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Valuation of biochemical and microbiological indicators in soil quality assessment

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Abstract

This thesis research aimed at valuating the suitability of biochemical and microbiological indicators in soil quality and soil health assessment, applying an interdisciplinary approach by means of different methodologies. Since soil quality assessment relies on a broad range of information useful to understand the role of different mechanisms in the soil ecosystem, the adequacy of different indicators has to be tested. A deep exploration of soil biota diversity, along with spatial and temporal variation in soil's features, intends to corroborate the hypothesis that biochemical and biological properties are the most suitable in assessing variations in soil quality. As the concept of soil quality encompasses both functionality and biological diversity, two cases of study are proposed and each of them aimed at the description of this two aspects. Focussing on soil functionality, the resilience of a soil contamined by xenobiotics was tested using biochemical indicators and microbial communities structure. On the other hand, focussing on soil diversity, the effect of different agricultural practices on soil microbial communities has been described by deeply investigating soil bacterial community.

The first case study examined the degree of interference of high soil copper contamination when an old vineyard is converted into a protected area, by investigating the resilience of a polluted soil using biochemical and microbiological indicators. The site is located in Ravenna Province (Emilia Romagna, Italy), within a intensively productive agricultural area. This study was organized into a two-factorial nested design to compare soil from an old vineyard under ecological restoration, to a soil from an intensively managed crop; the impact of management (conventional *vs* re-naturalized orchard) and position

within each orchard (tree-rows and strips) was analysed. Chemical and biochemical properties, with an insight to ecophysiological parameters and enzymatic activities, were evaluated. Bacterial and fungal communities were described by means of PCR-Denaturing gradient gel electrophoresis technique starting from total soil DNA. Total copper in soil sampled in rows of re-naturalized site amounted to 1000 mg kg^{-1}, whereas it did not exceed 80 mg kg^{-1} soil in the other treatments. Total organic carbon and all biochemical properties significantly improved in re-naturalized compared to conventionally cultivated site, while no significant differences were observed between tree row and strip, indicating that deep improvement of soil biochemical properties exceeded the negative impact of copper contamination. A shift of bacterial community composition, as well as increased bacterial diversity in soil under restoration, indicated a bacterial response to copper stress; to the contrary, soil fungi were less susceptible than bacteria, though a reduction of fungal DNA was detected. Findings suggest that ecological restoration of highly polluted agricultural soils leads to overcoming the reduction of soil functionalities linked to copper contamination. Moreover, biochemical and ecophysiological parameters demonstrated to be suitable indicators for soil functioning assessment in case of soils under ecological restoration.

As concerns the second case study, it aimed to describe the interactions between soil physico-chemical parameters, plant growth, and soil microbial diversity under six different agricultural practices, in apple orchards affected by replant disease. The applied approach gave an insight into soil microbiological component, focusing on soil bacterial diversity. Two apple orchards located at two different sites in South Tyrol, Italy, were selected as representatives of

perennial crops affected by replant disease. Physico-chemical and ecophysiological properties, along with soil microbial communities (evaluated with PCR-Denaturing gradient gel electrophoresis and high-throughput sequencing of the V1-V3 region of the bacterial 16S rRNA gene) were analysed. Considering a threshold of 1% relative abundance of OTUs for each soil, 39 bacterial phyla and 197 genera were detected in all soils. The phyla *Proteobacteria*, *Actinobacteria*, *Acidobacteria* and *Bacteroidetes* comprised between 24% and 71% of all sequences. Plant growth parameters were correlated with chemical, ecophysiological parameters and bacterial genus abundances. Findings revealed that in this study, *Rhodanobacter*, *Blastocatella*, *Arenimonas*, *Variovorax*, *Ferruginobacter*, *Demequina* and *Schlesneria* were the genera most affected by treatment type in terms of abundance. Furthermore, *Ferruginobacter*, *Demequina* and *Schlesneria* (genera not commonly described as involved in biocontrol) were strongly correlated with plant growth. Treatments were found to exert contradictory effects on bacterial communities and on ecophysiological parameters. Besides, the differences in the soil physico-chemical and ecophysiological parameters at the two sites, resulted in the soils being incomparable for an assessment of soil health based on microbiological indicators. On the other hand, variations in minor populations, or in unclassified sequences, made interpretations of interactions among bacterial species difficult. Nonetheless, correlation of bacterial genus abundances with plant growth supplied novel information about the involvement of certain microorganisms in apple replant disease. High-throughput sequencing can be proposed as a suitable method to deeply describe soil microbiological communities, allowing a

broader investigation of the soil microbiota and its inter-relationships, helping to overcome the lack of information.

Even though soil complexity makes the assessment of soil quality difficult, biological indicators are demonstrated to give a deep insight into soil functionality and stability. The investigation of soil biodiversity could better identify specific groups accomplishing a given function, considering that a multiple range of processes occurring in soil can affect diverse soil functions. Finding of this work revealed that not only extracellular enzymes, but also less abundant microbial populations, seem to play a crucial role in catalyzing ecological processes involved in soil fitness. In addition, results confirm that organisms can actively contribute to ecosystem services, by means of efficient exploitation of resources. Notwithstanding it is well ascertained that soil microbiota benefit of nutrient sources thanks to both high diversity and high abundance of species, the obtained results demonstrated that also minor populations or still unclassified species could contribute to soil functions by exerting unproportional ecological effects. The outlook provided by this work, suggests a broader application of next-generation DNA sequencing techniques for a deeper investigation of soil diversity and to perform comprehensive multivariate analysis and meta-analysis in order to better describe ecological inter-relations in soil. Furthermore, diverse soil features related to quality assessment, could be properly investigated by applying methodologies which reach different levels of detail. Application of this methodologies could boost a more exhaustive and reliable knowledge concerning the soil mechanisms and, thus, the identification of appropriate soil quality and health indicators.

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Introduction

Soil supports a broad range of ecosystem services (ES) by sustaining primary production and preserving environmental quality and human health (Glanz, 1995; Doran *et al*., 1996). Anthropogenic activities (soil erosion, environmental pollution, intensive agricolture, over-grazing, salinization, desertification) cause an increase in soil degradation and a loss of productive land (Oldeman, 1994). Although soil plays a pivotal role in ecosystem's functioning, only in the last years research efforts aimed to investigate the relationships between soil and ES (Adhikari and Hartemink, 2015). For this reason a deeper exploration of soil biota diversity and spatial variation in soil's properties has been performed applying an interdisciplinary approach. Critical soil functions are those related to nutrient cycling (storage and release into plant available forms), water fluxes (regulation of drainage, flow and storage of water and solutes), biodiversity (support to the growth of a variety of organisms), filtering and buffering (protection of air and water against xenobiotics), physical stability and support (soil structure as backing of vegetation growth) (Larson and Pierce, 1991; Dailey *et al*., 1997; Doran and Parkin, 1994; Seybold *et al*., 1999; Karlen *et al*., 1994; Harris *et al*., 1996; Singer and Ewing, 2000). Last but not least, soils also provide anchoring support for anthropogenic structures.

According to the need of more information useful to understand the role of different mechanisms in determining the quality of soils, and to test the suitability of different indicators, the present work contributes with two different case studies. For each case study, different methodologies have been applied

according to the soil quality feature to be stressed and to the required level of detail:

- Ecological restoration of a heavy Cu-polluted agrosystem: resilience of a soil contamined by xenobiotics tested using biochemical indicators and microbial communities structure, focussing on soil functionality;

- Effect of different agricultural practices on soil microbial communities: managed agrosystems soil has been described using biological indicators able to deeply investigate soil microbiota and thus to assess soil health, focussing on soil diversity.

The two studies corroborate the hypothesis that biochemical and biological properties are the more suitable in assessing alterations in soil quality and soil degradation. Even though soil complexity (also in biological terms) make the assessment of soil functionality difficult, biological indicators could give a deep insight to soil stability, due to biodiversity; moreover they could better identify specific groups accomplishing a given function. In the following chapters, current understanding of indicators and the eligibility of minimum data set befitting the two case studies are illustrated. Finally, the present work aims to justify the broad range of suitable indicators used and, in conclusion, it highlights the need of a robust and commonly accepted framework in soil quality assessment.

1. Assessing soil quality: conceptual framework and methodologies

1.1. Defining soil quality and soil health

Since the 1970s many definitions have been proposed for soil quality concept (in literature it appears first in the work of Warkentin and Fletcher, 1977), and many efforts have been done in order to explain its meaning clearly. In 1980s, soil quality was defined as "*the sustained capability of a soil to accept, store and recycle water, nutrients and energy*" (Anderson and Gregorich, 1984). Nowadays, a commonly accepted definition is "*a soil capable to function"* (Larson and Pierce, 1991). The above-mentioned definition considers the bias due to the specific goal of a scientist's investigation. Thus, a plausible flexibility in describing soil quality has been introduced (Bastida *et al.*, 2008). Not only the interest of a researcher, but also the weaknesses in valuating ES as an integrated system justify a less strict approach (Letey *et al*., 2003). Stressing the concept of fitness, soil quality is more thoroughly defined as *the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation*" (definition stated in Karlen *et al*., 1997). Soil quality became a fundamental in sustainability only in 1993, within the "Soil and water quality: an agenda for agriculture" report (National Research Council Committee, 1993).

Even though there is not a clear distinction between soil quality and soil health, the second one indicates *the capacity of the soil to function as a vital*

living system (Gómez-Sagasti *et al*., 2012; Doran and Zeiss, 2000). It is possible to assert that soil quality merely refers to soil function, whereas soil health describes soil as non-renewable and dynamic living resource. Healthy soil is capable to suppress diseases and biotic attacks, to be resistant and resilient in response to stresses and disturbance (van Bruggen and Semenov, 2000). Thus, considerations on soil health mainly focus on biotic component (Anderson, 2003), and gain even more importance because of the strong relationship with soil fertility. Biological stability (determined by resilience and resistance) depends on structure or diversity of the microbial community, on aboveground vegetation, soil properties and their effect on microbial communities, disturbance severity and soil history. Hence, soil response to stresses and disturbance arise from a combination of biotic and abiotic features that can be measured and used as indicators (Griffiths B.S. and Philippot L., 2013). Soil losses, excessive fertilization that imbalances nutrients, and pollution contribute to soil degradation causing negative effects on soil quality and soil health (Laishram *et al*., 2012).

1.2. Soil functionality and soil biological diversity

Soil functionality and soil biological diversity are the two features considered in assessing soil quality. Regarding functionality, it's important to pinpoint that there is no univocal relationship between a given soil function and a related mechanism: a multiple range of processes occurring in soil can affect diverse soil functions (Laishram *et al*., 2012). The main catalysts of ecological processes involved in soil fitness are the extracellular enzymes: they are connected with carbon sequestration, other nutrients cycling, plant growth promotion and bioremediation (Burns *et al*., 2013). Within functional classes, both

high diversity and high abundance of species grant an efficient exploitation of resources, and it implies that organisms can actively contribute to ES (Ferris, 2015).

Concerning stability of soil ecosystem, it is properly described by means of the estimation of soil microbial diversity (Bulluck *et al*., 2002; Neher and Darby, 2009). Species' assemblage diversity in soil comprehends organisms habitat below- and aboveground, at a micro-niche and at landscape scale. Since organisms perform several functions (which determine chemical, physical, and biological properties of soil), they supply many ES - support for primary production, climate control, control of pests and diseases, decontamination. There is a hypothetical interrelation between diversity and abundance of soil organisms and soil properties and functioning: this dependence seems to be not so certain, even though it's indisputable a strict connection between biodiversity and functionality (Bargett, 2002).

1.3. Anthropogenic activities as trigger in soil quality decline

Anthropogenic activities strongly affect soil biodiversity and ecology, especially whose related to agricultural management (Brevik *et al*., 2015). Mono-cropping, fertilizers and pesticide application and other practices, cause a decrease in soil biodiversity and a shift in structure and composition of soil biota (Figuerola *et al*., 2014; Berg and Smalla, 2009; Reeve *et al*., 2010). Soil resilience depends on soil type and vegetation, climate, land use, scale and disturbance regime: maintenance of recovery mechanisms after a disturbance is recognized to be a critical point for system restoration (Seybold *et al*., 1999).

Even though soil resilience plays a basic role in ecosystem functioning, its mechanisms have not been well defined nor well understood.

Taking into account this conceptual framework, the importance of soil in providing a broad range of ES, justifies the development of methods suitable for soil quality assessment. Comprehension of mechanisms which link soil biodiversity to soil function (also considering effects on aboveground biota) is an issue of great concern because it would provide efficient and sustainable environmental management practices. Research and political efforts in monitoring soils at different scales are needed since soil is recognized as one of the key factors of a sustainable agricultural management. The establishment of a commonly accepted methodology, has its practical basis in local and national policy (Adhikari and Hartemink, 2015). As reported in the previously mentioned “Soil and water quality: an agenda for agricolture” NRCC report (National Research Council Committee, 1993), soil quality protection was suggested as technical guideline serviceable to environment preservation. In order to apply the proposed guidelines, a broad set of suitable indicators have been investigated in the last decades.

2. Soil quality indicators

A soil quality indicator is defined as a *chemical, physical or biological property of soil that is sensitive to disturbance and represents the performance of ecosystem function in the soil of interest*; furthermore indicators are dynamic soil properties, because of their sensitivity over time and space (Doran and Parkin, 1996). Usage of indicators allows to assess soil functions (since they often can not be directly measured) and can be both qualitative and quantitative parameters. Indicators abide by the following criteria: reactiveness to management's and climate changes; significant correlation with specific soil functions (Bengtsson, 1998); usefulness in elucidating ecosystem processes, integrating soil physical, chemical, and biological properties and processes (Anderson, 1988; Doran and Parkin, 1996; Drinkwater *et al*., 1996; Bongers and Bongers, 1998; Oyarzun *et al*., 1998); be accessible and interpretable to many users (Doran, 2000; Doran and Parkin, 1996). Addictionally, simple indicators that strictly relate to a specific ecological process are fitting better than synthetic indexes which give a too much reductive insight of the investigated system.

2.1. Soil quality indicators: an overview

Considering the case-specific definition of soil quality, as stated by Sojka and Upchurch (1999), indicators of soil quality must be selected according to soil use and management, soil characteristics and environmental circumstances. Since it is possible to interpret a wide set of soil parameters from a descriptive point of view, a broad set of suitable indicators have been investigated in the last decades. Furthermore, it is important to identify a set of indicators mainly because the identified categories do not precisely align with the various soil

functions (Doran and Parkin, 1996). Integration of different parameters or indicators does not mean to create a synthetic index of soil quality: it allows to interpret diverse soil characteristics and to select the most representative for that soil. For these reasons this approach is the most widely used in research. On the contrary, the application of a synthetic index can simplify the system and, as consequence, give a biased description of the investigated soil and its evolution.

2.2. Typologies of soil quality indicators

There are three commonly used categories of soil indicators: chemical, physical and biological. Recent trend in soil research attempts to integrate all of them, mainly because the identified categories do not precisely align with the various soil functions (Doran and Parkin, 1996). Chemical indicators well describe nutrient and water cycling, buffering capacity; physical indicators are used for the assessment of stability and support, water flows and habitats; biological indicators give information about biodiversity, nutrient cycling and filtering capability. Organic matter, specifically soil carbon, transcends the three categories and has the most widely recognized influence on soil quality, being linked to all soil functions. It affects other indicators, such as aggregate stability (physical), nutrient retention and availability (chemical), and nutrient cycling (biological); and it is itself an indicator of soil quality (Doran and Parkin, 1996). In the present work, for measures related to soil carbon, the wording “ecophysiological parameters” will be used, according to definition given in Anderson and Dormsch, 1989.

2.2.1. Physical indicators

Physical indicators commonly investigated for soil quality assessment are texture, bulk density, porosity, and aggregate stability. These parameters are strongly correlated with hydrological processes like erosion, aeration, runoff, infiltration rate, and water holding capacity (Schoenholtz *et al*., 2000). In general, a soil has good physically features when it shows adequate rates of water infiltration, prevents surface runoff, shows cohesion, high aeration and root density, and ease for mechanization (Dexter, 2004).

Soil texture contributes to the balance between water and gases, and it is very stable over time. Therefore, bulk density and total porosity can better represent the effects of soil use and management on the water/air relationships (Beutler *et al*., 2002). Lower bulk densities are generally observed in soils under less anthropogenic interferences (Bini *et al*., 2013): native soils have higher content of organic matter that permit a better aggregation of soil particles, improving soil structure. As a result, macroporosity improves the soil permeability for water, air and roots (Tejada *et al*., 2006). The total soil porosity can be classified as textural, depending on the proportion of soil particles, and structural, depending on biopores and as macro-structure. While textural porosity is stable over time, structural porosity can be affected by cropping methods, compaction and soil management, causing a change in soil water retention curve (Dexter, 2004).

Soil aggregates are formed by particles smaller than 0.2 µm that group to form micro-aggregates (20-250 µm), which in turn are grouped to form macro-aggregates. Micro-aggregates are more stable and less affected by soil use and management. Moreover, they are responsible for long-term stabilization of soil

organic carbon. On the other hand, macroaggregates are more susceptible to soil management, and they have a strong relation with organic matter dynamics (Six *et al*., 2004).

2.2.1.1 Inter-relation between physical soil attributes and soil microbial communities

In soils with more inputs of organic residues, aggregate dispersion is usually less severe than in intensively managed soils. As a consequence, soils rich in organic matter show a greater microbial activity (Qin *et al*., 2010). Dispersion of aggregates causes a decrease in soil organic matter, as a consequence macroporosity and soil oxygenation are reduced, as well as the performance of decomposing microbiota and their access to the organic material (Degens *et al*., 2000; Tejada *et al*., 2006; Chodak and Niklinska, 2010). Soil aggregates affect aeration, permeability, nutrient cycling, and serve as refuge for microorganisms and soil fauna in microsites. Many organic substances (produced by several organisms as earthworms, arbuscular mycorrhizal fungi, bacteria and also the plants) as secretions, mucilages, mucigels, and cell lysates act as cementing substances and they can, in addition, stimulate microbial activity (Preston *et al*., 2001). Humic substances are fundamental for water and nutrients supply in soil: charges in their carboxylic and phenolic groups attract the water molecule and thus reduce its percolation through the soil profile, increasing soil water retention. As the content of available water is a determining factor of the microbial activity in soil, the soil physical attributes affecting water availability and aeration will also affect the soil microbial activity, since there is an inverse correlation between water availability and microbial activity (Geisseler *et al*., 2011). Thus, the impairment of soil microbial activity due to water limitations can

lead to losses of soil functionalities like synthesis and mineralization of soil organic matter and consequent effects on the biogeochemical cycles. Nevertheless, different microbial groups are differently affected by water restrictions in soil. For example, bacteria have restricted movement in drier soils in which water film is more intensively interacting with the soil particles (Wong and Griffin, 1976). Concluding, physical soil attributes and soil microbial communities are co-influenced (Degens *et al*., 2000), and both are affected by soil organic matter.

2.2.2. Chemical indicators

Chemical soil features are linked to the capacity to provide nutrients for plants and/or retaining chemical elements or compounds harmful to the environment and plant growth. In soil quality assessment, the most common parameters measured and interpreted are soil pH, cation exchange capacity (CEC), organic matter and nutrients content, which can give indication about the soil capacity for supporting high yield crops (Kelly *et al*., 2009). Since chemical attributes can be correlated with plant yields, the variations of a particular indicator can be easily interpreted. Interrelation between chemical parameters and soil productivity, is used in soil management because it is simple to improve soil chemical properties by liming and/or fertilizing. Soil chemical indicators can not only be applied in agricultural management but are also useful in assessing forest production and sustainability, nutrient cycles dynamics, plant biomass and organic matter (Schoenholtz *et al*., 2000).

