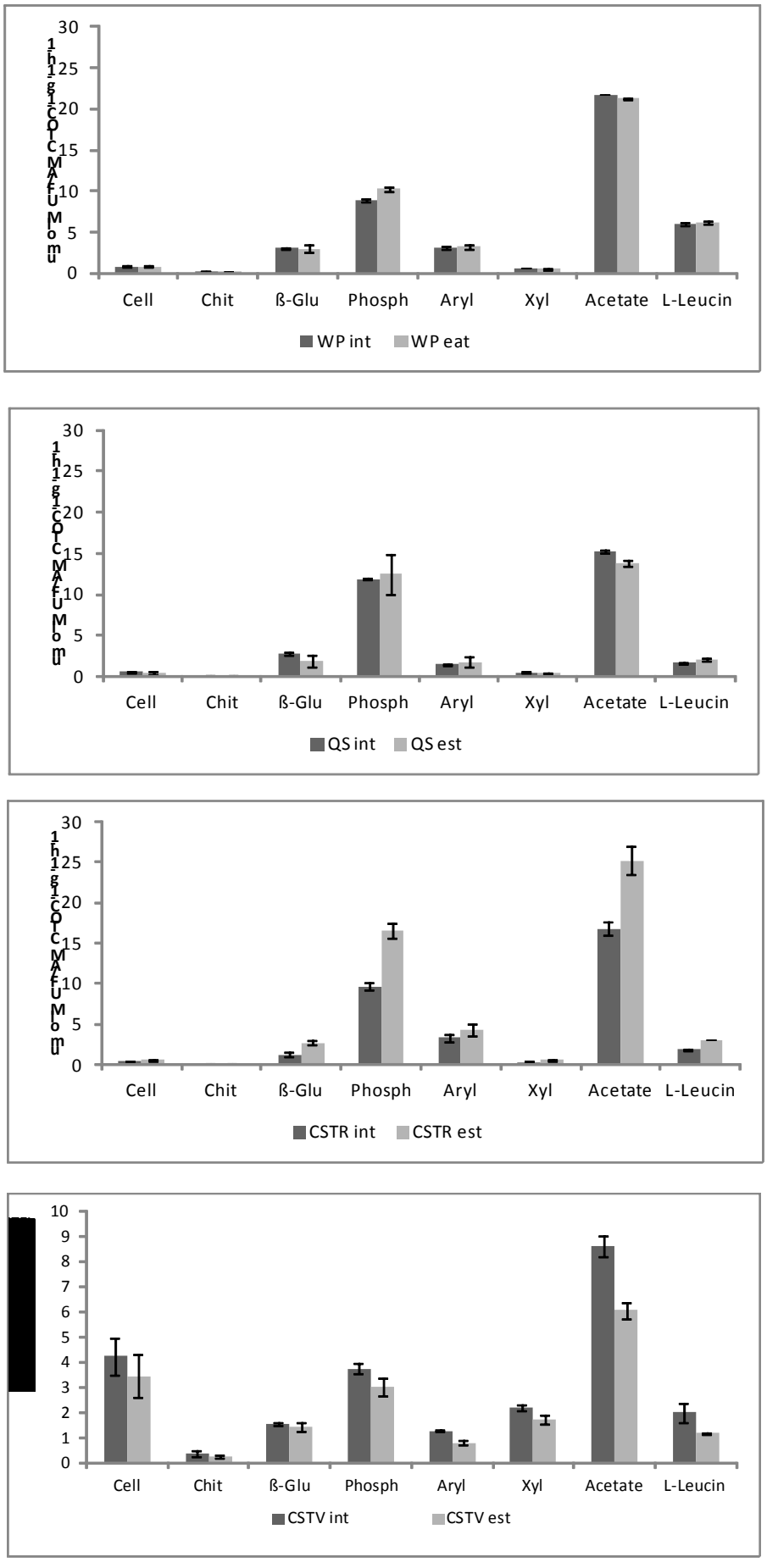
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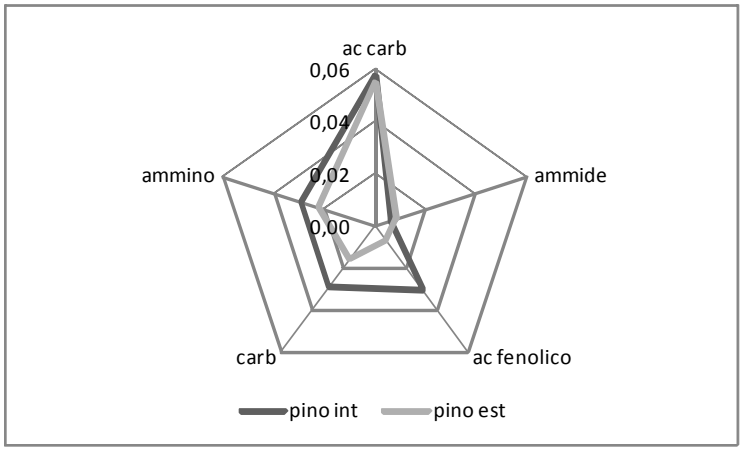
Figure 7.1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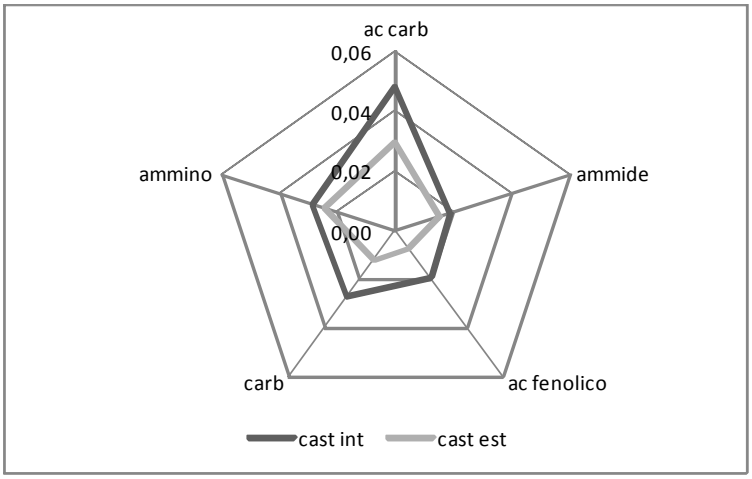