Idowu *et al*. (2008) suggested that in soils devoted to agricultural production, the most important chemical parameters to be assessed are pH,

available P, K, Cu, Fe, Mn, and Zn. Soil pH is a key indicator, since the direct inter-relationship it has with nutrient availability (it allows to predict the potential for nutrient availability in a given production system; Sousa *et al*., 2007) and also affects microbial activity. Soil organic carbon is also a key attribute in assessing soil health, generally correlating positively with crop yield (Bennett *et al*., 2010). The soil organic carbon affects microbial activity and, thus, important functional processes in soil (nutrient retention and availability, mainly N, water holding capacity, stability of aggregates; Silva and Sá-Mendonça, 2007). Interaction of carbon with chemical, physical and biological soil properties make carbon content an important feature in soil quality assessment, as crucial component of soil fertility. Organic matter in soil can exert controversial effects: high organic carbon content reduces pesticide efficiency, increasing the frequency of required applications. Xenobiotic sorption is enhanced by complexation with soluble organic matter: they are adsorbed on organic fractions and transported through soil or ground-water (Sojka and Upchurch, 1999). Since the pivotal role that carbon exerts on soil system, and since the complexity of carbon cycling in soil, carbon-related parameters (ecophysiological parameters) will be analyzed in a dedicated paragraph. Nitrogen is the most required plant nutrient, which is found in several chemical forms in soil and fosters a very dynamic cycling. Soil nitrogen is usually measured and expressed as mineral N, especially nitrate, organic N or potentially mineralizable N, as stored in the soil organic matter. Despite the importance in plant nutrition and environment, the use of nitrogen as parameter for assessing soil health is subjected to factors that affect its dynamics in soil, like climatic conditions, turning inadequate the diagnosis of the real availability for plants, based on soil chemical analysis (Cantarella, 2007). Phosphorus is also a

key nutrient for agricultural yields and is essential in assessments of soil quality. Soil solution represents a stock of bioavailable P in the form of orthophosphates; available P is also represented by microbial P and organic P.

Soil chemical parameters have been traditionally used for assessment of potentially available nutrients for crops, and are based on worldwide well-established analytical methodologies. Among them, organic matter, pH, and available nutrients and also some potential hazardous chemicals have been used to establish levels of soil health. On the other hand, these procedures can not help in a full understanding of ecological processes occurring in soil: for example, in a study by Melo and Marchiori (1999) very good levels of chemical indicators in a cropped soil are reported, but the biological indicators were far below the ones found in a native forest used as reference. Thus, the integration among indicators seems to be a more appropriate approach to assess soil health.

2.2.3. Biochemical and biological indicators

Organic carbon and immobilized minerals must be recycled in the ecosystem before being utilized by organisms in a continuous and sustainable life cycle (Schjønning *et al*., 2004). Thus, biological processes are essential for keeping the soil capacity for recycling carbon to the atmosphere and assure the continuance of photosynthesis, concomitantly with nutrient mineralization for plant and microbial nutrition. Healthy soils have the capacity to keep these processes working in a indefinitely sustainable way.

As previously mentioned, microbial component of soils is more sensitive than physical and chemical attributes to environmental changes and soil management (Masto *et al*., 2009), justifying an increasing application as soil

quality indicators. Organic matter modulates the biological activity as source of C, energy and nutrients that will be mineralized to CO_2 and minerals. Mineralization rate depends also on the quantity and quality of the organic material in soil (Zhang *et al*., 2006b). Simultaneously, the microbial biomass will immobilize C, N, P and other nutrients that can be easily released for plant use due to its rapid turnover (Bayer and Mielniczuk, 2008). The most used biological indicator is soil basal respiration in either forestry or agricultural soils (Bastida *et al*., 2008), thanks to its strong relationship with soil organic matter. A decrease in organic carbon inputs into the soil via surface or rhizosphere, are demonstrated to reduce soil respiration (Bini *et al*., 2013). Impacting managements affect soil biological activity by depressing it. The metabolic quotient (qCO_2) is an index given by the amount of CO_2-C released per unit of microbial biomass in time and represents the metabolic status of the soil microorganisms (Anderson and Domsch, 1993). Higher qCO_2 values usually indicate both microbial stress and inputs of easily degradable organic carbon that promptly stimulates the microbial activity (Dinesh *et al*., 2003).

In assessing alterations in soil quality and soil degradation, biochemical and biological properties are recognized to be the more suitable than the physical-chemical ones. Soil enzyme activities have been often suggested as sensitive indicators of soil quality: their suitability depends on the possibility to assess principal microbial reactions involving nutrient cycles, sensitivity to stresses and soil changes and ease to measure (Gianfreda and Bollag, 1996; Calderon *et al*., 2000; Drijber *et al*., 2000; Nannipieri *et al*., 2002). Soil biochemical properties related to carbon, nitrogen, phosphorous and sulfur cycles are used as indicators of soil quality, even though their efficiency is still

controversial mainly because of the lack of reference values, the antithetical behaviour in degraded soil and variations over time and space in expression levels (Gil-Sotres *et al*., 2005; Paz-Ferreiro and Fu, 2013). Since soil enzymatic activities link microbial population with nutrient dynamics (Sinsabaugh and Moorehead, 1994), and since they are different among soils, it is possible to use them as indirect measures of functional diversity (Caldwell, 2005).

Specific factors involved in determining community structure and functioning are still not well understood. Soil microbial properties are broadly used as soil quality indicators thanks to their high sensitivity and because they give an integrated information concerning environmental factors (Gómez-Sagasti *et al*., 2012). In defining suitable biological indicators, able to give information about soil species diversity, it is possible to use diverse methods in order to measure both abundance and diversity/function. Focusing on microbial communities, molecular methods have been developed in order to overcome the enormous number of microorganisms living in soil, of which only 1% can be cultivated (Torsvik and Ovreas, 2002). Soil microorganisms can be classified into functional groups according to the biological process they play in an ecosystem. All microorganisms that act in the N cycle (e.g., diazotrophic, nitrifying, denitrifying, ammonifying and proteolytic bacteria, etc.) and C cycle (e.g., cellulolytics, amilolytics, proteolytics, etc.) are examples of functional groups. In this case, the individual species is not the focus, but the function they play collectively in an environment. High microbial diversity in soil positively affects ecological processes after a disturbance, improving soil resilience (Kennedy, 1999). Functional redundancy is higher in less degraded soils (Harris, 2003), even though the composition of the plant community may favor the prevalence or

cause suppression of certain microbial functional groups in soil (Matsumoto *et al*., 2005).

2.3. Applicability of soil quality indicators

Physical parameters (i.e. soil texture, aggregation, moisture, porosity, and bulk density) and chemical parameters (i.e. total C and N, mineral nutrients, organic matter, cation exchange capacity) are commonly used as indicators and methodology subtended to their interpretation is well established. However, most of them generally have a slow response, when compared to the biological ones, such as microbial biomass C and N, microbiological biodiversity, soil enzymes, soil respiration, etc. In order to maintain soil health and productivity (not only in terms of economic sustainability, but also with increasing emphasis on reforestation and recuperation of degraded areas by the use of organic amendments, reintroduction of plants, soil fauna and microorganisms), the use of indicators is functional to soil description and soil evolution assessment. In assessing alterations in soil quality and soil degradation, biochemical and biological properties are thus recognized to be the more suitable than the physical-chemical ones, because their high sensitivity towards changes in soil. Physical and chemical feature change less over time and space, so they cannot describe soil evolution as consequence of anthropogenic activities. The soil biota is very dynamic and promptly affected by soil use and management or any other disturbance, conversely from most chemical and physical properties, which take longer to be changed. That is the reason why the edaphic organisms are good indicators of soil health, especially if the indicator corresponds to ecological processes occurring in soil. In order to better explain the complexity of the biological component of soil functionality, the related indicators can be classified

(referring to the present work) in biochemical, ecophysiological and microbiological indicators.

Efficiency of soil biochemical properties (among them enzymatic activities have a particular concern) as indicators of soil quality is still controversial mainly because of the lack of reference values. Moreover they are demonstrated to show antithetical behavior in degraded soil and variations over time and space in expression levels (Gil-Sotres *et al*., 2005; Paz-Ferreiro and Fu, 2013). Nonetheless, since soil enzymes activities link microbial population with nutrient dynamics (Sinsabaugh and Moorehead, 1994), and since they are different between soils, it is possible to use them as indirect measures of functional diversity (Caldwell, 2005).

Biochemical properties as indicators of soil quality show a high degree of variability in response to climate, season, geographical location and pedogenetic factors. This lead to contradictory interpretations when describing the effects of a contaminant or a given management on the soil quality (Gil-Sotres *et al*., 2005). Moreover, with the knowledge actually available, estimation of soil quality relying only on individual biochemical properties, simple indexes or ratios, cannot be considered trustworthy. Thus, a minimum data set of biochemical properties capable of describing the complexity of the soil system is required for each situation. Facing these challenges, the use of multivariate statistical techniques is a useful tool for selecting attributes for assessment of soil health. Considering methodologies used for diversity and functionality analysis in soils, profiling techniques are powerful tools, but they may cast doubts on the true relationship and contribution of the microbial diversity to the concept of soil quality. Also in

this case, a set of microbiological techniques is required for each situation and type of study, while assessing soil health.

Soil microbial properties are broadly used as soil quality indicators thanks to their high sensitivity and because they give an integrated information concerning environmental factors (Gómez-Sagasti *et al*., 2012). In defining suitable biological indicators, able to give information about soil species diversity, it is possible to use diverse methods in order to measure both abundance and diversity/function. Focusing on microbial communities, molecular methods have been developed in order to overcome the enormous number of microorganisms living in soil, of which only 1% can be cultivated (Torsvik and Ovreas, 2002).

3. Soil microbiota as key factor in biogeochemical cycling

Microorganisms show a strong interlinkage with biogeochemical cycling, confirming their pivotal role in soil development and preservation (Elliott, 1997; Pankhurst *et al*., 1997). Many problems in soil ecology research derive from the inaccuracy in measuring species richness and evenness and from the lack of information about the specific functions carried out by different microorganisms. Bias in assessing species richness and evenness is emphasized if ascertaining that the majority of species are rare, while the most abundant are only a few (Loreau *et al*., 2001). In describing the complexity of soil system, the following features –focussing on microbial characteristics - are the most relevant and they are clearly reviewed in Nannipieri *et al*. (2003). Soil communities are abundant and heterogeneous: Killham (1994) assumes that, in a temperate grassland soil, bacterial biomass amounts to 1-2 t ha^{-1} and fungal biomass to 2-5 t ha^{-1}. According to Stotzky (1997), soil is a "*structured, heterogeneous and discontinuous system*", furthermore the nutrients' and energy sources' scarcity lead microorganisms to occupy discrete and dynamic microhabitats. These exploited micro-niches are also known 'hot spots', zones of increased biological activity, depending on different physio-chemical properties in bulk soil and rhizosphere, or high concentration of organic matter. Concerning the localization of microorganisms, they live free in aquatic environment or attached to surfaces, in water films surrounding solid particles. Soil solid phase can absorb biological molecules such as proteins and nucleic acids: adsorption of extracellular enzymes by clay and by humic molecules guarantees protection against proteolysis and denaturation and, thus, the maintenance of their activity and

function. In extreme conditions, surfaces of mineral components can act as catalysts, prevailing over microbial activities during metabolic processes. The concept of microbial biodiversity points out two different aspects of taxonomic diversity: in relation to environmental impact and in relation to soil functioning. According to the above-mentioned concepts, soil ecosystem analyses concentrated (i) on energy transfer between pools and (ii) on quantification of xenobiotic.

In assessing carbon fluxes in soil, it is important to differentiate between four diverse microbial physiological states: active, potentially active, dormant and dead microorganisms. Even though all of them contribute to C cycling, only the active fraction is a driven factor of biogeochemical processes (Blagodatskaya and Kuzyakov, 2013). Active microorganisms compose only about 0,1% (it varies according to the resources input easily available) and are involved in the ongoing utilization of substrates and associated biochemical transformations. Potentially active microorganisms are in a state of *physiological alertness* (De Nobili *et al*., 2001) and can start utilization of available substrates within minutes to a few hours: this fraction contributes between 10 and 40% (up to 60%) of the total microbial biomass. Dormant microorganisms can contribute to biogeochemical processes in case of altered environmental circumstances. Dead microbiota, including lysed cells and microbial residues, are a source of easily available substrates and thus affect turnover of C and N. Active, potentially active and dormant microorganisms constitute the living microbiota. Despite all these fractions succeed in soil functioning valuation (when comparing treatments, environmental conditions, land use and management practices), attention might be paid to active microorganisms, as previously highlighted. The crucial role in C

and N cycling and the high sensitivity to environmental changes, justify the suitability in using microbial and biochemical features of soils as quality and health indicators. Approaches based on determination of cell components (e.g. DNA and molecular biomarkers), identify both active and non-active microorganisms without separating the two. This kind of approach, thus, fails in investigating the functions carried out. Conversely, approaches based on measuring the changes of different parameters (e.g. substrate utilization and product formation, such as respiration) during microbial growth and based on process rates could give a more precise description of the functions performed (Blagodatskaya and Kuzyakov, 2013). Encountered difficulty in measuring soil diversity makes the inter-relationships between microbial diversity and soil functioning, and between stability (resilience and/or resistance) and microbial diversity, still unclear (Nannipieri, 2003).

Soil functioning and fertility relies mainly on decomposition activity of the microflora: for this reason specific microbial-based indicators have been developed. Two different aspects of taxonomic species diversity can be investigated within soil microbial diversity: one related to the environmental impact, the other related to functioning. Microbial diversity seems to give robust indication about soil quality only if integrated in a minimum data set of soil quality indicators (Anderson T. H., 2003). Soil fertility depends on the inter-relation between microbial response to an abiotic environment and microbial functions during plant decomposition. In assessing soil health is thus important to identify indicators able to describe synthetically both aspects of microbial functioning (energy transfer between pools and quantification of xenobiotic compounds). Concerning this issue, ecophysiological parameters are reported to be suitable

indicators in soil quality valuation. Once clearly understood the role of microbiota in mineralization of organic substrates and in releasing of nutrients and other elements, new methods to deeply assess microbial functions in soil have been developed. In particular, direct microscopic observation lacks in efficiency when applied for the quantification of environmental impacts on the microflora (Anderson and Domsch, 2010). However, assessment of soil health taking into account microbiological parameters is still controversial. Specific factors involved in determining community structure and functioning are still not well understood.

3.1. Combining abiotic factors and microbial functioning: an ecophysiological approach

The relationship between dead primary products (litter), soil organic matter (C_{org}) and soil microbial biomass (C_{mic}) became a key issue in microbial ecology. Research started to study how carbon pools could affect microbial productivity letting auto-ecological studies gain even more importance: maintenance carbon requirements, growth rate, yield, turnover time have been discussed with respect to the soil microbial community (Parkinson *et al*., 1978; McGill *et al*., 1981). The term ecophysiology refers to the relationship between ecology and microbial physiology, implying an interlinkage between cell-physiological functioning under the influence of environmental factors. The investigation of soil organic carbon dynamics and it was firstly postulated in Anderson and Domsch, 1978.

The term ecophysiology refers to the relationship between ecology and microbial physiology, implying an interlinkage between cell and physiological functioning under the influence of environmental factors. Carbon pools could

affect microbial productivity: in order to confirm this hypothesis auto-ecological studies gain even more importance: maintenance carbon requirements, growth rate, yield, turnover time have been discussed with respect to the soil microbial community (Parkinson *et al*., 1978; McGill *et al*., 1981). It is still under debate the relationship between soil taxonomic diversity and functional features, in particular functional efficiency. Redundancy in exerting precise metabolic processes is a predominant attribute in microbial communities, but there are not evidences if a peculiar functional ability is carried out by a taxonomic group/species in a more efficient way than another. The assumptions behind the application of ecophysiological parameters in soil ecology studies refer to Odum's ecological theory: efficiency in energy utilization corresponds to an increase in diversity during ecosystem succession, it means that at maturity stage the lowest microbial community respiration and an increasing degree of below-ground species diversity are expected (Odum, 1969). Furthermore, each metabolic activity performed by an organism depends on available carbon sources. High microbial performance in energy utilization could be measured by determining community respiration per biomass unit, calculating the so-called metabolic quotient (qCO_2). As to that, evolution of CO_2 reflects the catabolic degradation under aerobic conditions. Metabolic quotient can be interpreted as follows: if community respiration is low, more carbon will be available for biomass production (high ratio microbial carbon (C_{mic}) to total organic carbon (C_{org}) (Anderson and Domsch, 1986b).

3.2. Ecophysiological parameters as key soil health indicators

As previously illustrated, decomposition activity of the microflora is the main mechanism determining soil functioning and fertility; for this reason,

microbial-based indicators have been developed. It seems that soil health cannot be properly described by assessing environmental impact and soil functioning separately, hence an ecophysiological profile could be a supplementary information to be integrated in a minimum data set of soil quality indicators (Anderson T.H., 2003). In assessing soil health and soil fertility, is thus important to identify indicators able to describe synthetically both aspects of microbial functioning: ecophysiological parameters seem to be fitting indicators in soil quality valuation. However, assessment of soil health taking into account microbiological parameters is still controversial. Nonetheless, there is still no evidence that assumes soil microbial taxonomical diversity as a prerequisite for functional efficiency.

Ecophysiological indices are based on physiological activities (respiration, carbon uptake, growth/death, etc.) performed by the total microbial biomass per unit time. As heretofore explained, a change in microbial community activity is the result of an impact affecting soil microbiota: thus the effect of the impact can be detected and quantified.

The ecophysiological parameters commonly calculated are the following, though the first two of them are the most explanatory and the most widely used:

- qCO_2: CO_2 output per unit biomass (Anderson and Domsch, 1978; Pirt, 1975);
- C_{mic}-to-$_{Corg}$ ratio: ratio of biomass carbon to total organic carbon (Anderson and Domsch, 1989).
- growth rate (m), yield (Y), turnover time (d) (Anderson and Domsch, 1986);
- qD: microbial-C-loss (Anderson and Domsch, 1978);

- V_{max} and K_m: carbon uptake kinetics (Anderson and Gray, 1990).

The qCO_2 is nowadays applied at different spatial scales, being the most reliable ecophysiological parameter used in soil microbial ecology. The use of qCO_2 as soil indicator was firstly taken into consideration by Anderson and Domsch (1986a), who reported that qCO_2 is also a sensitive indicator of the effect of different temperature regimes on soil microbial community. Insam and Haselwandter (1989) applied the concept of "*bioenergetic economisation with successive age of an ecosystem*" to investigate soils of receding glaciers. Metabolic quotient is also applied as stress indicator of soils contaminated with heavy metals (Fließbach *et al*., 1994).

C_{mic}-to-C_{org} ratio is site-specific and it is usually applied as stability indicator for environmental change. It also could be used as a reference value for comparing different soil systems: because of that, qCO_2 and C_{mic}:C_{org} ratio can be integrated each other in order to define the so-called "baseline performance" of a microbial community. C_{mic}-to-C_{org} ratio and metabolic quotient are the best fitting indicators for long-term studies. The microbial baseline performance is particular of a soil category and could help in roughing out an "ecophysiological profile" of a site. If it is possible to appreciate a strong deviation from a site-specific baseline value, it means that environment is changing and, consequently, a new soil community is establishing (Anderson, 2003). Indeed, Odum (1985) pointed out that "*repairing damage by disturbances requires diverting energy from growth and production to maintenance*".

In applying ecophysiological parameters as indicators, it is important to consider that C_{org} content is usually lower *in situ* conditions in comparison to those measured under optimal *in vitro* conditions (Anderson and Domsch,

1986a). The above-mentioned bias depends on the different age of the organisms in culture, that inevitably affects qCO_2. In spite of the difficulties encountered in distinguishing active microorganism from inactive ones and in properly simulating *in situ* conditions, it is possible to generally interpret the behavior of metabolic quotient and C_{mic}-to-C_{org} ratio in different soils. Continuous crop rotation, which implies heterogeneous inputs, shows lower community respiration (measured as a lower qCO_2) than monocultures (Anderson and Domsch, 1990). A decrease of metabolic quotient was also observed in case of highest plant diversity in litter input (Bardgett and Shine, 1999); along successional stages of reclaimed soil systems (Insam and Haselwandter, 1989); and in mature agricultural soils (Anderson and Domsch, 1990). Concerning agricultural soils, monoculture lead to significantly lower C_{mic}:C_{org} ratio in comparison to soils under continuous crop rotations. Percentage of C_{mic} to total C_{org} of soils under monoculture is 2.3% (mean value), conversely it raises to 2.9% for continuous crop rotation (in unamended plots) (Anderson and Domsch. 1989; Insam *et al*., 1989; Powlson *et al*., 1987). Even considering monoculture systems, the high C_{mic} content, if compared to the baseline performance, is due to the amount of input left on the crop, while in rotation systems it depends on higher chemical diversity of organic matter input that could favour those organisms able to optimize their metabolism according to the amount of carbon available. According to ecological bioenergetics theory it means that in complex systems soil microbiota are characterized by a significantly higher yield coefficient (Y) and higher metabolic quotient for CO_2 in “young” sites as compared to “matured” sites. Thus, there is an inverse relationship between C_{org} entering metabolism and C_{org} fixed in microbial cells: therefore it is reflected in C_{mic}-to-C_{org} ratio

(Anderson and Domsch, 1990). Considering chemical properties of soil, it is recognised that in acidic soils is observed an increase in qCO_2, on the contrary C_{mic}-to-C_{org} ratio decreases, if compared with neutral or calcareous soils.

4. Role of enzymes in ecosystem and soil functioning

In describing soil quality, two features have been pinpointed: stability (defined by both resilience and resistance) and diversity. Each of them is related to soil functionality by means of different mechanisms and processes. Metabolic requirements of microbial communities and available nutrients in soil inter-relate thanks to enzymatic activities. Capacity of soil microbiota to efficiently maintain these critical soil processes in case of biotic and abiotic stresses, disturbance, and ecological succession, seems to affect ecosystem productivity and stability more than taxonomic diversity. Soil biochemical properties refer to general parameters such as microbial carbon, dehydrogenase activity and nitrogen mineralization potential; they also include more specific measurements such as activity of many hydrolytic enzymes, such as phosphatase, urease and β-glucosidase (Gil-Sotres *et al*., 2004). Enzymes, thanks to their involvement in soil nutrient cycling and energy transfer, properly describe biochemical processes dynamics in soil. Hence, their activity indicates the biological capacity of a soil to carry out the biochemical processes which are important to maintaining the soil fertility (Galstian, 1974; Dkhar and Mishra, 1983; Burns, 1986; Garcia *et al*., 1994), thus they can be used as simple indices or in combination.

Enzymes in soil are the key element that links resource availability, microbial community structure and function, and ecosystem processes (Caldwell, 2005). Soil enzymes are differentiate in biotic enzymes (associated with viable proliferating cells) and abiotic enzymes. Biotic enzymes are located intracellularly (in cell cytoplasm and in the periplasmic space) and at the outer cell surfaces. Enzymes in soil solution are generally short-lived because physical adsorption,

denaturation or degradation cause their inactivation (Burns, 1986). Abiotic enzymes are those excreted by living cells during cell growth and division from extant or lysed cells but whose original functional location was on or within the cell (Skujinš, 1978). They can occur adsorbed to clay surfaces and complexed with colloids through adsorption, entrapment, or copolymerization during humic matter genesis (Boyd and Mortland, 1990). Complexed enzymes belong to an extracellular enzyme pool and coact with the diffusion of substrates to the active enzyme site (Burns, 1982). Thus, it is possible to assert that soil is a sink and source of indigenous and persistent enzymatic capacity (Galstian, 1974; Burns, 1986; Lähdesmäki and Piispanen, 1992; Busto and Perez-Mateos, 1995). Soil enzymatic pool, and its consequent activity, depends also on land use history, since enzymes are produced by living organisms which contribute to the biological soil formation.

Mechanisms underlying enzyme synthesis and activation are currently investigated: chemical, physical and biological properties are supposed to interact with substrate turnover and with the enzymes' distribution. It is commonly accepted that factors affecting enzymatic reactions are pH, ionic state, temperature (enzyme reaction rate is temperature-dependent) and the presence or absence of inhibitor and activator (Burns, 1978; Tabatabai, 1994). The limitant factor of enzyme reaction is the temperature: high values (T>50°C) cause the denaturation of protein structure of enzyme (Campbell and Smith, 1993). Furthermore, enzyme active site requires for particular cations (such as Ni for urease). Presence of the inhibitor reduce enzyme activity, while it increases when an activator occurs (Mathews and van Holde, 1995). Moreover it is still unclear how extracellular enzymes cope with inhibitory and competitive properties of the

soil matrix, and which strategies subtend to effective substrate utilization (Burns *et al*., 2013). Actually, soil enzymology is gaining importance because climate change and anthropogenic impacts on the environment can affect microbes and their extracellular enzymes. Shifts in enzyme soil diversity are not well understood but their investigation could provide a deeper knowledge in soil stability and functionality.

Enzymes convert complex organic molecules to assimilable compounds (sugars, amino acids, NH_4^+, HPO_4^2) by macromolecular depolymerisation, accomplishing this function by reacting through different mechanisms that differentiate them into six categories: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (Voet and Voet, 1995). The prevailing enzyme categories in soil are hydrolases, then oxidoreductases, transferases, and lyases (Dick and Tabatabai, 1992). The most generic classification takes into account the source: soil enzymes can be extracellular (such as arysulphatase) or endocellular (such as dehydrogenase). Extracellular enzymes and accumulated enzymes derived from abiotic sources; enzymes belonging to the first group are continuously released (Skujins, 1978). According to the enzyme localization, it is possible to differentiate nine states in which they can be found in soil system: adsorption (Esminger and Gieseking, 1942); microencapsulation, cross-linking, copolymer formation (Boyd and Mortland 1990), entrapment, ion-exchange, cross-linking and covalent attachment (Dick and Tabatabai, 1992). Each of this phenomena protects extracellular enzyme activity from external disturbances, in particular enzyme activities are significantly correlated with the organic matter content of soils. Soil organic matter is proven to protect enzymes from microbial attack (Conrad, 1940, demonstrated that urease is protected from microbial

denaturation by SOM), furthermore enzymes can complex with humic acid forming complexes which are more stable to heat and enzymatic degradation than free enzymes (Serban and Nissenbaum, 1986). The bio-macromolecules breakdown requires the combined activities of many different microorganisms, because of their complexity in structure and diversity. Besides, potential organic xenobiotics (such as polycyclic aromatic compounds) require extracellular catalysis prior to microbial uptake and catabolism (Martin *et al*., 2009).

Soil functioning is highly dependent from enzyme activities because they release available nutrients to plants, participate in decomposition of organic inputs and transformation of organic matter, have a role in N_2 fixation, nitrification and denitrification processes and in detoxification of pollutants (Dick 1997). The complexity of enzymatic reactions involves not only the substrate metabolism, but also the capability in responding to changes in structural diversity and in nutrient availabilities. Soil enzyme activities have correlations with physico-chemical features, microbial community structure, vegetation, disturbance and succession. Soil enzymes have a crucial role in carbon (β-glucosidase and β-galactosidase), nitrogen (urease), phosphorous (phosphatases), and sulphur (sulphatase) cycles. The importance of soil enzymes was first stated by Dick and Tabatabai (1992): "*soil enzymes are useful in describing and making predictions about an ecosystem's function, quality and the interactions among subsystems*". Later, this principle has been applied in the set up of a soil quality indicators framework. Bandick and Dick (1999) suggest to use enzyme as indicators because of "*their relationship to soil biology, ease of measurement and rapid response to changes in soil management*". Moreover enzymatic activities can be investigated at different scales of resolution, from the landscape to soil particle size fractions.

4.1. Enzymatic activities as soil quality indicators

Enzyme-mediated processes started to be deeply investigated when it was possible to use them as specific indicators of functional diversity. Soil enzymes as soil quality indicators can be differentiate into: (i) pollution indicators, (ii) ecosystems perturbations indicators and (iii) agricultural practice indicators (Burns, 1977). Unlike is the case for physiological or taxonomic diversity of the soil microbial biomass (which describe potential activities), functional diversity of soil enzymes is related to the actual activities and it is usually applied in assessment of major nutrient cycles. Functional enzyme diversity can be investigated applying interacting sets of information (both quantitative and qualitative parameters) that can be made up by: (i) measurements of reactions against target substrates, (ii) investigation of different reaction mechanisms within a given enzyme function, and (iii) the possible determination of enzyme sources. The major nutrient cycling investigated in soil are those related to carbon, nitrogen and phosphorous. It has been recognized that there are specific compounds against which classes of enzymes are active. As regards carbon cycling in soil, Sinsabaugh *et al*. (1992) identified different cellulases and β-glucosidases, whereas Kirk and Ferrell (1987) recognized polyphenol oxidases and peroxidases as catalysts for ligninolytic activities. Within the nitrogen cycle, hydrolysis of different amino acid groups acts on proteins and peptides (Ladd and Butler, 1972; Tabatabai *et al*., 2002). Ammonium can also derive from C–N bonds thanks to urease activity. Phosphodiesterase and phosphomonoesterase activities lead to mineralization of phosphate from organic esters. Phosphodiesterase uses tissue-based substrates, whereas phosphomonoesterase act on soil organic phosphates pool (Dalal, 1977).

Specific inhibitors or substrates are useful in assessing enzyme functional diversity by considering that catalysis occurs at specific reactive sites. Assays which consider inhibitors activity have been developed for proteases (Morihara, 1974) and peptidases (Ladd and Butler, 1972; Sinsabaugh *et al*.,1992). In order to gain a deeper understanding of which taxonomic groups can directly access a specific nutrient resource (performing therefore a specific enzymatic activity), different molecular methods have been tested (Kelly, 2003; Wellington *et al*., 2003). By means of these methodologies, it is possible to determine specific functional genes and their expression, and, as consequence, to describe energy and nutrients pathways in soil. Proteomics provides information on enzyme potential and expression, and integration of these methods with conventional approaches could describe the linkage activity-source taking into account taxonomic categories; i.e., bacteria and fungi (Caldwell, 2005). Concerning the application of combined assays, extracellular enzymes from bacterial sources generally have neutral-alkaline optima while fungal and plant enzymes have acidic optima: by using this test it is possible to differentiate some enzyme origin (i.e. phosphatases, proteases). This methodology founds on the principle that every enzyme has an optimum pH value and lower the activity up and below this optimum (Tabatabai, 1994). Rhee *et al*. (1987) estimated that fungi contributed approximately 86% of soil cellulase activity, while bacteria are the major source of proteases (Mayaudon *et al*., 1975). Investigation of functional diversity within soil enzymes, can be conducted in different ways, according to the aim of the research. The analysis can be based on specific enzyme activities against C (cellulose), N (protein) and P compounds. A further level of detail can be reached by investigating different enzymatic reaction within each nutrient cycle (cellulase

and phenoloxidase for carbon, protease and amidase for nitrogen or phosphomono- and diesterases for phosphorus). A given enzyme activity can be then differentiate by inhibitor class providing a deeper insight within a functional group. Specific enzyme activities involving carbon, nitrogen and phosphorous compounds, are usually evaluated as differences in activity. Ratios between and within major C-, N- and P- processing enzymes can provide insight into the microbial community response to changing nutrient resources and the relative importance of different nutrients, and changed under different soil and vegetation regimes (Caldwell *et al*., 1999).

4.1.1. Enzymatic activities as heavy metal pollution indicators

Enzymatic activities are differently affected by xenobiotics: pollutants can exert an appreciable influence on enzyme-mediated processes. Since every xenobiotic act on enzymatic activities in contrasting and peculiar ways (heavy metals, hydrocarbons, acid precipitation, industrial nitrogen and sulphur gaseous compounds), soil enzymes are used as pollution indicators. This application has been widely used to evaluate effects of agricultural management, as well as any ecological alteration due to xenobiotic pollution (Burns, 1977). Besides, soil enzymes could be use as suitable indicators in every case of land-use change (Acosta-Martinez *et al*., 2007; Trasar-Cepeda *et al*. 2008a; Gil-Sotres *et al*. 2005); devegetation and revegetation (Bastida *et al*. 2006); forest fires (Camci Cetin *et al*. 2009), and changing climatic conditions (Sowerby *et al*. 2005). Generally, heavy metals suppress soil enzyme activities, on the contrary pesticides can have a positive or negative effect (of different magnitude) depending on the type of pesticide. Industrial amendments or contaminants exert a similar controversial effect on enzymatic activities, according to the type of enzyme. The order of

enzyme activities level can be summarized as follows: non-polluted soils > polluted but restored > polluted but un-restored (Hinojosa *et al*. 2004). Agricultural soils show an higher arylsulfatase, β-glucosidase, phosphatase, urease, dehydrogenase, and fluorescein diacetate hydrolase activities compared to non-cultivated soils. Shifts in enzymatic activities depend on type, concentration, application duration and number of pesticides and amendments in soils (Gianfreda *et al*., 2005). Heavy metals can also drop microbial and enzyme activities by interacting with catalytic groups, altering protein conformation or competing with other metals involved in the formation of enzyme-substrate complexes (Eivazi and Tabatabai, 1990).

4.1.2. Enzymatic activities as agricultural management indicators

Agricultural practices (irrigation, tillage, fertilizers and pesticides application, plant residues application, and using crop rotations) modify soil functioning by affecting nutrient turnover and microbiological activity (Ladd 1985; Dick *et al*. 1987; Tabatabai, 1994). This kind of effect seems to directly depending on changes in soil biological properties due to these practices (Curci *et al*. 1997), but it is still disputed (Dick, 1992; Gil-Sotres *et al*., 2005). Regarding tillage, many researchers have discussed how tillage, broking up soil aggregates, determines an increase of enzymatic activities due to the exposure of new surfaces (Khan, 1996; Latif *et al*., 1992; McGill *et al*., 1986), whereas others argue that soil physical disruption decrease organic matter content and the consequent decline in enzyme activities (Carter, 1986; Dick, 1994; Jensen *et al*., 1996). Moreover, enzyme activity was found to be the most strongly depressed soil property under intensive agronomic use compared with other biochemical parameters (Saviozzi *et al*., 2001). In establishing a soil pollution indicator based on enzyme activity,

the following characteristics has to be taken into account: (i) pollutant sensitivity, (ii) reflecting ability of different levels of pollution, (iii) response either increases or decreases, (iv) sensitivity of different pollutants, (v) discriminating between pollutant effect and prior degradation of the polluted soil and (vi) differentiating all pollutants based on different degrees of soil degradation they cause (Doran and Parkin, 1994). Because of the high level of complexity that characterize the interrelation enzyme activities to soil, many authors (Beck, 1984; Perucci, 1992; Sinsabaugh, 1994; Stefanic, 1994) suggested to use soil enzymes as soil quality indicators in combination with other physico-chemical, biochemical and biological indicators.

5. Soil degradation and organic carbon depletion

The intensive agricultural management leads to a status of soil degradation well reported. Soil degradation can be defined as the process leading to the loss of the self-organization ability of the soil constituents, due to either natural causes or human activities. Self-organization process means that soil tends to reach a state of equilibrium characterized by minimized entropy (Addiscott, 2010; Targulian and Krasilnikov, 2007). Organisation for Economic Co-operation and Development (OECD) proposed to define soil degradation as a process concerning the loss of soil functions and referring to the processes by which soil does not properly fit for a specific purpose. Several factors could negatively affect soil function and be recognized as degradation processes: i) mechanical erosion; ii) decline in organic matter; iii) contamination; iv) sealing; v) compaction; vi) soil biodiversity loss; vii) salinization; viii) floods and landslides; ix) desertification; x) acidification (European Commission, 2006a; European Commission, 2012). Degradation processes investigated in the following paragraphs concern organic carbon depletion, and contamination in the form of high nitrate content and heavy-metals (especially copper).

According to Baldock and Skjemstad (1999), soil organic matter refers to "*all of the organic materials found in soils irrespective of its origin or state of decomposition*". Included are living organic matter (plants, microbial biomass and faunal biomass), dissolved organic matter, particulate organic matter, humus and inert or highly carbonised organic matter (charcoal and charred organic materials). The functional definition of soil organic matter excludes organic materials larger than 2 mm in size". Organic matter in productive agricultural soils

ranges between 3 and 6%. Organic fraction derived from plant and animal residues, and the active fraction contribute to soil fertility because the breakdown of these fractions results in the release of plant available nutrients. Since the humus fraction is the final product of decomposition (stable organic matter), it has less influence on soil fertility. However, stable organic matter has to be considered in fertility assessment and management because it contributes to soil structure, soil tilth, and affects soil chemical properties such as cation exchange capacity. Soil organic matter (SOM) positively affects agricultural soil by accomplishing a variety of functions (Millar and Turk 1943, Donahue *et al*. 1983): i) aggregate stability enhancement; ii) water infiltration, soil aeration and water holding capacity improvement, preventing runoff; iii) downturn in adherence of clay and surface crusting, making soil easy to work; iv) cation exchange capacity improvement, thanks to diverse functional groups which can hold and then release nutritional reserves; v) plant nutrients adsorption; vi) buffering capacity improvement; vi) nutrient source for soil biota; vii) soil microbial biodiversity and activity enhancement, promoting soil suppressiveness.

Stable organic matter content in soil depends on organic material inputs: crop residues, animal manure, compost, cover crops and perennial grasses and legumes. Material with high contents in carbon accelerates soil organic matter stabilization. Organic matter, in addition, is the main source of CO_2 in nature and one of the most abundant reserve of all the nutrients that may be fully assimilated by plants. Concerning the interaction between C and N cycling, Van Groenigen *et al*. (2006) stated that increases in soil OM levels are restricted when soil nitrogen supply is limiting. This mechanism can be explained by plant-microbe competition for nutrients that can accelerate soil OM breakdown and consequently soil

degradation (Barron-Gafford *et al*., 2005).

Soil organic matter degradation is commonly reported the Mediterranean region, where the climatological and lithological conditions, together with anthropogenic activities, (Bastida *et al*., 2006; García-Orenes *et al*., 2012; Lazcano *et al*., 2013; Lopez Bermudez and Albaladejo, 1990) is recognized to be responsible of a loss in soil quality. Many author pinpointed that soil degradation fundamentally refers to a loss in terms of soil quality (Dick and Tabatabai, 1993; Trasar-Cepeda *et al*., 1998; Ros *et al*., 2003).

6. Heavy metal pollution in soils

Heavy metals content in soils is determined by measuring total elementary content of metals and by describing path and mechanisms of their transport. Heavy metal transport in soil is an issue of great concern because xenobiotics are absorbed in soils and delivered to groundwater. Thus, toxicity of heavy metals can be assessed by estimating the mobile fraction, the readily soluble fraction, the exchangeable fraction, or the plant available fraction of the heavy metal content of a soil. Hence, these diverse fractions can be directly used as indicators of toxic effects on soils and water ecosystems (Sharma *et al*., 2009). Furthermore, according to their origin, heavy metals show different behaviour in soil: metals from anthropogenic sources generally have a greater mobility in soil if compared to those from natural origin where the metals are strongly associated with soil components (Baize, 1997).

In assessing metal bioavailability, total concentration of the pollutant is not a reliable parameter because it does not reflect the actual metal concentration to which soil microbiota is exposed. Xenobiotics bioavailability depends on diverse soil properties, such as texture and pH. A few studies have been performed, and they lack in considering a broad range of pollutant concentrations for a proper definition of dose-response mechanism. Furthermore, do not employ standard methods, making them not comparable each other. In light of these considerations, it seems difficult to identify key factors affecting the expression of toxicity or to identify thresholds of toxicity (Giller *et al*., 2009). To overcome these drawbacks, investigation on zinc, copper, nickel and cobalt effects on microorganisms, invertebrates and plants using a wide range of soils

and dose-responses, has been performed in many studies. These research efforts provide a deep soil characterization, including the soluble metal concentrations in the soil solution, and standard methods for the bioassays (Smolders *et al*., 2004; Rooney *et al*., 2007; Criel *et al*., 2008; Li *et al*., 2009).

The parameter which shows the strongest correlation with heavy metal pollution is the cation exchange capacity (CEC). Cation exchange capacity is a function of pH, clay fraction and soil organic matter, which are key factors in metal bioavailability. Furthermore, it explains most of the variation in toxicity thresholds based on total metal concentrations between soils. In general, toxicity decreased linearly with increasing CEC. Weakness in considering CEC as a general toxicity indicator relies on the fact that plants and invertebrates show this correlation more consistently than microbes (Smolders *et al*., 2009). This evidence confirms the difficulty in predicting toxicity to soil microbes, since they seem to function in a diverse way than other organisms. On the contrary, it is commonly accepted that microbiota show a greater sensitivity to heavy metal stress than animals or plants living within the same soils (Giller *et al*., 2009). Heavy metals predominantly tested are zinc, copper, nichel and cobalt and obtained results are referred within standard experimental setting to freshly amended and field-aged soils. Studies have been performed using different plant and invertebrate tests and microbial processes such as nitrification and carbon substrate decomposition. Different types of organisms show different grades of sensitivity and within microbes some species are relatively sensitive to metals (such as rhizobia; Chaudri *et al*., 2008) and some insensitive (nitrifiers and organisms involved in degradation of carbon compounds; Mertens *et al*., 2006).

Many authors observed that metal pollution affect in a negative way establishment and growth of soil microbial biomass (Brookes and McGrath, 1984; Barajas-Aceves, 2005). Furthermore, decrease in the total amount of biomass depends on a decrease in the substrate utilisation efficiency: this phenomenon is due to a higher energy cost of microorganisms subject to metal stress (Chander and Joergensen, 2001; Chander *et al*., 2002). Even though the amount of microbial biomass may be a sensitive indicator of metal stress, its suitability in environmental monitoring as an indicator of soil pollution is limited because of its high spatial variability and shortcomings in its measurement (Broos *et al*., 2007).