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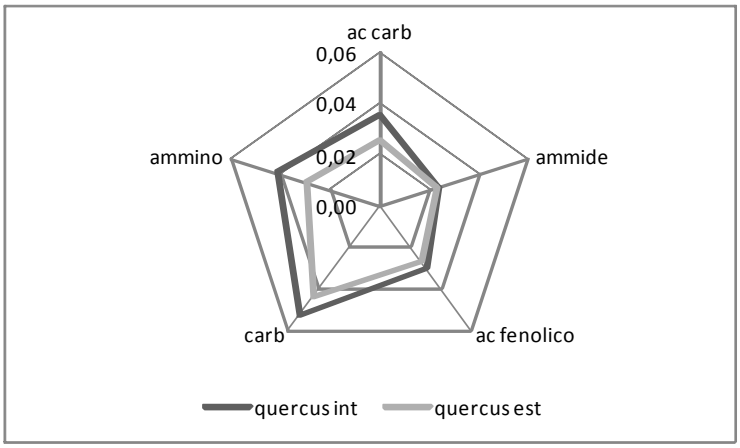
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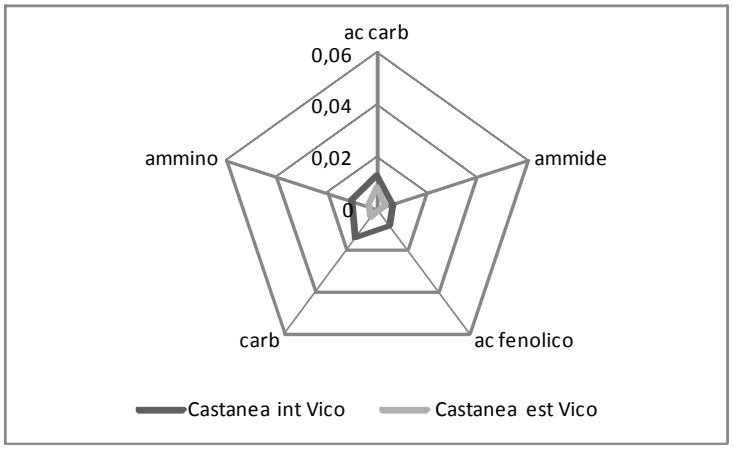
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Figure 7.2