Controversial evidencies seem to characterize interactions between heavy metal and soil: acute toxicity or disturbance (short-term assays) affect microbial response in a divergent way if compared to chronic toxicity or stress phenomena (long-term assays). Thus, in order to deeper understand the mechanisms behind soil resilience to heavy metal toxicity, it seems to be more appropriate to solely rely on long-term field experiments (Giller *et al*., 2009). In this kind of approach, it is important to pinpoint that native soil microbes are probably well-adapted to the concentration of pollutants (McLaughlin and Smolders, 2001), since they have had enough time to adapt genotype over several years. The above-described scenario consists in a long-term contaminated crop where metals reached an equilibrium within the soil solid fraction. Along these lines, it is possible to assert that metal resistant or tolerant species can occur by adaptation or selection. Baath *et al*. (1991) found a slight decrease of qCO_2 with increased heavy metals: the qCO_2 mean value was 3.6 for slightly polluted soils (~400 pg Cu g^{-1}), and only 3.2 for heavily polluted soils (approx. 10.000 pg Cu g^{-1}). Brookes and McGrath (1984) found a higher qCO_2 on

polluted as compared to uncontaminated soils. Chander and Brookes (1991) and Bardgett and Saggar (1994) reported a doubling of qCO_2 upon heavy metal contamination through sewage sludge amendment, and Ortiz and Alcaniz (1993) found an elevated qCO_2 after sewage sludge amendment which partially may be attributed to heavy metals. This contrasting results suggested that heavy metals affect differently the metabolic quotient according to soil pH, texture and organic matter content.

6.1. Copper pollution in soil

Copper is a highly reactive metal, which results in large quantities in industrial processes discharge. An important source of copper as pollutant of soils and water comes from agricultural practices, in particular from pesticides and fungicides. Copper contamination exerts effects both to soil biota and plants, depending on parent material, soil physio-chemical properties and aboveground plants. Copper mobility and availability depend on soil pH: at pH values below 6 its solubility increases and thus it becomes readily available; at pH values above ~7.5 the solubilisation of SOM and subsequent formation of Cu-SOM complexes foster copper migration in soil (Komárek *et al*., 2010). However, copper toxicity is more severe in acid soils, in particular if they exhibit a low cation exchange capacity (Gupta and Aten, 1993). Organic matter, iron, manganese oxides and hydroxides complex with copper to a greater degree, in comparison to clay minerals (specifically and non-specifically adsorbed). Clay minerals, organo-clay association and particulate organic matter (POM) are the predominant carrier phases of copper in soils (Besnard *et al*., 2001). Copper concentrations in contaminated soils are reported to be higher in proximity of the vine plants and in

the upper layers where they can reach values above 200 mg Cu kg^{-1}. In growing area where Bordeaux mixture has been intensively applied, copper concentrations above 1000 mg kg^{-1} have been reported (Flores-Vélez *et al*., 1996). Superficial localization of copper is due to strongly immobilization exerted by SOM and Fe-, Mn- (hydr)oxides (Flores-Vélez *et al*., 1996; Brun *et al*., 1998, 2001; Narimanidze and Brückner, 1999; Pietrzak and McPhail, 2004).

Sorption and complexation properties determine the great mobility of this metal in soils (Parat *et al*., 2002; Bradl, 2004; Fernández-Calviño *et al*., 2009b). The main sinks of copper are soils rich in Fe-(hydr)oxides and organic matter, especially humic and fulvic acids. Soil organic matter exerts one of the most effective retention mechanism for Cu in soils by coordinating with carboxyl or amine ligands (Strawn and Baker, 2008, 2009). Copper complexated with SOM shows a lower toxicity compared to free Cu^{2+} (Karlsson *et al*., 2006) and it makes organic matter less vulnerable to biodegradation (Parat *et al*., 2002). Organic matter affects Cu mobility in two different ways: particulate organic matter adsorbs Cu^{2+}, while soluble SOM complex Cu^{2+}, and consequently increases its solubility, especially at pH values above ~7.5 (Arias *et al*., 2006; Fernández-Calviño *et al*., 2008a; Martínez-Villegas and Martínez, 2008). According to soil texture, the formation of soluble organic complexes reduces the lability of Cu but under field conditions, it could enhance its mobility through the soil, especially in soils rich in coarse light organic fraction, in fine clay fraction and in sandy soils (Martínez and McBride, 1999; Martínez *et al*., 2003).

Carbonates can also affect copper activity: in calcareous soils Cu mobility depends largely by the surface precipitation of $CuCO_3$. This mechanism is typical

of alkaline soils containing high concentrations of carbonates, commonly observed in many vineyards, and lead to the release of Ca^{2+}, Mg^{2+}, Na^{+} and H^{+} into the soil solution (Ponizovsky *et al*., 2007). As result, newly-formed Cu phases ($Cu(OH)_2$, $CuCO_3$/$Cu_2(OH)_2CO_3$, CuO, sulfates (e.g., from the Bordeaux mixture) precipitate in the soil (Ponizovsky *et al*., 2007; Komárek *et al*., 2009b; Strawn and Baker, 2009). Copper sulfates, such as $CuSO_4$ (chalcocyanite) and $CuSO_4 \cdot 5H_2O$ (chalcanthite), are highly soluble and require very high Cu concentrations to form in soils (Lindsay, 1979). Amounts of Cu applied as agricultural practice does not depend on the level of carbonates, consequently the fractions of Cu in the soil, and the percentage of inactive Cu, indicating that $CaCO_3$ does not affect soil health, and neither the capability of soils to sequestrate Cu. Cu toxicity, even in calcareous soils, seems to be more related to soil texture (Toselli *et al*., 2009).

Total Cu concentrations ranging between 100 and 150 mg kg^{-1} represents the threshold above which copper can negatively influence plant growth and soil functioning (Kabata-Pendias and Pendias, 2001; Ippolito *et al*., 2010). Since Cu occurs in different forms in soils and its general low bioavailability to plants and microorganisms, Cu toxicity is at great extent site-specific (Adriano, 1986). Furthermore it is worthy to remind that soil organic matter and pH affect Cu immobilization, as previously mentioned.

Tillage, erosion and xenobiotics' contamination is known to provoke a decline in soil fertility, which in turn make soil to be easily affected by contamination in a closed-loop process. Vineyard soils are usually susceptible to contamination because of their weakness in terms of functionality: this feature is depending on soil degradation with respect to biochemical properties (Miguéns *et al*., 2007). During the last few decades, in Europe some areas devoted to

vineyards have been abandoned: this land use change caused soil erosion and subsequent dispersion of pollutants into diverse ecosystems (Nóvoa-Muñoz *et al*., 2007a; Fernández-Calviño *et al*., 2008a). According to EC regulation 473/2002, Cu-based fungicides are allowed in prescribed doses (8 kg Cu ha^{-1} which should be further decreased to 6 kg Cu ha^{-1} after four years of vine cultivation). Among the permitted compounds there are $Cu(OH)_2$, $3Cu(OH)_2 \cdot CuCl_2$, $CuSO_4$ and Cu_2O, which are recognized as indispensable in vine deseases control. Instead, synthetic organic fungicides are banned because the concern their residues have in environmental and human health. Cu-based fungicides have also been extensively used in hop fields (Schramel *et al*., 2000; Komárek *et al*., 2009a), coffee (Loland and Singh, 2004), apple (Li *et al*., 2005), avocado orchards (Van Zwieten *et al*., 2004) and during the cultivation of several vegetables (Adriano, 2001). Considering areas in the world where copper contamination is more severe, it is noticeably that it is linked to higher pesticide doses applied in more humid regions due to the more intense development of downy mildew (Komárek *et al*., 2010). Even though Cu-based pesticides have been widely use in agriculture, the main application of these products is onto vineyards. Desease control applied to vines implicates pollution not only by copper, but also by zinc (from fungicides, manure and compost applications; Weingerl and Kerin, 2000; Ramos, 2006), arsenic (from herbicides, insecticides or geological background, Villaescusa and Bollinger, 2008; Nóvoa-Muñoz *et al*., 2007b), lead (from atmospheric depositions and insecticide application; Mihaljevič *et al*., 2006; Komárek *et al*., 2008; Frank *et al*., 1976) and cadmium (from phosphate fertilizers; Komárek *et al*., 2008).

Concerning the impact of copper on European agrosystems, there is still

under debate the thresholds to be adopted, especially how those limits have to be adapt according to the climatic conditions and main crops in different European agricultural areas, primarily in Italy and Spain. Organic management of grape and tomato can not exclude Cu-based fungicide to protect the crops from downy mildew. Several factors cause Cu-accumulation in the upper layers of those cultivated soils: long-time applications, non-biodegradability of copper compounds, long biological half-life of copper and its low mobility in soils. Copper contamination of soils has been reported in traditional European wine-producing countries such as Portugal (Magalhaes et al., 1985), Italy (Deluisa et al., 1996), France (Brunet al., 1998), Spain (Fernandez-Calvino et al., 2010) and Serbia (Ninkov et al., 2014).

Since viticulture has represented an important agricultural practice in many countries, in order to sustain production, long-term use of organic and inorganic pesticides has been required. As consequence, it caused increasing concentrations of their residual compounds in the environment (Komárek *et al*., 2010). Copper compounds are commonly used as fungicides and they have been broadly used in vineyards to the extent of controlling vine (*Vitis vinifera* L.) fungal diseases, such as vine downy mildew. *Plasmopara viticola*, the pathogenic agent of vine downy mildew, is a fungus which started spreading across Europe at the end of 1800. Bordeaux mixture ($Ca(OH_2)$ + $CuSO_4$) has been usually applied onto vineyards in vine downy mildew control, and, additionally, other copper compounds have been used in the last decades ($3Cu(OH)_2 \cdot CuCl_2$; $CuSO_4 \cdot 3Cu(OH)_2$, Cu_2O, $Cu(OH)_2$). Long-term application and consequent wash-off from plant surface determined severe copper accumulation in vineyard soils. According many authors, copper content in the upper layers of arable land soils

ranges from 5 to 30 mg kg^{-1}, while in vineyard soils it can reach between 200 to 500 mg kg^{-1} (Geoffrion, 1975; Deluisa *et al*., 1996; Brun *et al.*, 1998).

Copper contamination affects microbial communities in vineyard soils both in numbers and in variability (Dumestre et al., 1999; Díaz-Raviña et al., 2007; Dell'Amico et al., 2008; Lejon et al., 2008). Mechanisms subtended to changes in microbial activities and structure have to be deeply clarified: changes in microbial functionality seems to diminish mineralization rates of organic xenobiotics such as pesticides. Moreover, Cu pollution of soil seems to be quite heterogeneous, mainly depending on micro-areas into the soil where organic matter accumulates and thus adsorbs copper cations. Presence of hotspots has been corroborated by evidences elucidating the capability of microorganisms to avoid copper accumulations in soils or that they are preferably colonized by metal-tolerant species (Yamamoto et al., 1985; Jacobson et al., 2007). Even though soil organic matter exerts controversial effects on copper mobility, it seems to moderate Cu toxicity to microorganisms (Lejon et al., 2008). As well as others heavy metals in soil, changes in microbial and enzymatic activities largely depends on textural features since there is an inverse relationship between colloids in soil and severity of heavy metals contamination. Cu-contamination can cause a shift in bacterial communities inhabiting the top soil layer (Dell'Amico *et al*., 2008; Lejon *et al*., 2010). Conversely, in long-term experiments on Cu-polluted soils, demonstrated that pH and soil management exert a greater effect on the composition of bacterial communities than those exerted by copper content (de Boer *et al*., 2012; Mackie *et al*., 2013; Fernandez-Calvino *et al*., 2010).

Copper is reported to both stimulate and depress soil enzymatic

activities. Dehydrogenases activity, indirect indicator of the activity of the microorganisms in the soil, is generally depressed by high concentration of Cu in soil. The same effect is exerted on urease activity, which is reported to be decreased by copper contamination. While dehydrogenases and ureases are inactivated by copper, the same studies pinpoint that alkaline phosphatase and acid phosphatase are less affected by Cu contamination (Wyszkowska *et al*., 2003; Wyszkowska *et al*., 2005). The response of these enzymes is, however, depending on copper concentrations applied, and none of the concentrations inhibits acid and alkaline phosphatase more than 50% (Wyszkowska *et al*., 2005). However, this study underlines that the extent of the negative effect of soil Cu contamination depends, among other factors, on the type of soil and on soil management.

7. Microbial diversity and functioning

Since a wide range of microorganisms is involved in critical soil functions, microbial diversity in soil plays a crucial role in the maintenance of soil health and quality. It is still under debate the linkage between soil taxonomic diversity and soil functional features, in particular if species diversity could be related to functional efficiency. Functional redundancy is a predominant attribute in microbial communities, but there are not evidences if a peculiar functional ability is carried out by a taxonomic group/species in a more efficient way than another (Nannipieri *et al*., 2003).

Plant type, soil type, and soil management regime affect soil microbial diversity and inter-relationships among these factors, in turn, affect soil health. Plant type and soil type are the two main drivers of soil microbial community structure by means of complex mechanisms (Garbeva *et al*., 2004). Soil microbial communities are often difficult to fully characterize, because of their huge phenotypic and genotypic diversity, heterogeneity, and crypticity. Bacterial populations in soil top layers can go up to more than 109 cells per g soil, of which less than 5% have been studied (Torsvik *et al*., 1990). Direct DNA-based methods can help in overcoming this limitation. In microbial terms, “biodiversity” describes the number of different species and their relative abundance in a given community in a given habitat. In molecular-ecological terms, “biodiversity” can be defined as the number and distribution of different sequence types present in the DNA extracted from the community in the habitat. The term “community structure” implies that information is included on the numbers of individuals of different recognizable taxa. Even though these terms are often used interchangeably in

soil ecology, the concept of microbial diversity indicates the number of species present and the evenness of their distribution. A habitat with a larger number of species is more diverse, whereas an evenly distributed community is more diverse than an unevenly distributed community with the same number of species (Hedrick *et al*., 2000).

7.1. Assessment of soil microbial diversity

Six methods have been developed for the evaluation of biodiversity in soil: cultivation-based methods; cultivation-independent methods; clone libraries; microbial community fingerprinting techniques; metabolically active communities analysis; polyphasic approach (Garbeva *et al*., 2004).

Cultivation and isolation are traditional methods used for recovering diverse microbial groups. Since culture media favor copiotrophic organisms, these methods are not able to represent *in situ* functional diversity in a microbial community. Moreover, only a small fraction of the microbial cells in soil are accessible to study. Cultivation-independent methods are molecular techniques based on PCR or RT-PCR of specific or generic targets in soil DNA or RNA. The 16S and 18S ribosomal RNA (rRNA) or their genes (rDNA) represent useful ecological markers for prokaryotes and eukaryotes, respectively. Clone libraries allow to identify and characterize the dominant bacterial or fungal types in soil. In order to obtain reliable results, this method has to be integrated with rarefaction analysis, calculation of coverage values, or other statistical techniques. Microbial community fingerprinting techniques include denaturing or temperature gradient gel electrophoresis (DGGE/TGGE), amplified rDNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP), single-strand

conformational polymorphism (SSCP), and ribosomal intergenic spacer length polymorphism (RISA). PCR-based methods are demonstrated to be susceptible to potential biases due to nucleic acid extractions and PCR amplifications. Metabolically active communities can be investigating using rRNA-based methods. The polyphasic approach integrates culture-dependent techniques and culture-independent methods.

7.2. Soil microbial diversity and suppressiveness to plant pathogens

As previously elucidated, microbial communities in soil are affected by several factors, among of them three seem to impact the most:

- Plant type, since they are the main suppliers of energy sources;
- Soil type, since it determines the habitable niches in soil, thanks to soil structure and texture;
- Agricultural management regime.

Pathogen- or disease-suppressive soils limit the survival or the growth of the pathogens. General suppression often reduces fungal and nematode attacks, whereas specific suppression is often effective against only one or a few pathogens. In specific disease suppression, the monoculture of one crop, can lead to an initial increase in disease followed by a spontaneous decline in disease. Long-standing suppression is a biological condition naturally associated with the soil; while induced suppression is initiated and sustained by crop monoculture or by the addition of inoculum of target pathogen. The mechanisms underlying soil suppressiveness are not always clear, and involve both biotic and

abiotic factors. Nevertheless several studies indicated that mechanisms within the microbial activity of soil are responsible for the suppression of pathogens. Just to give an example, *Pseudomonas*, *Burkholderia*, *Bacillus*, *Actinomycetes* (among bacteria) and *Trichoderma*, *Penicillium*, *Gliocladium*, *Sporidesmium*, nonpathogenic *Fusarium* spp. (among fungi) have been commonly identified as microbial genera antagonists of soilborne plant pathogens. The mechanisms by which these microorganisms make soil suppressive can be the microbial activity or soil respiration (competition mechanism) (Hoitink and Boehm, 1999; Van Os and Van Ginkel, 2001), microbial community diversity and composition (Garbeva *et al*., 2006; Perez- Piqueres *et al*., 2006), population size of certain microbial groups like Actinomycetes or oligotrophic bacteria (Workneh and van Bruggen, 1994; Tuitert *et al*., 1998); presence of antibiotic genes correlated with soil suppressiveness (Raaijmakers and Weller, 1998; Garbeva *et al*., 2006). Suppressive soils have also been the source for several antagonistic microorganisms (non-pathogenic *F. oxysporum* (Alabouvette *et al*., 1979), *Verticillium biguttatum* (Jager *et al*., 1979), *Pythium nunn* (Lifshitz *et al*., 1984), and *Pseudomonas* spp. (Raaijmakers and Weller, 1998). However, in many pathosystems, the relevant mechanism behind soil suppressiveness is not yet understood. In most cases, one soil characteristic correlates positive as well as negative with suppressiveness, depending on the pathogen and the agroecosystem involved. As a consequence, the agricultural practices enhancing soil suppressiveness are hard to determine. Predicting the precise effects of the agricultural practices on suppressiveness for each disease and soil type is still hard to determine (Janvier *et al*., 2007).

There is a huge amount of studies dealing with this issue (not reviewed in

this work), revealing common findings: there is a positive correlation between microbial diversity and the disease-suppressive capacity of soil; and a broad knowledge about microbial communities in soils is an essential requirement in understanding the soil suppressiveness. Knowledge concerning the soil characteristics influencing soil suppressiveness towards specific diseases is still lacking. Van Bruggen and Semenov (2000) suggested that the microbial community structure and its resilience after application of various disturbances or stresses could be the main mechanism driving the disease suppression in soil. Soil health can be influenced by cropping and management practice: cover crops, compost application, tillage, crop rotation. Effective agricultural practices result in the lack of positive selection of the pathogen and provides time needed for the biological destination of pathogen inoculum by antagonists residing in soil.

8. Ecological restoration of a heavy Cu-polluted agrosystem: Podere Pantaleone case study

8.1. Introduction

The intensive agricultural management which characterizes Po valley (in which the study site is located) leads to a status of soil degradation well reported (Costantini *et al*., 2013). Soil degradation can be defined as the process leading to the loss of the self-organization ability of the soil constituents, due to either natural causes or human activities. Self-organization process means that soil tends to reach a state of equilibrium characterized by minimized entropy (Addiscott, 2010; Targulian and Krasilnikov, 2007). Organisation for Economic Co-operation and Development (OECD) proposed to define soil degradation as a process concerning the loss of soil functions and referring to the processes by which soil does not properly fit for a specific purpose. Several factors could negatively affect soil function and be recognized as degradation processes: i) mechanical erosion; ii) decline in organic matter; iii) contamination; iv) sealing; v) compaction; vi) soil biodiversity loss; vii) salinization; viii) floods and landslides; ix) desertification; x) acidification (European Commission, 2006a; European Commission, 2012). Degradation processes investigated in the present work concern organic carbon depletion, and contamination in the form of high nitrate content and a heavy-metals (especially copper).

In the proposed case study, soil of an abandoned and uncultivated for 35 years vineyard, was studied in order to assess the effects exerted by high Cu-contamination on biochemical and microbiological properties. Long-term

abandonment triggered a process of ecological restoration which transforms it into a protected area for an *in situ* preservation of indigenous plant and animal species, located in the middle of an intensive fruit tree growing area. Soil quality assessment was conducted identifying suitable indicators and it was based on previous studies conducted on Po Valley and on the study site. The experiment was set up in an intensive agricultural system, within a conventionally managed area vocated to fruit farming. The experimental design it was organized into a two-factorial nested design to analyze the impact of management (conventional *vs* re-naturalized orchard) and position within each orchard (tree-rows and strips). Chemical and biochemical properties along with bacterial and fungal communities, evaluated with PCR- DGGE starting from total soil DNA, were analyzed.

8.1.2. Preliminary survey

The study site is located in the Po valley in Northern Italy, a productive agricultural area in Italy. The western and central part of Po valley is characterized by a sub-continental climate which fosters arable crops and forage production; the eastern part shows Mediterranean climate and it is traditionally cultivated with fruit tree crops and fresh vegetable. Topsoil in the Po valley shows a good level of macroporosity (enhanced by cultivation and accumulation of organic matter); the horizons from 30 to 70 cm in depth are slightly rubified and gradually pass to the fine sandy fluvial parent material. The aquifer level is around approximately one meter from the surface. The millennial physical, biological, and chemical transformation of the unweathered sand into soil has fostered ES, especially those related to organic matter content.

Thanks to its effectiveness and low cost, inorganic copper is used as a broad-spectrum fungicide and bactericide in several crops; intensive and protracted application of copper compounds caused its accumulation in soil (please refer to paragraph 4.3.). In Italy, soil total Cu concentration was reported to range between 2 and 375 mg/kg (Mantovi *et al*., 2003). The potential risk of soil contamination with Cu prompted the European Union to establish a threshold in copper application rate to fruit trees, which is 100 mg kg^{-1} (Commission Regulation N° 473/2002). In Emilia Romagna region, copper sprays are widely used to treat pear orchards (*Pyrus communis* L.), vegetable crops, and grapevine (*Vitis vinifera* L.). As a consequence a higher copper accumulation in pear orchard and vineyard soils was expected (Toselli *et al*., 2009). The preliminary survey on chemical properties of the study site, revealed that copper content in soil samples taken from the re-naturalized subsite was largely above the normed threshold.

8.2. Material and methods

8.2.1. Study site

The Pantaleone property, located in the Municipality of Bagnacavallo, Ravenna Province, Italy (11°58'E, 44°25'N; 6-11 m asl) was a family-run farm up to the 1950s, counting a surface of approximately 7 ha. The Pantaleone property included a vineyard, managed according to the traditional technique called "married vine", where one woody plant acted as a support for one or two vines spaced at 3 m on the planted row. In order to control downy mildew (*Plasmopara viticola*) the Bordeau mixture ($Ca(OH)_2$+$CuSO_4$) was applied, while sulfur was used for inhibit powdery mildew (*Uncinula necator*). Until the '80, when the farm

was totally abandoned, Podere Pantaleone began its ecological restoration: in 1989 it became a protected area in 1989 and then, in 2006, was included in the Natura 2000 European ecological network of protected areas, as a 'Site of Community Importance' in the Mediterranean biogeographical region (http://www.poderepantaleone.it). Abandoned vineyard evolved into a forest system along time, while strips were about 4 m wide with a permanent vegetative ground cover.

The soil was sampled in the re-naturalized Pantaleone property (RE-NAT) and in a nearby cultivated orchard, representative of the conventional soil management (CONV) in that area. The two adjacent sites had similar soil origin and vegetation; indeed, both were tree crops with tree-rows alternated to strips with vegetation cover. The cultivated peach orchard was conventionally managed: permanent vegetation cover on strips and periodical tillage for controlling weeds on the tree-rows and chemical control of pests and foliar diseases. Both soils (conventional and re-naturalized) are classified as Calcaric Cambisol according to the World Reference Base for soil resources (FAO, 2015). Soil texture was silty-loam according to USDA texture triangle. Soil sampling was performed in three points, randomly chosen, for each treatment at a depth of 20 cm. Samples on rows were collected under a canopy of three trees, while those of strips were collected in the middle of corresponding inter-rows. Each sample was divided into several subsamples to be handled and stored according to technical requirements of relevant analysis.

8.2.1. Chemical analysis

For each replicate, soil was air dried, milled, and sieved at 2 mm before

chemical analysis. Texture was determined by pipet method (Sheldrick and Wang, 1993). Chemical properties were determined according to SSSA methods (Sparks, 1996). Actual and current pH was determined in water and 1M KCl (1:2.5, m:v), respectively; total carbonates were determined using a volumetric calcimeter; total organic carbon (TOC) was determined by dichromate oxidation with external heat; total nitrogen with the Kjeldahl method; available phosphorous was extracted with 0.5 M sodium bicarbonate at pH 8.5, and determined with ascorbic acid method (Olsen method); total sulfur and metals were extracted with aqua regia and hydrogen peroxide solution using a microwave oven, and determined by atomic emission spectroscopy inductively coupled plasma (EOS-ICP). Bioavailable metals were extracted with DTPA solution in agreement with Lindsay and Norwell (1978) and determined by atomic emission spectroscopy inductively coupled plasma (EOS- ICP). Water extractable (1:10, mass-to-volume, for 30 min at room temperature) total phenols were measured by Folin-Ciocalteu method (Oliver *et al*., 1951). Humic substances (HS) were extracted with 0.1 M sodium hydroxide + 0.1 M sodium pyrophosphate solution (Na + PP), purified and quantified in agreement with Ciavatta and Govi (1993). Separation of humified (humic acids, HA, and fulvic acids, FA) from non-humified compounds (NH) was performed on solid polyvinylpyrrolidone according to Ciavatta *et al*. (1990). Three parameters of humification (humification index - HI; degree of humification - DH%; hunification rate – HR%) were calculated as follows:

$$HI = \frac{NH}{(HA + FA)} \qquad DH\% = \frac{HA + FA}{TEC} * 100 \qquad HR\% = \frac{HA + FA}{TOC} * 100$$

Copper content was determined in Na + PP, and water (1:10, mass-to-volume, for 30 min at room temperature) extracts by EOS-ICP.

8.2.2. Biochemical analysis

Biochemical analysis were carried out on subsamples of fresh soil, sieved at 2 mm. This set of analysis allow the determination of the relevant ecophysiological parameters and enzymatic activities. Soil microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) were determined using the fumigation-extraction method proposed by Vance *et al*. (1987). Fumigated and non-fumigated soil samples were extracted with 0.5 M K_2SO_4, and analyzed for organic C and total N using an elemental analyzer (OC-VCPH/CPN, Shimadzu, Kyoto). C and N measured in non-fumigated soil extracts was used as assessment of labile C (C_{ext}) and N (N_{ext}) pools. Soil basal respiration rate (SBR) was measured in agreement with ISO 16072:2002 method. An aliquot of moist soil, equivalent to 20 g of air dried soil, was weighted into airtight glass jars, and the water content was adjusted to 70% of water holding capacity (WHC). The samples were incubated at 25° C for 4 weeks, and the evolved CO_2 was trapped in plastic vials containing 1.5 ml of 0.05 M KOH and measured at regular intervals during the incubation. Trapped CO_2 was quantified by titration after precipitation of carbonate with 1.5 ml of 0.75 M $BaCl_2$ using 0.005 M HNO_3. The CO_2 evolution of the 28st day was used as a measurement of the basal respiration. The metabolic quotient (qCO_2) was calculated as the ratio between SBR and C_{mic} (Anderson and Domsch, 1990).

Soil enzyme activities were assayed on triplicate samples using colorimetric methods in agreement with SSSA methods (Dick, 2011). Dehydrogenase activity was assessed as described by Von Mersi and Schinner (1991), β-glucosidase activity with the method based on para-nitrophenol assay

suggested by Eivazi and Tabatabai (1988), acid and alkaline phospho-monoesterase activity as described in Eivazi and Tabatabai (1977), protease activity with the method by Ladd and Butler (1972), urease activity according to Kandeler and Gerber (1988), and catechol oxidase activity as described by Perucci *et al*. (2000). Total microbial activity was also indirectly assessed by fluorescein diacetate hydrolysis method proposed by Schnürer and Rosswall (1982).

8.2.3. Soil microbial community

From each of replicate, a subsample of 25 g of soil was air dried at room temperature for 12 h and stored at 80°C. Total DNA was extracted from a 0.50 g of soil using a commercial kit (Power Soil DNA kit, MoBio Laboratories, Inc. Carlsbad, CA, USA) according to manufacturer's instructions.

A PCR was performed in order to amplify bacterial 16S rRNA gene. The set of primers 63f (5'CAGGCCTAACACATGCAAGTC3', forward) and 518r, (5'ATTACCGCGGCTGCTGG3', reverse), give an amplified fragment of 495 bp and was chosen because of its suitability in ecological and systematic studies (El Fantroussi *et al*., 1999). Subsequent DGGE analysis was performed inserting a 40-nucleotide GC clamp to the 5' end of the forward primer, in order to prevent amplicons' denaturation (Muyzer *et al*., 1993). The PCR mixture was prepared using 1x buffer (20 mM Tris pH 8.4, 50 mM KCl), 2.0 mM $MgCl_2$, 200 mM of each dNTP, 1.25 U of Taq polymerase, and 10 pmol of each primer (Taq polymerase kit; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 100 ng of total DNA and sterile water to a final volume of 50 ml. Samples were amplified as follows: 94°C for 3 min; 94°C for 45 s; 60°C for 30 s; 72°C for 1 min (20 cycles); 94°C for

45 s; 55°C for 30 s; 72°C for 1 min (10 cycles), a final extension at 72°C for 5 min (TGradient Thermal Cycler; Biometra GmbH, Goettingen, Germany). DGGE was performed as described by Muyzer *et al*. (1993), using a D-code system (Bio-Rad Laboratories, Hercules, CA, USA). PCR samples (200-250 ng) were loaded on 6% polyacrylamide gels in a 1x TAE buffer with a denaturation gradient ranging from 40% to 80%. Electrophoresis was run at a constant voltage 75 V at 60°C. The DGGE analysis was repeated three times to confirm the pattern. After electrophoresic run for 16 h, the gel was stained with GelRed™(Biotium Inc, Hayward, CA, USA) at 10,000x dilution in 1x TAE for 30 min, washed in water for 20 min, and photographed using an Alpha Image UV illuminator (Alpha Innotech, San Leandro, CA, USA).

In order to describe fungal communities, a nested PCR amplification was performed in order to reduce non-specific binding in products (Manici and Caputo, 2010), using the two primer sets ITS1F/ITS4 (White *et al*., 1990; Gardes and Bruns, 1993) and ITS1F-GC/ITS2 (Anderson and Cairney, 2004). For Ascomycetes detection, primers ITS1F/ITS4 A (Larena *et al*., 1999) and ITS1F-GC/ITS2 were chosen; for Basidiomycetes detection, ITS1F/ITS4 B (Gardes, and Bruns, 1993) and ITS1F-GC/ITS2 primers were used. PCR reactions were performed with 100 ng of total soil DNA. The first amplification was performed with ITS1F-ITS4, the PCR mixture used contained 1x buffer (20 mM Tris pH 8.4, 50mM KCl), 1.7 mM $MgCl_2$, 200 mM of each dNTP, 2.5 U of Taq polymerase and 10 pmol of each primer (Taq polymerase kit; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and sterile water to a final volume of 50 ml. Samples were amplified as follows: 94°C for 3 min, 94°C for 30 s; 55°C for 30 s; 72°C for 30 min (35 cycles), and a final extension at 72°C for 10 min (TGradient Thermal Cycler;

Biometra GmbH, Goettingen, Germany). Amplicons were diluted 1:10 in sterile water and 1 ml of each dilution used as a template for the subsequent amplification with primer ITS1F-CG/ITS2 as described above. DGGE was performed as described previously for bacteria with some modification: PCR samples (200-250 ng) were loaded on 8% polyacrylamide gels in a 1x TAE buffer with a denaturation gradient ranging from 20% to 50%, and electrophoresis was run at a constant voltage 50 V at 60°C for 16 h. Relative quantification of Ascomycota and Basidimycota was performed using total soil DNA extracted and PCR with ITS1F-ITS4A and ITS1F-ITS4B were performed as above. Relative quantification of the PCR products was performed with electrophoresis by loading 10 ml for each sample and using Low mass DNA Ladder (Invitrogen) as reference.

8.2.5. Data analysis

All results from chemical and biochemical analysis were reported on dried soil basis. Analysis of variance was used to compare chemical and biochemical parameters between land management and sampling position, accounting the non-independence of management and position. Parametric ANOVA assumptions were verified through Bartlett's test for homogeneity of variances and Shapiro-Wilks test for normality of distributions. If necessary the data were transformed following the Box-Cox procedure. Parametric data were analyzed using the program R software (R Core Team, 2015). All mean separation tests were performed using Tukey HSD test at $P<0.05$. Simple linear correlation analysis was performed in order to measure the degree of association between parameters. The DGGE images of microbial communities were

processed with Gel Compar II software (Applied Maths, Belgium), removing background noise with a minimum profiling greater than 0.5%. Data inferred from DGGE fingerprinting were organized in a presence/absence data matrices and processed to compare diversity and composition using the PAST program software for data analysis in paleoecology (Hammer *et al*., 2001). Diversity of each treatment (represented by three replicates each) was estimated with Chao 2 and Jackknife diversity indexes. Two-way non-parametric multivariate analysis of variance (NP-MANOVA) using Euclidean distance was applied to the presence/absence data matrix of bacterial and fungal communities. Euclidean distance was adopted also for Analysis of similarities test (ANOSIM test) and to Canonical Correspondence Analysis (CCA).

8.3. Results

8.3.1. Chemical properties

Investigated soils are classificated as sub-alkaline, showing pH values of 7.65 in CONV and 7.39 in RE-NAT plots. Sampling position did not differ as regards pH, on the contrary a significant difference occurred between managements (Tab. 1). Total carbonates were significantly different both in management and in sampling position, showing the highest carbonate value in CONV (Tab. 1).

Total organic carbon (TOC) content, which was higher in RE-NAT samples compared with cultivated (CONV) soils in rows and stripes, while rows of re-naturalized (RE-NAT) were characterized by TOC of 41% higher than stripes (Tab. 1). Total carbonates and humic substances (HS) content showed a response to soil management consistent with that observed for TOC. Total

nitrogen (TN) showed three times higher values in RE-NAT than in CONV soils (Tab. 1) and C:N ratio was slightly, but significantly, higher in re-naturalized than conventionally managed soils (Tab. 1). Contrary to available P, total S was largely higher in RE-NAT (460 mg kg^{-1}) than in conventionally managed soil (260 mg kg^{-1}), without any significant difference between sampling sites. Total copper (Cu) and lead (Pb) differed significantly between both soil management and sampling site (Tab. 1). Total Cu was much higher in re-naturalized than orchard soil, mainly due to a high amount of copper localized in the rows of the old vineyard, which amounted to 994 mg kg^{-1} compared to 61 mg kg^{-1} in strips. The total copper concentration was consistent with those of DTPA-extractable soil Cu. In RE-NAT strips, CONV rows and CONV strips, copper content did not differ significantly. Similar to Cu, total soil Pb was higher in RE-NAT soils than CONV soils, and it was higher in rows than in strips. The lead (Pb) content in organic matter, and water extractable fractions fractions were not reported because lower than instrumental detection limit.

Table 1. Means of main chemical parameters determined in the soil under different types of land management and sampling position in orchard.

	pH	Total $CaCO_3$	TOC	Total N	C:N ratio	HS	HR%	DH%	HI
Management									
CONV	7.46	132	14.7	1.46	10.1	5.76	39	26,9	2,83
RE-NAT	7.29	90.5	38.7	3.21	12.2	19.2	47,4	30,2	2,56
P	***	**	**	**	**	**	**	*	*
Sampling position									
Row	7.38	103	31.4	2.72	11.3	15.8	45,8	29,8	2,38
Strip	7.38	119	21.9	1.95	10.9	9.08	40,7	27,3	2,69
P	ns	**	ns	ns	ns	ns	*	ns	ns
Factors interaction									
P		ns	ns	ns	ns	ns	*	ns	ns

	Available P	Total S	Total Pb	Pb_{DTPA}	Total Cu	Cu_{DTPA}	Cu_{OM}	Cu_{H2O}
Management								
CONV	39.8	257	16.1	1.48	88.7	14.8	16.6	0.25
RE-NAT	23.1	461	26.7	3.98	527	123	132	0.78
P	*	*	***	***	***	***	***	*
Sampling position								
Row	35.4	400	25.5	3.62	547	128	134	0.88
Strip	27.6	319	17.3	1.84	68.8	10.1	14.8	0.15
P	ns	ns	**	***	***	***	***	**
Factors interaction								
P	ns	***	ns	***	***	***		*

ns: not significant, *P >0.05, **P <0.01; ***P <0.001.

TOC = total organic carbon; CuDTPA, PbDTPA = DTPA-extractable Cu or Pb (mg kg-1 soil); CuOM = Na4P2O7-extractable Cu (mg kg-1 soil); CuH_2O = water-extractable Cu (mg kg-1 soil); HS = humic substances (mg kg-1 soil); HR% = humification rate; DH% = humification degree; HI = humification index.

8.3.2. Biochemical properties

The land abandonment for decades of the Pantaleone farm determined higher values of soil 0.5 M K_2SO_4 extractable C (C_{ext}) and extractable N(N_{ext}), microbial biomass N (N_{mic}) and microbial biomass C (C_{mic}), as reported in Table 2. Soil organic C content correlated positively with C_{ext} (0.97), C_{mic} (0.87), HS (0.99). Positive correlation can be also observed between soil total N with N_{ext} (0.96), and N_{mic} (0.52). Soil C_{ext} was much higher in RE-NAT than in CONV, while it did not differ significantly for sampling position. Similar results were observed for N_{ext}, with higher content in RE-NAT than in CONV soils. Additionally, the C_{ext}-to-N_{ext} ratio was higher in RE-NAT than in CONV soils.

Table 2. Means of ecophysiological parameters determined in the soil under different types of land management and sampling position in orchard.

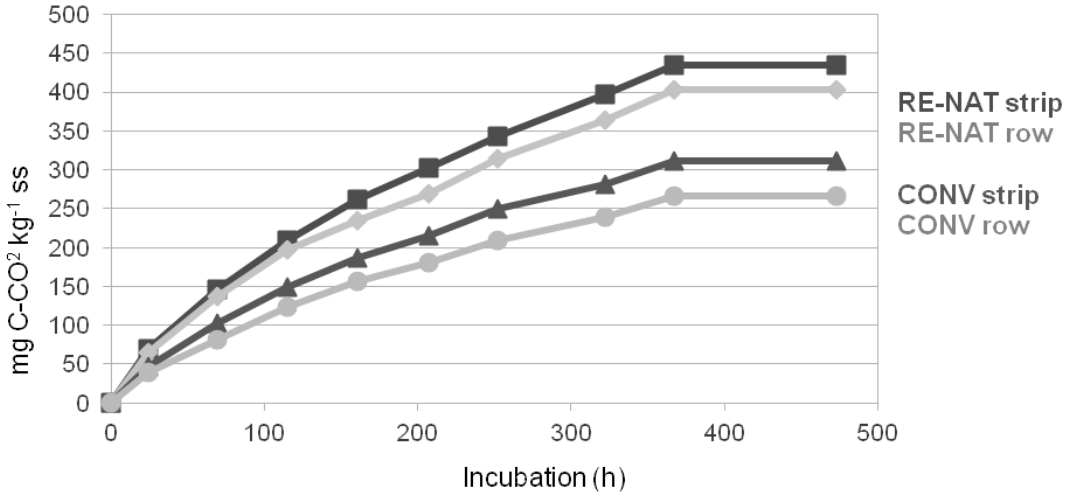
	C_{ext}	**N_{ext}**	**C_{mic}**	**N_{mic}**	**SBR_R**	**qCO_2**	**C_{ext}:N_{ext}**
Management							
CONV	85.2	8.35	103	20.5	0.65	6.04	10.3
RE-NAT	300	15.5	161	41.7	0.85	5.7	20.2
P	***	*	*	*	Ns	Ns	***
Sampling position							
Row	215	14.2	156	26.5	0.72	4.8	13.8
Strip	170	9.70	108	35.6	0.78	6.95	16.7
P	ns	ns	*	ns	ns	*	ns
Factors interaction							
P	ns	ns	**	ns	ns	ns	ns

ns: not significant, *P >0.05, **P <0.01; ***P <0.001.

C_{ext} = K_2SO_4-extractable C (mg kg^{-1} soil); N_{ext} = K_2SO_4-extractable N (mg kg^{-1} soil); C_{mic} = microbial biomass C (mg kg^{-1} soil); N_{mic} = microbial biomass N (mg kg^{-1} soil); SBR = soil basal respiration rate (mg C-CO_2 kg^{-1} soil h^{-1}); qCO_2 = metabolic quotient (mg C-CO_2 g^{-1} C_{mic} h^{-1}).