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Chapter 8. Diversity indexes

8.1 Diversity indexes

8.1.1 Index of Biological Fertility (IBF) and Simpson-Yule

Foresters have always relied on a knowledge of chemical and physical properties of soils to assess capacity of sites to support productive forests, in particular they have traditionally measured soil productivity using tree growth or wood yield (Schoenholtz et al., 2000). Others in the forestry community frequently indicate soils as simply ”part of the forest”, as opposed to a separate resource in its own right, and have not generally invoked the concept of soil quality (and indirectly its diversity) as a component of sustainable forestry (Burger and Kelting, 1998).

In this work different diversity indexes were applied, starting by the index of biological fertility (IBF), proposed by Pompili et al. (2008), based on the sum of the scores given to different chemical and biochemical parameters that are reported below:

$$IBF = Cbas + Ccum + Cmic + S.O. + qCO_2 + qM \text{ (ISPRA, 2008).}$$

Cbas is the basal respiration, Ccum is the cumulative respiration, Cmic is the microbial biomass carbon, S.O. is the organic matter, qCO_2 is the metabolic quotient and qM represents the mineralization quotient.

The index ranges are enclosed between 1 and 30, and helps to classify soils in the following classes of biological fertility: 1) stress conditions, 2) pre-stress conditions, 3) average, 4) good and 5) high.

IBF was calculated for each of the different objectives of this work and the results obtained are reported in table below (8.1):

Table 8.1: IBF calculated for the different aims of research

Aim 1 **Effect of management**	*IBF*	**Aim 2** **Effect of site**	*IBF*	**Aim 3** **Effect of tree species**	*IBF*	**Aim 4** **Effect of canopy structure**	*IBF*
Coppice	20 *a*	M. Peglia	19 *a*	W. Pine	22 *ab*	W.P. int	20 *a*
Aged Coppice	19 *a*	M. Rufeno	25 *b*	Chestnut	23 *b*	W.P. est	20 *a*
		Lago di Vico	26 *b*	Oak	25 *c*	CSTR int	24 *a*
						CSTR est	22 *b*
						QS int	23 *a*
						QS est	25 *a*
						CSTV int	21 *a*
						CSTV est	21 *a*

As a first analysis it is possible to observe that all soils present a high level of Biological Fertility, in fact, all of them obtained a high score indicating entering in the class 5.

However IBF failed to discriminate the different theses within the four aims of this study. It should be emphasized that this index was first proposed and calibrated with agricultural soils. This work was one of the first cases in which IBF was used in forest ecosystems and probably it should be re-adjusted when applied to forest soils. This could imply either widening the range of some biochemical properties (i.e. respiration) or adding new parameters such as the substrate induced respiration (SIR).

With the aim to follow a sort of hierarchical approach to calculate microbial diversity indexes, we calculated a microbiological index to test a part of the diversity that usually is omitted by foresters. According with Eaton and Farrell (2004) to identify variations in the functional diversity, as indicated by variations in C source utilization profiles and the "evenness" of catabolic responses to different C sources in soil microbial population-based diversity studies, we calculated the Simpson Yule index (Kennedy and Smith 1995; Staddon et al., 1997). The same index can be calculated using enzymatic activities data and/or community level physiological profile (CLPP) data obtained by means of Biolog or MicroResp (Lagomarsino et al., 2009).

Table 8.2 shows how Simpson-Yule was sensitive to discriminate the different theses within each aim of this work. Both enzymes and MicroResp approaches were effective in highlighting differences, however the trend obtained were opposite.

Table 8.2: Simpson-Yule index calculated for the different aims of research

Aim 1	*Enzymes*	**Aim 2**	*Enzymes*	**Aim 3**	*Enzymes*
Coppice	7,11 *a*	M. Peglia	6,55 *a*	W. Pine	7,66 *a*
Aged Coppice	5,48 *b*	M. Rufeno	6,19 *a*	Chestnut	7,05 *b*
		Lago di Vico	5,59 *b*	Oak	6,61 *c*
Aim 1	*MResp*	**Aim 2**	*MResp*	**Aim 3**	*MResp*
Coppice	21,64 *a*	M. Peglia	8,08 *a*	W. Pine	6,05 *a*
Aged Coppice	23,22 *b*	M. Rufeno	12,24 *b*	Chestnut	11,02 *b*
		Lago di Vico	12,87 *b*	Oak	12,42 *c*

In fact where microbial functional diversity estimated with enzyme activities showed the highest values when calculated with MicroResp showed the lowest. Similar contrasting results were also obtained by Epelde et al. (2008) who found significantly lower values of H' obtained from enzyme activities and higher values of H' obtained from EcoPlates™ in polluted soils.

It is well known that microbial functional diversity represents the capacity to perform different ecological processes and to use a wide array of substrates (Kandeler et al., 1996). This is achieved through different biological processes that can be directly linked (oxidative processes, eg. respiration) or indirectly linked (hydrolytic processes, eg. extracellular enzymes) to microbial cells metabolism. Consequently calculating microbial diversity indexes using enzyme activities or community level physiological profile methods could likely provide information on different components of microbial functional diversity. We here hypothesize that, measuring diversity using both methodological approaches, we obtained information on different ecological processes occurring in soil: on one hand the exemplification of complex organic substrates obtained after enzymatic hydrolysis and on the other the direct utilization of simple substrates by microorganisms (CLPPs). Lagomarsino et al. (2007) agree that CLPP-MicroResp™ is considered a direct measurement of microbial communities' catabolic profile providing thus an instant photograph of microbial physiology.

In the case of the first aim of this work (effect of forest coppicing) we can hypothesize that enzymes were specifically activated to perform the hydrolysis of complex polymers into simple ones. This kind of substrates may prevail on the forest soil after trees cutting as wood debris and litter which mainly consist in cellulose, lignin, tannins etc. On the other hand under natural conditions, the largest source of labile carbon (C) inputs to the soil is root exudates (Kuzyakov, 2010; Bertin et al., 2003); the quantity and character of root exudates released by trees show a broad variety of low-molecular weight organic compounds, including sugars, amino acids, organic acids and phenolics (Grayston et al., 1996). These compounds are used in CLPPs techniques for measuring microbial catabolic performances and may be thus more intensively used in a natural forest soil.

The above hypotheses, of course, if confirmed by further experimental evidences, may supply additional information on the relative contribution that enzyme activities and CLPP techniques provide for the evaluation of microbial functional diversity. They may also open an interesting research issue to the pursuit of a synthetic index that takes into account two different, but complementary, components of microbial functional diversity.

8.1.2 Microbial functional diversity in forest soils is more affected by tree species or by the lithological substrate?

In this work we found that soil microbial diversity in natural forest ecosystems was significantly influenced by the lithological substrate and the different tree species.