The metabolic quotient (qCO_2), or specific soil basal respiration rate, did not differ in management, but it significantly differed in interaction between management and sampling position and was significantly lower in Cu contaminated soil (3.3 mg C-CO_2 g^{-1} C_{mic} h^{-1}) than all other treatments (Tab. 3). CO_2 evolution in investigated soils along a 28 days period is plotted in Figure 1.

Figure 1. CO_2 evolution determined in the soil under different types of land management and sampling position in orchard.



As far as concerns protease activity, acid and alkaline phosphomonoesterase activities were higher in RE-NAT soil; urease and cathecol oxidase activity of RE-NAT were lower than CONV soils; finally β-glucosidase, fluorescein diacetate hydrolase, and dehydrogenase activities did not differ (Table 3). Finally, aiming at synthesizing whole soil functional response to treatments, a multivariate analysis (two-way MANOVA using Bray-Curtis distance) performed on all recorded enzymatic activities only showed significant differences in management ($P < 0.05$). Table 3 and Figure 2 report, respectively, two-way ANOVA results and means of enzymatic activities for the four investigated soils.

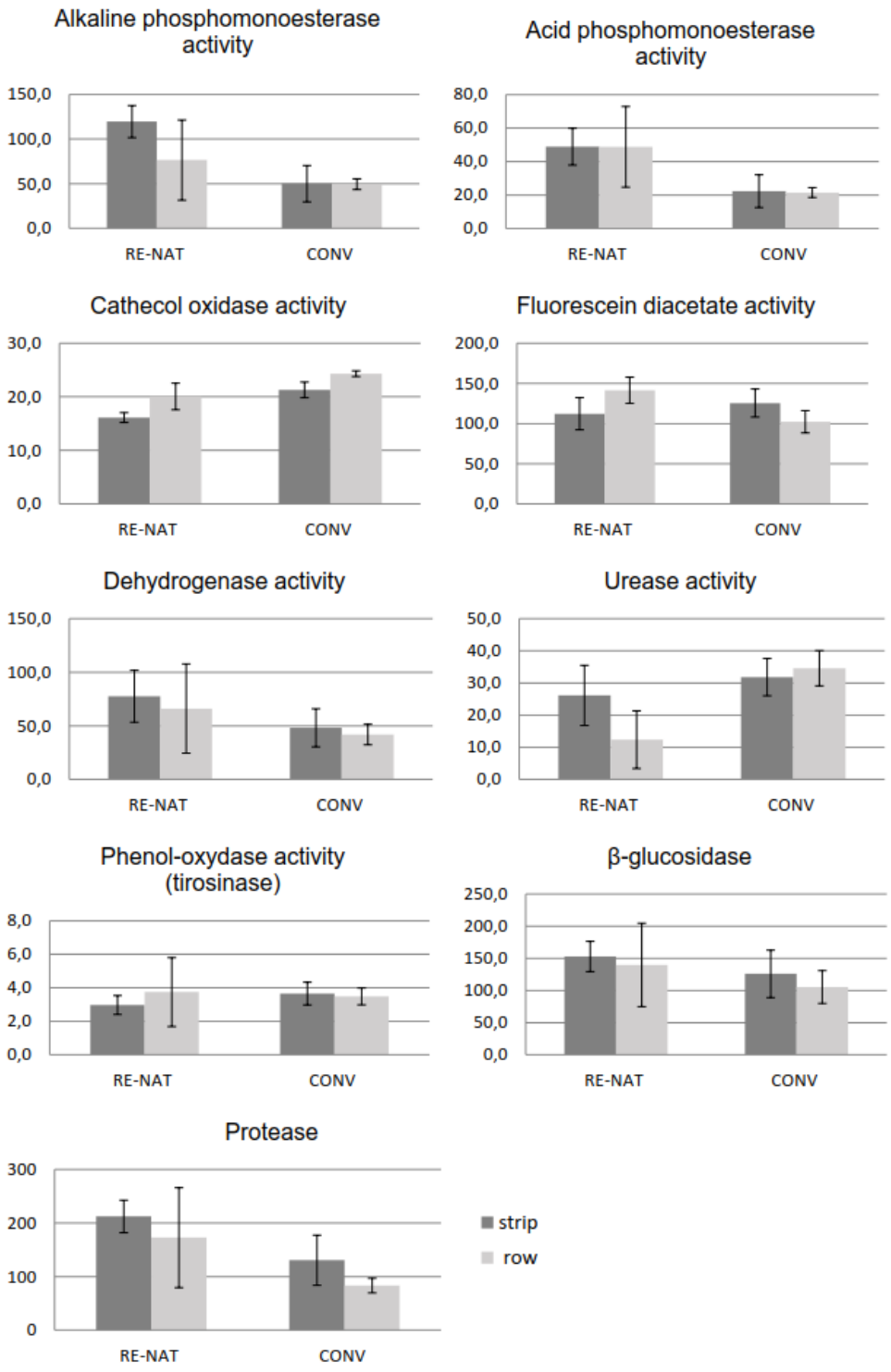
Table 3. Means of the enzymatic activities determined in the soil under different types of land management and sampling position in orchard.

	FDA	**β-glu**	**Prot**	**Urease**	**Acid PME**	**Alk PME**	**DeH**	**Catox**
Management								
CONV	114	116	107	33.2	113	258	45	22.8
RE-NAT	127	146	193	9.08	246	489	71.8	18.1
P	ns	ns	*	ns	ns	*	ns	**
Sampling position								
Row	122	123	128	23.4	183	322	53.9	22.2
Strip	119	139	171	28.9	176	424	62.8	18.7
P	ns	ns	ns	ns	ns	ns	ns	*
Factors interaction								
P	ns	ns	ns	*	ns	ns	ns	ns

ns: not significant, *P >0.05, **P <0.01; ***P <0.001.

FDA = fluorescein diacetate hydrolysis activity (mg kg^{-1} soil h^{-1}); β-glu = β-glucosidase activity (mg kg^{-1} soil h^{-1}); Prot = protease activity (mg kg^{-1} soil h^{-1}); Urease = urease activity (mg $N\text{-}NH_4$ kg^{-1} soil h^{-1}); Acid PME = acid phosphomonoesterase activity (mg kg^{-1} soil h^{-1}); Alk PME = alkaline phosphomonoesterase activity (mg kg^{-1} soil h^{-1}); DeH = dehydrogenase activity (mg kg^{-1} soil h^{-1}); Catox = catechol oxidase activity (mmol kg^{-1} soil h^{-1}).

Figure 2. Mean values of enzymatic activities activity, in tree rows and strips of re-naturalized (RE-NAT) and conventional (CONV) orchards. Bars indicate standard error.

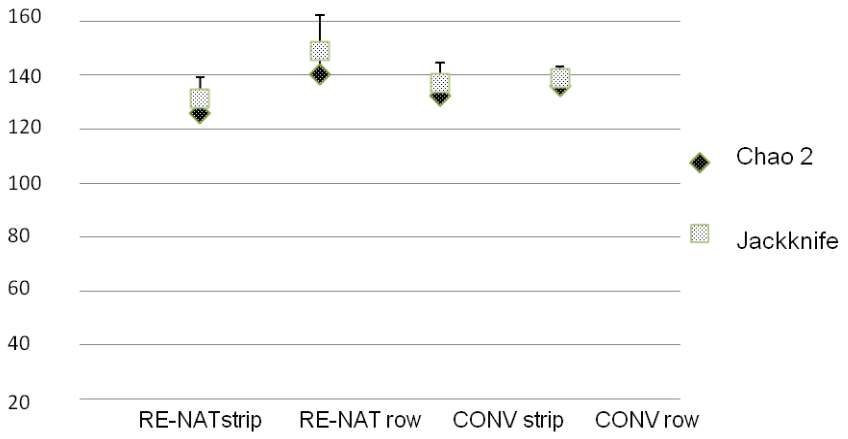


Fluorescein diacetate hydrolysis activity (mg kg^{-1} soil h^{-1}); β-glucosidase activity (mg kg^{-1} soil h^{-1}); Protease activity (mg kg^{-1} soil h^{-1}); Urease activity (mg N-NH_4 kg^{-1} soil h^{-1}); Acid phosphomonoesterase activity (mg kg^{-1} soil h^{-1}); Alkaline phosphomonoesterase activity (mg kg^{-1} soil h^{-1}); Dehydrogenase activity (mg kg^{-1} soil h^{-1}); Catechol oxidase activity (mmol kg^{-1} soil h^{-1}).

8.3.3. Soil microbial community

Two way non parametric MANOVA inferred from bacterial communities DGGE fingerprinting showed significant differences in management ($P > 0.001$), sampling position ($P > 0.01$) and interaction ($P > 0.01$) between management and position. Analysis of similarity confirms these results. Chao 2 diversity index of bacterial communities in tree rows of RE-NAT area was higher than the other treatments, and Jackknife diversity index. Graphical comparison of Chao 2 and Jackknife diversity indexes (Figure 3).

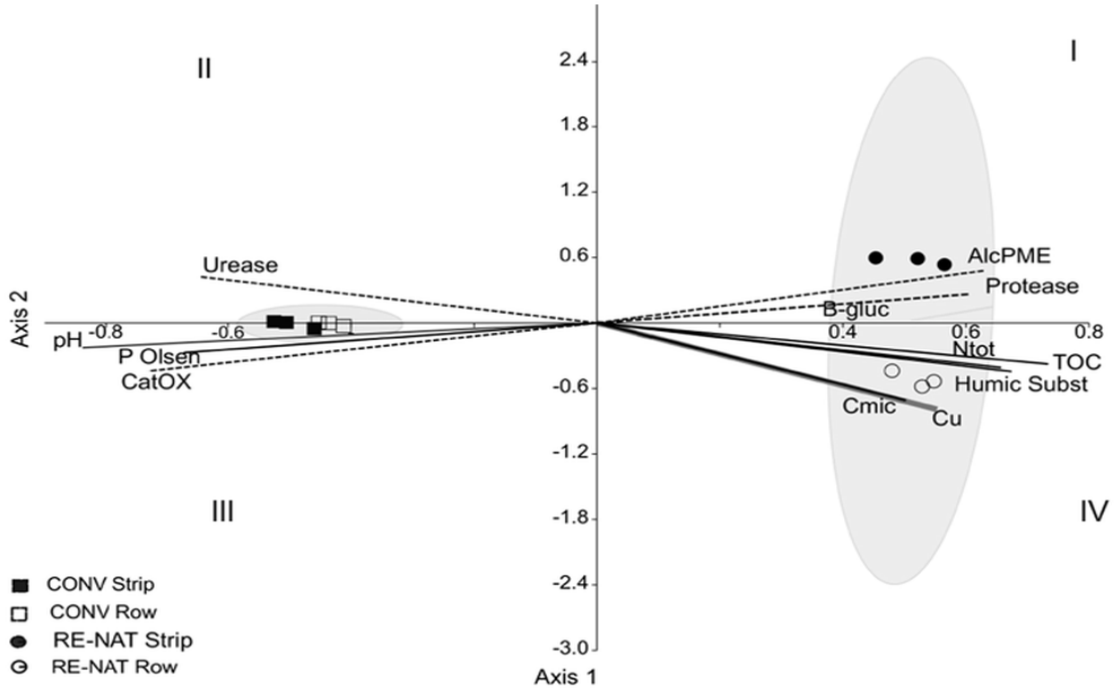
Figure 3. Chao 2 and Jackknife diversity indexes calculated for bacterial community



Canonical correspondence analysis (CCA) grouped bacterial communities of RE-NAT and CONV in two opposite quadrants, where samples of row and strips of CONV management overlapped in a small area indicating high similarity (samples were grouped according 95% of similarity). Conversely, rows and strips of RE-NAT felt in two opposite quadrants (III and IV), confirming the significantly different bacterial composition between samples of rows and strips.

Vector representing copper content oriented toward samples of rows (RE-NAT row) indicated that it affected bacterial composition in these samples (Figure 4).

Figure 4. Triplot of canonical correspondence analysis performed on bacterial communities and chemical and biochemical parameters for all soils (Axis 1= 0.19; Axis 2= 0.11; overall accounting for 75% of variance. Ellipses grouped (P< 95%) samples of re-naturalized (RE-NAT) and conventional management (CONV). Dash lines represent enzymatic activities.



Fungal community structure was analysed by visual analysis of PCR resulting pattern, thanks to the marker reference used during electrophoretic run (data not shown). Ascomycota and Basidiomycota differed significantly in management. Ascomycota differed also in sampling site and interaction between factors, but fungal community composition did not appear in any case to be affected by Cu contamination. Dissimilarity of Ascomycetes between the four

treatments, showed that only communities in CONV management differed significantly between rows and strips and that larger variability was found in rows than in strips. Ascomycota were more abundant in CONV than in RE-NAT soils. To the contrary, abundance of Basidiomycota was higher in RE-NAT.

8.4. Discussion

Since soil organic matter in soil is a key issue in agricultural management, this parameter, along with other ecophysiological parameters were measured in order to interpret carbon and nitrogen cycles in the investigated soils. Available P in CONV (40 mg kg^{-1}) is higher than in RE-NAT soil (23 mg kg^{-1}) indicating the use of inorganic P fertilizers in conventional agriculture. Indeed, available phosphorous, which represents bioavailable P in neutral and calcareous soils, is often used as an indicator of agricultural impact on the environment (Sharpley *et al*., 1994). Although the highest TOC in re-naturalized soil should foster a higher macronutrient availability, the use of P fertilizers as well as animal manure and other practices, may be responsible for a general increase of soil P (Beare *et al*., 2005). Based on previous studies which report positive correlations between total S concentration and soil OM content (Bettany *et al*., 1980; Provenzano *et al*., 2011), the highest total S in the abandoned vineyard may be linked to the higher soil OM content and pH reached with the re-naturalization process. The difference significant between CONV and RE-NAT management for available phosphorous (P), and total sulfur (S), confirmed the effective re-naturalization process in the Pantaleone reserve.

C:N ratio was slightly, but significantly, higher in re-naturalized than conventionally managed soils, confirming that the ecological restoration process

increased SOC input with a positive net balance (Magdoff and Weil, 2004). C:N ratio gives indication about the decomposability of soil organic matter, helping in the assessment of plant N availability, crop residue decomposition (particularly residue-cover on the soil) and crop nutrient cycling (mainly N cycling). Additionally, the C_{ext}-to-N_{ext} ratio was higher in RE-NAT than in CONV soils, confirming that land abandonment increases C labile pool and changes the soil system from a C-limited to N-limited state (DeLuca and Keeney, 1993). This parameter is a sensitive measurement of the soil substrate which drives soil microbial activity: if its value is higher than 20, it indicates that the soil has a high microbial activity (Haney *et al*., 2012). Whereas RE-NAT soil showed a water-extractable organic C:N ratio near to 20 independently from sampling site, the whole re-naturalized area reached such high soil microbial activity that C availability was not a limit. The significant difference of C_{mic} between rows and strips was due to the differences recorded within the RE-NAT area. This may be principally explained by a longer long-term abandonment in rows than strips, as well as by a higher TOC content in rows linked also to a different vegetal cover between tree-rows (nearly forested area) and strips (herbaceous cover). TOC levels were consistent with humic substances content, which was two to four times higher in the re-naturalized area than in conventionally managed orchard. Degree of humification and humification rate are significantly higher in RE-NAT soil, indicating that the conditions are favourable to soil organic matter humification. The metabolic quotient (qCO_2), did not differ in management, but it significantly differed in interaction between management and sampling position and was significantly lower in copper contaminated soil. In general, in a heavy metal stressed ecosystem, the qCO_2 values are expected to increase compared

to an unstressed ecosystem (Anderson and Domsch, 2010). Metabolic quotient calculated in this study suggest that re-naturalization was responsible for a consistent reduction of qCO_2 regardless of the high Cu contamination. Based on these findings, soil restoration performed with abandonment of tree-rows seems to have overcome the Cu contamination effect on qCO_2. This may be explained by the higher soil OM content reached in the RE-NAT rows, as suggested by previous studies (Khan and Sculli, 2000). Increase in hydrolytic activity is confirmed by soil organic C, C-to-N ratio and C_{ext}-to-N_{ext} ratio values in soils under re-naturalization. Soil organic C content correlated positively with C_{ext} (0.97), C_{mic} (0.87), HS (0.99). Positive correlation can be also observed between soil total N with N_{ext} (0.96), and N_{mic} (0.52).

Protease, acid and alkaline phosphomonoesterase (N and P cycle enzymes), were the most involved in soil organic C increase. Their higher value in RE-NAT soils further confirmed an N and P limitation in this ecosystem (Bandick and Dick, 1999). Higher urease activity in CONV soils can easily be explained as a response to the use of urea or organic fertilizers in this soils (Kandeler and Eder, 1993). In these soils, a lower phosphomonoesterase activity reflects a negative response to inorganic P fertilization. Similarly, when conventional management leads to a loss of soil OM, in particular the most labile fraction, a greater catechol oxidase activity is expected, like in this case study (Sinsabaugh, 2010). Multivariate analysis performed on all recorded enzymatic activities only showed significant differences in management ($P < 0.05$). This finding is consistent with previous observations on the relationship between management and soil enzymatic activity (Bandick and Dick, 1999; Stursova and Baldrian, 2011).

Top soil in rows in the Pantaleone protected area could be classified as highly Cu contaminated, according to Annex I A of European Directive 86/278/CEE which indicates concentration of 50-140 mg kg^{-1} total Cu in cultivated soils as a threshold. As observed for Cu concentration in soil, DTPA-extractable soil copper in rows of the re-naturalized area largely exceeded the threshold for toxicity in plants and the maximum level accepted in plants for animal feeding. However, these toxicity thresholds were estimated in plant growth assay in controlled conditions with increasing copper doses artificially added to natural soils (Zhang *et al*., 2013; Ippolito *et al*., 2010), whereas there is little evidence on the relationship between plant toxicity and DTPA-extractable Cu in long term Cu contaminated soils. Chopin *et al*. (2008) reported that the Cu accumulation ratio (vegetation/ soil) in *Vitis vinifera* L. in the Champagne region was below 0.2, and the transfer coefficient (aerial parts/fine roots) was below 0.15. On the other hand, the DTPA-extractable Cu was similar to that present in $Na_4P_2O_7$ extracts, confirming that the main available fraction of Cu in soil is correlated with OM and that Cu bound to the organic fraction reduces the adverse environmental effects on soil biota (Fernandez-Calvino *et al*., 2008). Finally, the low amount of water soluble Cu observed in re-naturalized rows was consistent with findings of low mobility as already observed (Wang and Staunton, 2006; Chopin *et al*., 2008). Surveys on soil biological parameters of highly Cu contaminated vineyards in several important wine producing areas, as well as standard ecotoxicity tests, performed on plants, microbial activity (nitrification) and invertebrates showed that soil fertility parameters and biological responses in all cases were more importantly explained by other soil properties than Cu (de Boer *et al*., 2012; Fernandez-Calvino *et al*., 2010; Mackie *et al*., 2013; Ruyters *et*

al., 2013).

Lead values were lower than 100 mg kg^{-1}, which is the recommended threshold value for environmental risk in neutral-alkaline soils (Gawlik and Bidoglio, 2006). This Pb distribution in soil treatments was consistent with that of DTPA-extractable soil Pb. Since lead concentration values did not exceed the natural background, it was not to be considered a primary factor in microbial changes. Finally, traces of Pb at concentrations similar to those observed in tree-rows of the Pantaleone reserve had been already observed in Cu contaminated vineyards (Chopin *et al*., 2008; Wightwick *et al*., 2008).

Copper contamination was responsible for a shift in bacterial community composition as well as a larger genetic variability: this may be due to variable effects of Cu on bacterial communities depending on substrates and in situ Cu concentration (Dell'Amico *et al*., 2008; Ippolito *et al*., 2010; Maier *et al*., 2000). Lejon *et al*. (2010) reported a depressing effect of Cu on bacterial diversity in soils poor in organic matter, conversely bacterial diversity increased in soils enriched with organic amendments. These findings confirmed the resilience of soils rich in organic matter, which tends to reach an equilibrium in terms of metabolic activity and an improved bacterial ability to adapt to stress conditions (Ippolito *et al*., 2010; Singh, 2015). Regarding bacterial communities, mechanisms underlying soil resilience are linked to changes in community structure and selection of tolerant populations. CCA showed that pH mostly impacted on composition of bacterial communities in CONV, TOC was one of the soil properties playing a major role on bacterial composition in RE-NAT (Chaudhry *et al*., 2012; Maier *et al*., 2000) and that total N availability and microbial biomass are consistent with TOC (Anderson and Domsch, 1990, 2010).

Those findings are in line with the increase of bacterial diversity in rows of Pantaleone reserve (RE-NAT), in which adaptation process was observed under natural conditions and in long term Cu contaminated soils. Differences between managements were definitely due to a series of changes in environmental parameters which were determined by two differing managements. Based on the response of diversity, bacteria were more sensitive to copper contamination than fungi.