A step forward in this work has been done applying a multiple regression analysis (MRA) using quantitative (y) and qualitative (x) variables (XL-Stat), with the final aim to assess if soil microorganisms were more affected by tree species or by the lithological substrate.
Figures 8.1, 8.2, 8.3 and table 8.3 show the MRA results for IBF and Simpson Yule indexes (calculated with enzyme activities and CLPP), microbial biomass carbon and other parameters.
The diversity indexes calculated with enzyme activities or MicroResp, the IBF and the main chemical and biochemical parameters were differently effective in showing significant changes due to the two factors of variations (site and tree species). Enzyme activities, microbial biomass, respiration and pH seem to be the most sensitive parameters confirming their role as reliable indicators of soil quality (Nannipieri et al., 2003).

Table 8.3 Multiple regression analysis applied for different chemical and biochemical analyses. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns not significant

Parameter	Site	Species
IBF	***	--
CE enzymes	***	***
CE MicroResp	--	***
Microbial biomass	***	***
Microbial quotient (*qmic*)	***	***
Metabolic quotient (*qCO2*)	--	**
Mineralization quotient (*qM*)	***	--
Basal respiration	***	***
pH	***	***
TOC	***	*

8.3 Figures caption

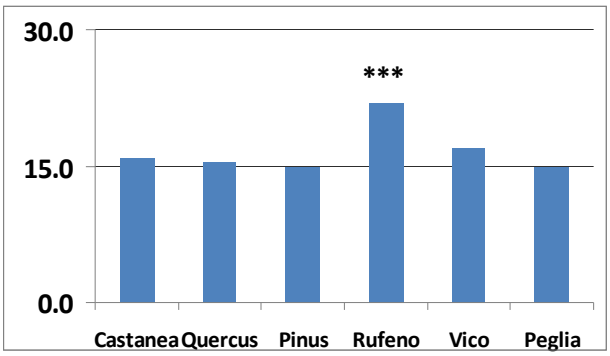

Figure8.1 Multiple regression analysis calculated for IBF

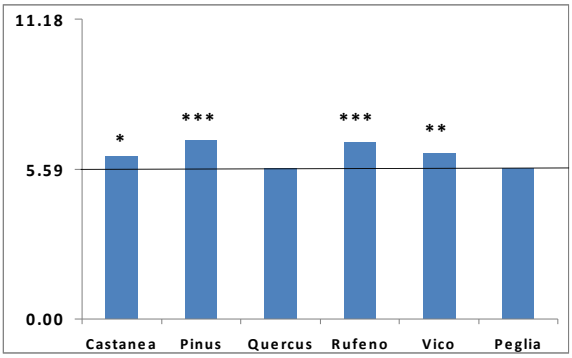

Figure8.2 Multiple regression analysis calculated for CE enzymes

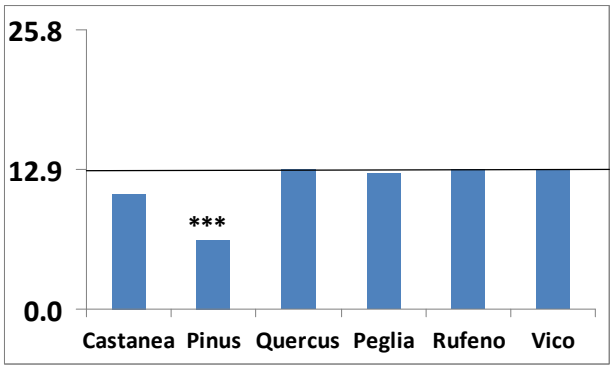

Figure8.3 Multiple regression analysis calculated for CE CLPP-MicroResp

8.4 References

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Chapter 9. Conclusions

9.1 Conclusions

This work aimed to study microbial diversity in soils of natural forests to assess a specific site fingerprint which may represent a benchmark in monitoring soil quality. Such an approach has been scarcely considered in the current literature and this research may thus represent a first attempt to provide a baseline characterization of forest soils microbial diversity under natural conditions.

The main objectives of this thesis were:

1. To assess soil microbial functional diversity in a coppiced forest system;
2. To assess soil microbial functional and genetic diversity in relation to the lithological substrate;
3. To assess soil microbial functional diversity in relation to different tree species;
4. To assess soil microbial functional diversity in relation to tree canopy structure;
5. To calculate different microbial indexes.

In relation to the above aims the following final observations were drawn:

1. The oak forest coppicing significantly increased soil microbial pool and metabolism probably due to quali-quantitative changes of root products and litter that provided enhanced availability of C substrates. Microbial functional diversity, assessed by means of CLPP (MicroResp™ and Biolog™) and enzyme activities was affected by forest management.

2. The results suggested that under the same plant cover, *Quercus cerris,* and the same topographic conditions in the three sites, the parent material and the specific pedogenic processes strongly influenced the functional characteristics of soil microbial communities. Lago di Vico soil potentially showed the best fertility conditions for the growth and metabolism of microorganisms, conversely this site was characterized by a "static system" showing the lowest microbial activities. This suggested that microbial metabolic performances in forest soils reflect the combined effect of plant cover influence and the specific site features (soil type, bedrock, soil moisture…). Also the genetic approach reflected a significantly different amount of microbial DNA in the three sites.
3. Microbial functional diversity was significantly different under the three plant species (*Q. cerris, C. sativa* and *P. strobus*) pointing thus to three different

systems:Microbial biomass under *Quercus* seemed to be particularly active showing high respiration rates, high carbon substrates utilisation, high efficiency in the use of carbon substrates (low qCO_2). *Castanea* soil showed intermediate values of biomass, activities, diversity and a homogeneous use of carbon substrates. *Pinus* soil showed low biomass, low activities, low use of carbon substrates but a general higher enzymatic activity. This could indicate high recalcitrance of litter and carbon compounds that need to be degraded and hydrolysed before being utilised.

4. Different trends were observed under conifer and deciduous plants indicating that morphology and type of canopy affect the properties of the soil below. In particular while for the broadleaves we observed a general decreasing gradient of most biochemical and microbiological properties from the stem to the outer part of the canopy, the opposite, in some cases, or no significant differences were found under pine. The spatial diversity of microbial communities in forest soils seems to depend also on additional factors beyond the type of plant cover. We found in fact that the specific features of Lago di Vico soil buffered any differences in microbial biomass spatial diversity or, at least, these where evidenced, by very low values if compared to the other sites, only by means CLPP and enzyme approaches. The influence of canopy structure on soil microbial diversity may thus result from the combined effects of the soil lithological properties, physicochemical characteristics, litter quantity and quality.

5. The IBF showed that the soils of the three Natural Reservations belong to the same class of biological fertility (High); however, in this study, it was evidenced that this index should be re-calibrated for forest soils. Among the diversity indexes tested, the Simpson Yule proved to be the most effective in discriminating among the different case-studies. Nevertheless the use of enzyme activities and of MicroResp lead to different results suggesting that these techniques may point to different components of microbial functional diversity. This last methodological aspect may also open an interesting research issue to the pursuit of a synthetic index that takes into account two different, but complementary, components of functional diversity. Finally the multiple

regression analysis highlighted that, depending on the parameter used the diverse plant species and/or sites may exert different influences. Enzyme activities, microbial biomass, respiration and pH resulted to be the most sensitive parameters, being significantly affected by both factors of variation, confirming thus their role as reliable indicators of soil quality and health.

The present thesis was therefore a preliminary attempt to lay the foundations for the constitution of a database and/or a proposal of a set of methodologies aimed to describe soil microbial diversity in natural forests. This could help to dispose of a background knowledge of the natural status of forest soil microorganisms to be used as a benchmark in studies aimed to infer soil quality level in response to natural or anthropic stress.