The relative differences in DNA concentration of Ascomycota and Basidiomycota further confirmed that soil management mainly affected both Phyla, as already reported about soil management impact on soil fungi (Bailey *et al*., 2002; van der Wal *et al*., 2006). The large variability of Ascomycetes in tree-rows may be due to periodical tillage on the surface layer for controlling weeds and reducing the development of vegetal cover. The highest abundance of Ascomycota in cultivated soils is due to the effectiveness of this group of fungi in decomposing holocellulose in preference to lignin (Osolko and Takeda, 2002) which is linked to a need for readily available energy sources of this phyla (Ma *et al*., 2013). Management, in particular soil tillage, impacted significantly on fungal composition and there was no evidence of the Cu effect on fungi. Conversely, a higher abundance of Basidiomycota in natural rather than cultivated soil is a typical trend observed in studies into tillage impact on soil microbial communities, considering the higher ability to decompose lignin and to improve soil aggregation and aggregate stability (Caesar-TonThat *et al*., 2010). Basidiomycetes are particularly susceptible to mechanical soil disturbance, since tillage boosts mitospostic fungi (*Fusarium*, *Aspergillus*, *Penicillium* species and other microfungi), which are able to quickly colonize soil with spores and smaller

mycelial structures (Caesar-TonThat *et al*., 2010). A prevalence of Ascomycetes in tilled soil and Basidiomycetes in undisturbed soil, agrees with other previously discussed indicators of soil disturbance and again proved a successful ecological restoration of the abandoned vineyard. Copper contamination reduced both Ascomycota and Basidiomycota in Cu contaminated soils as compared to the uncontaminated RE-NAT soils. That non-specific impact on fungal communities may suggest that Cu can interfere on C metabolism and fungal biomass development; this theory is consistent with the lower specific respiratory activity (qCO_2) recorded in Cu contaminated soils. However, as fungi represent the largest part of microbial biomass (in volume), particularly in non-cultivated soils (Bailey *et al*., 2002), and microbial biomass was highest in Cu contaminated soil, there is sufficient evidence to suggest that abiotic changes determined by land abandonment exceeded the impact of high copper soil concentration.

9. Effect of different agricultural practices on soil microbial communities: case study of soils affected by replant disease

9.1. Introduction

According to many studies, fertilizer application affects the physical properties, fertility and organic matter content of soils, as well as ultimately, crop yield (Pernes-Debuyers and Tessier, 2004, Mallarino and Borges, 2006, Belay *et al*. 2002; Cai and Qin, 2006). Conventional farming is known for its ability to increase crop yields, however, it also causes soil degradation, soil and water pollution (Reganold *et al*., 1987), and results in the development of microbial species with xenobiotic resistance by transferring genes or mobile genetic elements (Springael & Top, 2004). In contrast, the effects of organic practices on soil quality are still under debate. Several researchers have demonstrated that organic amendments increase microbial biomass and diversity (Belay *et al*. 2002; Chu *et al*. 2007; He *et al*. 2008; Insam and Merschak, 1997; Sharma *et al*., 1998; Zhong and Cai 2007), while others reported that organic amendments have, on the short term, no significant effects on soil microbiological properties (Sarathchandra *et al*., 2001; Miller and Miller, 2000). Shifts in the microbial community and its functioning are, nonetheless, considered to depend more closely on the chemical and biochemical changes resulting from fertilizer application (Chu *et al*. 2007, McAndrew and Malhi, 1992; He *et al*., 2007, Pernes-Debuyers and Tessier 2004) than on the nutrient content of the fertilizer itself. Microbial diversity in agricultural soils is a critical factor in maintaining soil health

(Garbeva *et al*. 2004) as it is assumed to be related to stability and resilience towards disturbances.

Apple replant disease (ARD) is a common disease of apple orchards found in most apple growing areas. Symptoms arise, for the majority of the cases, within the first three months after the replanting of apple trees, and are more widespread in orchards or sites where apple trees were grown previously (Mazzola and Manici 2012). Although ARD has been investigated in numerous studies spanning the last decades, its etiology is still poorly understood. Both biotic and abiotic factors (extreme pH values, abiotic stresses, phytotoxins, poor soil fertility, heavy metal contamination) are considered to be involved in ARD development, although it is believed that biotic components play a predominant role in the disease (Mazzola, 1998). While the role of fungi in ARD has been deeply investigated, but still remains unsolved (Kelderer, 2012; Tewoldemedhin *et al*., 2011), even less is known about the role of bacteria. The findings regarding the involvement of *Actinomycetes*, *Bacillus*, *Streptomyces* and *Pseudomonas* in ARD are controversial (Mazzola and Manici, 2012; Utkhede *et al*., 1992, Zhao *et al*., 2009).

High-throughput sequencing is an increasingly applied method in microbial ecology because it can potentially detect a larger portion of the culturable and non-culturable microorganisms in a sample than has been previously possible (Acosta-Martínez *et al*., 2008; Roesch *et al*., 2007). The widespread application of molecular techniques has allowed researchers to gain a more extensive knowledge about the ecology of soils (Shange *et al*., 2012),

thus helping to better understand the effects of agricultural practices on the soil microbiota and consequently the functioning of the community members.

The aim of this study was to describe the interactions between soil chemical parameters, plant growth, and soil microbial diversity, under different fertilization regimes in apple orchards. In investigating the various factors involved in ARD, plant growth reduction (the main indicator of apple replant disease; Mazzola and Manici, 2012) was correlated with soil chemical and ecophysiological parameters as well as with bacterial populations. Two apple orchards, both located in Italy, were selected as representative of perennial crops affected by ARD. The hypothesis was that fertilizer type and application could exert a shift in soil chemical and biological parameters linked to ARD.

9.2. Material and methods

9.2.1. Study site and experimental design

The present study was conducted on soils collected from two sixth generation apple orchards situated in South Tyrol (Italy) that have been monitored since 2009 within an open-field study set up by the Laimburg Research Centre (Ora, South Tyrol, Italy). The medium term study was established in order to evaluate the effect of different fertilizers, timing and rate of fertilizer application, as well as of different tillage techniques within ARD-affected crops. One experimental orchard belongs to an open-field experiment located in Latsch (46° 37′ 0″ N, 10° 52′ 0″ E; 700 m a.s.l.), where the cultivar Golden Delicious is grown on M9 rootstock. In the open-field experiment situated in Laimburg (46° 21′ 0″ N, 11° 18′ 0″ E; 220 m a.s.l.), the cultivar Braeburn is grown on M9 rootstock.

A two-factorial experiment in a randomized block design with three replicates was performed. The factors were soil treatment (corresponding to a combination of fertilizer type and tillage technique) and sampling site (corresponding to the orchard's site, Laimburg or Latsch). The medium term study was started in 2009, immediately after apple planting.

9.2.2. Soil treatments

The treatments (fertilizers and tillage techniques) used in this study are listed in Table 4.

Table 4. Treatments included in the study and applied to soils at both sites (Laimburg and Latsch).

Abbreviation used	Treatment	Description of applied amendment	Application rate	Tillage technique
K	Untreated control	---	---	Ploughing-brushing
AS	Ammonium sulfate	Nitrogen fertilizer used in conventional farming	24 g N/tree	Ploughing-brushing
C	Compost	Biowaste compost	2 kg compost/tree	Ploughing-brushing
ON	Organic nitrogen fertilizer	Applied in pellet form, contains soya, corn meal, and horn and other animal-derived materials	24 g N/tree	Ploughing-brushing
CS	Compost + fermented slurry from a biogas plant	Combination treatment of biowaste compost (C) with fermented slurry from a biogas plant	23 g N/tree (+ 2 kg compost/tree)	Ploughing-brushing
OF	Fungi-derived fertilizer	Granules of fungal biomass	24 g N/tree	Ploughing-brushing
OG	Organic nitrogen fertilizer greened	Greened plot amended with organic fertilizer (ON)	24 g N/tree	Mulching

Ammonium sulfate is a nitrogen fertilizer that has been widely used in conventional farming. In contrast, the other fertilizers applied in this study are commonly used in organic agricultural practices. A complete description of the treatments has been reported by Kelderer *et al*. (2012). Fertilizers were applied annually in spring (last fertilizer application April 2014).

9.2.3. Soil analysis and plant growth test

Soils were sampled from a depth of 0 - 30 cm in July 2014 prior to soil being directly sieved to 2 mm. For all treatments at both sites, three soil replicates were sampled. Soil samples were immediately stored at 8 °C, except for a small quantity for molecular analysis which was stored at -20 °C.

Soil pH was measured using a glass electrode in a 2:3 (w/v) suspension of air dried soil in deionized H_2O, while potential pH and electrical conductivity (EC) were measured in a 2:3 (w/v) suspension in 0.01 M calcium chloride. Water holding capacity (WHC) was measured according to Harding and Ross (1964). Soil water content was determined gravimetrically by drying 10 g of fresh soil at 105 °C for 24 h. The soil organic matter (SOM) content was determined by mass loss on ignition in a muffle furnace at 550 °C for 5 h (Carbolite, CWF 1000). Total C and N contents were analyzed in dried samples using a CHN-analyser (TruSpec CHN Makro/LECO). Soil basal respiration was measured as CO_2 evolution from fresh soils at a constant temperature of 22 °C using an automated system of continuous flow infrared gas analysis (IRGA; Heinemeyer *et al*. 1989). Measurements were taken for 16 h at hourly intervals. Using the IRGA, microbial biomass (C_{mic}) was determined by substrate-induced respiration (SIR) after the addition of 1% glucose (dry matter basis) to the soil samples. CO_2 evolution was

measured for 8 h after amendment in order to obtain the “maximum initial respiratory response” from which microbial biomass was calculated (Anderson and Domsch, 1978). The metabolic quotient (qCO_2) was calculated from the basal respiration and C_{mic}.

Total shoot length (cm) and dry matter (g) of shoots were measured 85 d after planting. Plant growth test were conducted in pot trials simulating in field conditions and results were expressed as the sum of 6 plants for each replicate. Dry matter of shoots was measured on a fresh matter basis, drying shoots at 80 °C until the weight was constant.

9.2.4. Molecular analysis of the bacterial and fungal communities using PCR-DGGE

Total soil DNA was extracted from 300 mg of fresh soil using the NucleoSpin® Soil Kit (Macherey-Nagel), according to the manufacturer's protocol . DNA quantity and quality (260/280 and 260/230 ratios) were checked using a NanoDrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

16S rDNA bacterial fragments of the total genomic DNA extracted from soil were amplified for DGGE (denaturing gradient gel electrophoresis) using the primers 984 forward and 1378 reverse, according to Heuer *et al*. (1997). A GC clamp (Muyzer *et al*., 1995) was attached to the forward primer. PCR amplification was performed as follows: 3 min denaturation at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 62 °C, and 2 min at 72 °C. Cycling was completed with a final extension at 72 °C for 10 min. The reaction mixture was prepared using template DNA (ca. 20 ng), and final concentrations of 100 nM of

each primer, 5 X MyTaq Buffer (Bioline Reagents, London, UK), 0.2 mM dNTPs, 0.4 mg ml^{-1} bovine serum albumin (BSA) and 0.038 U μl^{-1} of MyTaq DNA polymerase (Bio-line Reagents, London, UK). Approximately 2.5 µl PCR product was loaded on a polyacrylamide gel (8% acrylamide solution, 40% formamide and 7 M urea) with a vertical denaturing gradient from 40% to 70%. The electrophoretic run was performed in 1 X TAE buffer at 100 V for 16 h at a constant temperature of 60 °C, in an INGENYphorU system (Ingeny International BV, Goes, Netherlands). Gels were stained with silver nitrate using the Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech, Freiburg, Germany), air dried and scanned for subsequent image analysis. Digitalized DGGE images were analysed for the presence/absence of bands using GelCompar II® (Applied Maths).

The ITS1 region of fungal rDNA was amplified according to Bougoure and Cairney (2005) using the primers ITS1-GC forward and ITS2 reverse. Amplification was performed as follows: 1 min denaturation at 94 °C, followed by 35 cycles of 1 min at 95 °C, 2 min at 55 °C, and 2 min at 72 °C. Thermal cycling was completed with a final extension step at 72 °C for 10 min. Amplification reactions were prepared as described above, except that 0.05 U μl^{-1} of MyTaq DNA polymerase (Bio-line Reagents, London, UK) was used. A volume of 2.5 µl PCR product was loaded in a polyacrylamide gel (7% acrylamide solution, 40% formamide and 7 M urea) with a vertical denaturing gel gradient from 30% to 50%. The electrophoretic run was performed as described above.

9.2.5. PCR amplification and Illumina high throughput sequencing

PCR amplification of DNA extracts and high throughput sequencing was

performed at GATC Biotech (Konstanz, Germany) on a MiSeq Illumina sequencer, using a 300 bp paired-end approach. The V1-V3 region of the 16S rRNA gene of bacteria was amplified using the primers 27F and 534R (Wilson *et al*., 1990 and Kalanetra *et al*., 2004). Each of the primers was synthesized together with a sequencing adaptor (A adapter 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3' and B adapter 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3') and the forward primer was in addition modified with a MID tag, so that multiple samples could be pooled together for sequencing. Bacterial 16S rRNA gene reads were analysed using the MOTHUR v.1.34.4 software pipeline (64 bit executionable) according to the Standard Operating Procedures for MiSeq (Kozich *et al*., 2013) after an initial trimming. Unique bacterial sequences were aligned against the SILVA rRNA gene database and trimmed such that only overlapping sequence was considered. Chimeras were removed using the chimera.uchime command. Pre-clustering was conducted at 1%, and rarefaction curves were generated using a 97% identity cut-off. Operational taxonomic units (OTUs) were binned at 97% identity. The number of sequences included in analyses (1,590,042 from count file) was based on the lowest number of sequences found in all samples. Three diversity estimates (InvSimpson diversity, Shannon diversity and Chao richness estimate) and Good's coverage were calculated for bacteria.

9.2.6. Data analysis

Chemical and ecophysiological parameters were tested for normality using the Shapiro–Wilk test and by examining histograms derived from descriptive statistics. Two-way analysis of variance (ANOVA), LSD post-hoc test; $p \leq 0.05$, and principal component analysis (PCA) of soil chemical and

ecophysiological parameters were performed using Past (Software for data analysis in paleoecology; Hammer *et al*., 2001) and R (R Core Team, 2015). Plant growth test results were subjected to (ANOVA). Significant sources of variation were subjected to a mean separation test using Fisher's least significant difference (LSD) protocol *post-hoc* test ($p \leq 0.05$).

Molecular analysis of the bacterial and fungal communities using PCR-DGGE was performed using GelCompar II, Version 4.0 (Applied Maths, Ghent, Belgium). DGGE cluster analyses were conducted using the criteria optimization and position tolerance of 1%. Background noise was removed before searching for bands with minimum profiling greater than 0.5%. Dendrograms derived from the DGGE analysis were subjected to analysis of similarity using PAST. Illumina high-throughput sequencing data were analyzed using the PAST and R software programs. OTU analysis based on Bray-Curtis similarity matrix and abundance values were log-transformed. Rarefaction curves as well as richness, diversity and evenness indexes were also calculated. Transformed OTU relative abundances were submitted to non-metric multidimensional scaling (NM-DS). NM-DS similarity index 3D ordinations of OTU data (considering a threshold of 1% relative abundance for each sample) for each site was performed on a Bray-Curtis similarity matrix, followed by analysis of similarity (ANOSIM) and similarity percentage analysis (SIMPER) analysis. Log-transformed abundance data were also used to set up a similarity matrix using Bray-Curtis distance, and non-parametric multivariate analysis of variance (NP-MANOVA) was performed. Management effect on soil chemistry and on bacterial communities was tested by performing canonical correspondence analysis (CCA) on log-transformed data. Linear relations between plant growth and relative abundance of genera

corresponding to >10 in at least one sample were inferred by performing a Pearson correlation (threshold considered was -0.7≤ r ≤0.7).

9.3. Results

9.3.1. Soil physico-chemical and ecophysiological parameters, plant growth test

Chemical and ecophysiological parameters measured in all soils, as well as the results of plant growth are reported in Table 5. Soils collected from Laimburg and from Latsch showed significant differences in terms of physico-chemical parameters. The pH value of the two soils was found to differ significantly (ANOVA: $F=79.08$; $p<0.001$), but there were no significant differences according to treatment within the two sites. EC ranged from 128-201 and 76-120 µS cm^{-1} in Laimburg and Latsch soils, respectively. EC was significantly different according to treatment ($F=2.5$; $p<0.05$) and site ($F=83.2$; $p<0.01$). The interaction of site and treatment was not significantly different. The WHC ranged from 0.12-0.2 and 0.18-0.36 ml H_2O g^{-1} in Laimburg and Latsch samples respectively: according to ANOVA, WHC differed significantly among treatments ($p<0.05$) ANOVA indicated that SOM was found to be significantly affected both by site ($F=260.5$; $p<0.01$) and treatment ($F=2.64$; $p<0.05$), without interaction between the two factors, however. SOM ranged from 4.62% to 5.17 in Laimburg and from 7.48 to 10.06% in Latsch. Similar results were obtained for TOC: values significantly differed both by site ($F=260.5$; $p<0.01$) and treatment ($F=2.64$; $p=0.05$), with no significant interaction between the two factors.

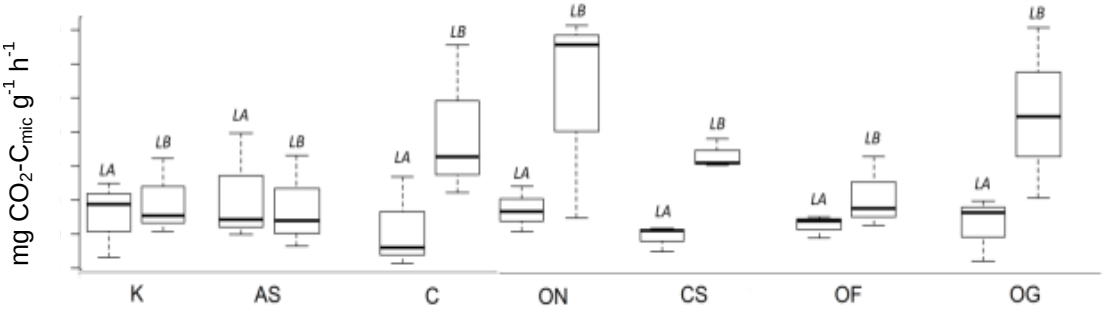
Table 5. Means of physico-chemical parameters and plant growth measurements measured in all soils.

Site	Treatment	pH (H_2O)	pH ($CaCl_2$)	EC	% WC	WHC	% SOM	%C	%N	C/N	SBR	C_{mic}	qCO_2	C_{mic}/C_{org}	Plant growth	
															% Dry matter	Shoot length
Laimburg (sandy-silt soil)	**K**	7.26 (0.14)	6.92 (0.04)	128 (10.6)	27.2 (2.03)	0.17	4.72 (0.32)	3.03 (0.12)	0.13 (0.02)	21.9 (3.51)	1.55 (0.76)	494 (74.6)	3.04 (1.09)	18 (2.92)	46.9 (5.4)	34.7 (12.9)
	AS	7.31 (0.06)	7.06 (0.01)	201 (57.8)	26.6 (3.76)	0.17	4.76 (0.46)	3.07 (0.22)	0.11 (0.03)	25.9 (6.92)	1.16 (0.6)	406 (29.4)	2.83 (1.32)	14.7 (0.44)	46.2 (6.6)	29.6 (10.8)
	C	7.35 (0.05)	7.08 (0.02)	155 (12.5)	26.2 (1.37)	0.2	4.9 (0.15)	3.09 (0.18)	0.15 (0.01)	18.8 (2.4)	2.36 (1.72)	435 (124)	5.03 (2.21)	15.2 (4.21)	43.7 (5.8)	20.8 (8.7)
	ON	7.55 (0.03)	7.07 (0.02)	142 (10.4)	28.8 (0.81)	0.12	4.89 (0.49)	3.23 (0.26)	0.18 (0.04)	15.7 (1.72)	3.23 (1.88)	508 (90)	6.06 (3.03)	18.1 (3.94)	47 (4.5)	27.8 (9.6)
	CS	7.50 (0.05)	7.14 (0.04)	175 (22.5)	28.5 (1.47)	0.19	5.17 (0.17)	3.24 (0.07)	0.18 (0.03)	16.6 (3.69)	1.77 (0.08)	405 (30.6)	4.37 (0.41)	13.5 (1.14)	46.9 (5.3)	30.6 (8.6)
	OF	7.62 (0.03)	7.13 (0.01)	158 (25.7)	27.6 (2.50)	0.2	4.62 (0.67)	3.16 (0.34)	0.16 (0.01)	17.2 (3.07)	1.28 (0.43)	400 (41.1)	3.19 (1.01)	15 (1.78)	43.4 (4.5)	26.3 (8.6)
	OG	7.61 (0.04)	7.27 (0.03)	142 (11.5)	28 (2.91)	0.2	4.95 (0.59)	3.07 (0.31)	0.18 (0.05)	16.5 (3.02)	2.75 (1.52)	473 (80.3)	5.56 (2.43)	16.4 (1.53)	43.6 (5.1)	27.5 (10.1)
Latsch (sandy-loam soil)	**K**	7.18 (0.09)	6.74 (0.29)	85 (1.7)	26.3 (2.7)	0.24	7.82 (1.32)	3.49 (1.04)	0.28 (0.08)	17.0 (5.23)	1.90 (0.48)	775 (213)	2.64 (1.09)	16.8 (2.26)	46.2 (4.7)	37.5 (10.5)
	AS	6.67 (0.07)	6.15 (0.37)	76.3 (8.5)	24.6 (2.4)	0.19	7.48 (1.84)	2.61 (0.18)	0.22 (0.03)	20.2 (7.41)	1.39 (0.44)	473 (183)	3.18 (1.55)	11 (3.49)	45.6 (2.7)	40.2 (7.9)
	C	6.96 (0.15)	6.40 (0.44)	96.7 (12.5)	25.0 (0.8)	0.18	8.32 (1.46)	3.26 (0.86)	0.27 (0.08)	18.6 (5.99)	1.62 (0.49)	799(196)	2.24 (1.31)	16.4 (1.32)	44.7 (2.6)	35.5 (10.7)
	ON	6.91 (0.06)	6.59 (0.11)	86.3 (20.8)	24.7 (1.0)	0.22	7.68 (1.2)	2.78 (0.86)	0.23 (0.08)	20.0 (5.92)	2.21 (0.08)	814 (177)	2.81 (0.65)	18.1 (1.64)	45.5 (4.2)	36.9 (11.1)
	CS	7.18 (0.03)	6.84 (0.03)	120 (28.7)	28.2 (1.2)	0.36	10.1 (1.24)	4.71 (1.3)	0.36 (0.07)	16.1 (1.47)	2.05 (0.76)	986 (263)	2.04 (0.36)	16.7 (2.52)	42.8 (3)	42.2 (8)
	OF	7.03 (0.18)	6.37 (0.12)	102 (24.2)	26.5 (1.6)	0.29	9.21 (0.56)	4.02 (0.59)	0.32 (0.04)	16.8 (1.66)	1.97 (0.19)	831 (100)	2.38 (0.31)	15.5 (1.49)	47.5 (3)	42.1 (8.6)
	OG	6.98 (0.04)	6.41 (0.09)	86 (13.0)	28.1 (1.6)	0.2	7.95 (0.07)	3.89 (0.37)	0.34 (0.01)	13.7 (0.57)	2.2 (0.81)	928 (34.1)	2.38 (0.9)	20.1 (0.58)	47 (3.2)	40.8 (8.3)

Note: Standard deviation is shown in brackets. Abbreviations: EC- electrical conductivity (µS cm^{-1}); WC- water content; WHC- water holding capacity (ml H_2O g^{-1}); SOM- soil organic matter; C- total carbon; N- total nitrogen; C/N- carbon/nitrogen ratio; basal respiration (µg CO_2-C g DM^{-1} h^{-1}); C_{mic}- microbial biomass (µg Cmic g DM^{-1}); qCO_2- metabolic quotient (mg CO_2-C g^{-1} h^{-1}); C_{mic}:C_{org}- microbial carbon:organic carbon ratio; shoot length (cm). K- Untreated control; AS- Ammonium sulfate; C- Compost; ON- Organic nitrogen fertilizer; CS- Compost + fermented slurry from a biogas plant; OF- Fungi-derived fertilizer; OG- Organic nitrogen fertilizer greened.

According to ANOVA, there were no significant differences in the soil basal respiration among treatments or between the two sites. In contrast, microbial biomass did differ significantly among treatments ($F=4.27$; $p<0.01$) and between sites ($F=103.47$; $p<0.01$). The interaction of the two factors regarding microbial biomass (treatment and site) also differed significantly ($F=2.95$; $p<0.05$). Plots amended with organic N fertilizer and compost showed basal respiration values that were significantly lower in Latsch compared to Laimburg. Soil treated with ammonium sulfate and compost combined with fermented slurry were comparable in terms of microbial respiration at the two sites. The carbon-to-nitrogen ratio was significantly different among the treatments ($F=2.723$; $p<0.05$) but not between sites, and there was no significant interaction of the factors. An explanatory box plot obtained from qCO_2 data is reported in Figure 5. The metabolic quotient was similar in untreated control plots and ammonium sulfate treated plots at both sites. Soils from organically fertilized plots indicated that those from Laimburg had higher qCO_2 values and that there was a broader variability within replicates than soils from Latsch. This parameter was significantly different between samples ($F=3.52$; $p<0.01$) according to one way ANOVA results. Taking into account the two factors site and treatment, analysis of variance showed that there was a significant difference between sites ($F=24.17$; $p<0.01$) but not among treatments, and there was no significant interaction between the two factors.

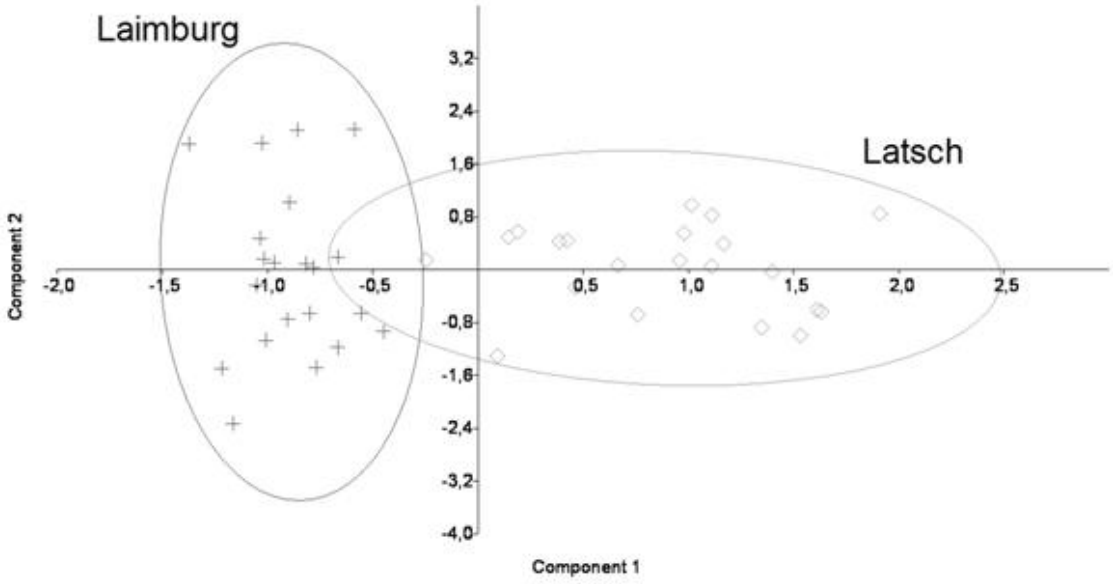
Figure 5. Box plots of metabolic quotients of all soil treatments at both Laimburg (LB) and Latsch (LA).



Abbreviations: LB= Laimburg; LA= Latsch. K- Untreated control; AS- Ammonium sulfate; C- Compost; ON- Organic nitrogen fertilizer; CS- Compost + fermented slurry from a biogas plant; OF- Fungi-derived fertilizer; OG- Organic nitrogen fertilizer greened.

Physico-chemical and ecophysiological parameters were subjected to PCA of log-transformed data (based on covariance matrix) in order to confirm the findings of ANOVA. Figure 6 shows the PCA plotted results where samples were grouped together by 95% concentration level ellipses. The soils were found to be significantly different according to the first axis which explains 47.6 % of the variance (eigenvalue = 0.11; Jolliffe cut-off = 0.011). Laimburg and Latsch samples grouped separately on opposite sides of the graph. Comparable variability within site can be seen.

Figure 6. Plot of PCA performed on log-transformed chemical and ecophysiological parameters, based on covariance matrix (eigenvalue 0.11; bootstrapping 1000; Jolliffe cut-off 0.011)



Shoot length and percent dry matter were measured for 6 plants for each plot. Shoot length was found, according to ANOVA, to be significantly different among management type ($F= 8.5$; $p<0.01$) and between sites ($F= 76.6$; $p<0.01$), and higher values were reported for Latsch soils. In contrast, the dry weight (TS) of the shoots was not significantly different between soils, nor significantly correlated to any physical-chemical property.

9.3.2. Molecular analysis of microbial communities

PCR-DGGE was performed to assess the fungal and bacterial diversity in soils. Dendrograms based on the presence/absence of bands showed that samples collected from the same site clustered together, and that there was no

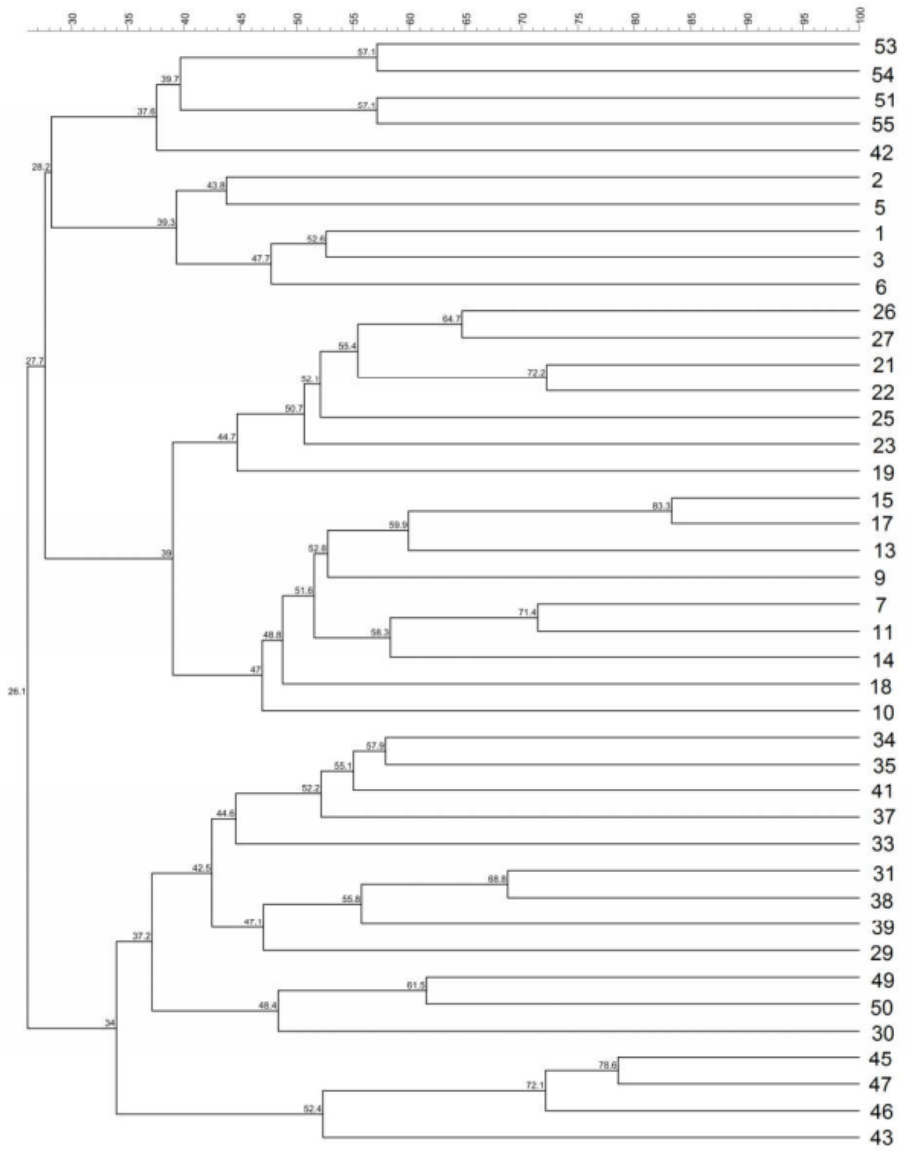
effect of treatment on the grouping (in Figure 7 dendrogram obtained for bacterial communities; in Figure 8 dendrogram obtained for fungal communities).

Figure 7. Dendrogram based on the presence/absence of bands obtained by PCR-DGGE amplification of bacterial 16S rDNA gene. Cluster analysis based on Pearson correlation similarity coefficient was conducted using the criteria optimization and position tolerance of 1%.

Abbreviations: LA- Laimburg; LA- Latsch; K- Untreated control; AS- Ammonium sulfate; C- Compost; ON- Organic nitrogen fertilizer; CS- Compost + fermented slurry from a biogas plant; OF- Fungi-derived fertilizer; OG- Organic nitrogen fertilizer greened. Numbers indicate replicates. LB-K= 1; 2;3. LB-AS= 5; 6; 7; LB-C= 9; 10; 11. LB-ON= 13a; 14a; 15. LB-CS= 17a; 18a; 19a. LB-OF= 21a; 22a; 23b. LB-OG= 25b; 26a; 27b. LA-K= 29; 30; 31. LA-AS= 33; 34; 35; LA-C= 37; 38; 39. LA-ON= 41; 42; 43. LA-CS= 45; 46; 47. LA-OF= 49; 50;

51. LA-OG= 53; 54; 55.

Figure 8. Dendrogram based on the presence/absence of bands obtained by PCR-DGGE amplification of fungal ITS1 region. Cluster analysis based on Jaccard distances was conducted using the criteria optimization and position tolerance of 1%.



Abbreviations: LA- Laimburg; LA- Latsch; K- Untreated control; AS- Ammonium sulfate; C- Compost; ON- Organic nitrogen fertilizer; CS- Compost + fermented slurry from a biogas plant; OF- Fungi-derived fertilizer; OG- Organic nitrogen fertilizer greened. Numbers indicate replicates. LB-K= 1; 2;3. LB-AS= 5; 6; 7; LB-C= 9; 10; 11. LB-ON= 13a; 14a; 15. LB-CS= 17a; 18a; 19a. LB-OF= 21a; 22a; 23b. LB-OG= 25b; 26a; 27b. LA-K= 29; 30; 31. LA-AS= 33; 34; 35; LA-C= 37; 38; 39. LA-ON= 41; 42; 43. LA-CS= 45; 46; 47. LA-OF= 49; 50; 51. LA-OG= 53; 54; 55.

High-throughput sequencing targeting the bacterial 16S rRNA gene performed using Illumina technology resulted in 1,590,042 sequence reads. These were assigned to an OTU according to a 97% sequence similarity level resulting in 326,371 OTUs. 108,791 OTUs were taken into account for the subsequent analysis (a threshold of 1% relative abundance for each sample were considered). Unclassified sequences, in all soils, were 1,144,763, representing 72% of all sequences detected. One-way ANOVA performed on total sequence reads (in each treatment, considering both sites) suggested a significant difference among treatments ($p=0.0001$; Tukey's pairwise test). The same result emerged for total OTUs ($p=0.0001$; Tukey's pairwise test). The number of sequence reads, OTUs, coverage, Shannon index, Invsimpson index and Chao index obtained for each sample are reported in Table 6. Ecological indices revealed that Laimburg soils had a higher diversity of microorganisms than the Latsch soils.

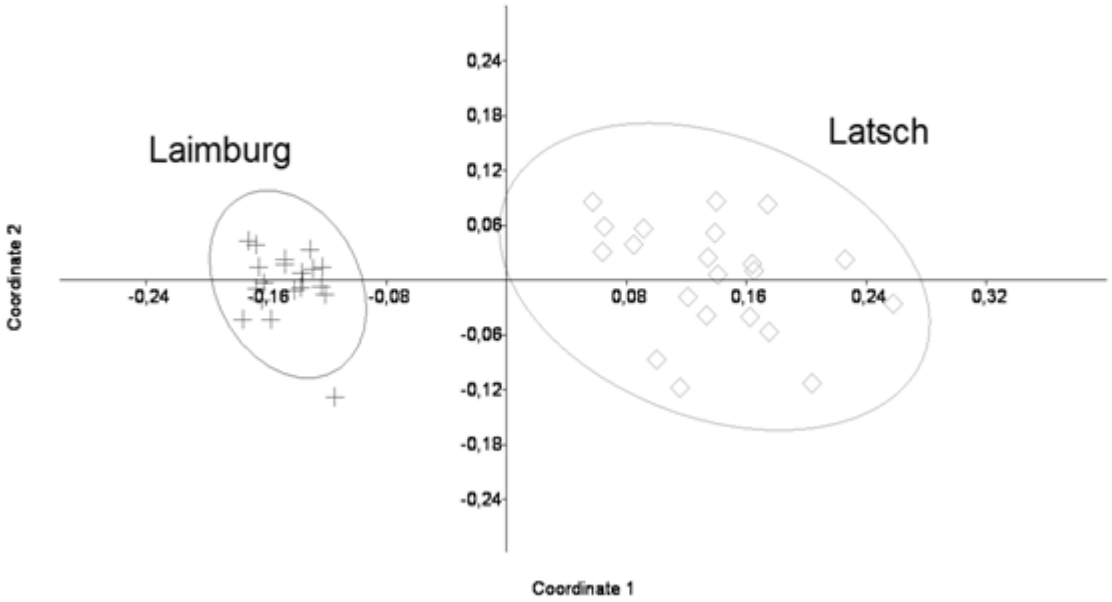
Table 6. Number of sequences analysed, observed diversity richness (OTUs) and diversity indexes of the 16S rRNA gene bacterial libraries obtained for clustering at 97% identity.

Site	Treatment	Reads	OTUs	Coverage	INVSIMPSON	CHAO (x10^2)	SHANNON
Laimburg	**K**	35052	7798	0.84	558 (534; 585)	132 (125; 139)	7.55 (7.53; 7.57)
	AS	40119	8320	0.84	537 (513; 564)	128 (122; 136)	7.55 (7.53; 7.58)
	C	32173	7954	0.81	528 (503; 556)	151 (144; 160)	7.61 (7.59; 7.64)
	ON	37746	8179	0.83	480 (458; 506)	137 (129; 145)	7.51 (7.48; 7.53)
	CS	44163	8595	0.85	522 (499; 548)	126 (120; 133)	7.52 (7.50; 7.54)
	OF	32153	7384	0.82	475 (453; 498)	136 (129; 145)	7.46 (7.44; 7.49)
	OG	38664	8458	0.82	506 (483; 532)	143 (135; 151)	7.54 (7.51; 7.56)
Latsch	**K**	41487	7646	0.86	564 (540; 589)	121 (114; 128)	7.43 (7.41; 7.46)
	AS	34276	7145	0.84	435 (412; 460)	124 (118; 132)	7.42 (7.39; 7.44)
	C	42200	7784	0.85	575 (551; 601)	120 (114; 128)	7.45 (7.43; 7.47)
	ON	35381	6925	0.84	499 (478; 522)	122 (115; 129)	7.35 (7.33; 7.37)
	CS	40535	7639	0.85	580 (556; 605)	121 (114; 129)	7.44 (7.42; 7.47)
	OF	29704	6569	0.82	522 (498; 548)	130 (122; 137)	7.46 (7.44; 7.48)
	OG	46362	8395	0.86	467 (446; 489)	130 (123; 139)	7.35 (7.33; 7.37)

Note: the numbers in brackets are the bounds on the lower and upper 95% confidence intervals for that average. K- Untreated control; AS- Ammonium sulfate; C- Compost; ON- Organic nitrogen fertilizer; CS- Compost + fermented slurry from a biogas plant; OF- Fungi-derived fertilizer; OG- Organic nitrogen fertilizer greened.

Species detection rates ranged from 81% to 86% in all investigated soils, consistent with the obtained rarefaction curves (not shown). NM-DS similarity ordinations on OTU data (considering a threshold of 1% relative abundance for each sample) were performed on the Bray-Curtis similarity matrix. Laimburg samples showed a lower diversity compared to Latsch soils (Figure 9). The two sites are found on opposite sides of the graph and axis 1 explains 93% of the variability.

Figure 9. NM-DS similarity index ordinations on OTU data (considering a threshold of 1% relative abundance for each replicate) performed on a Bray-Curtis similarity matrix (Stress=0.068; Axis 1 0.93; Axis 2 0.06)

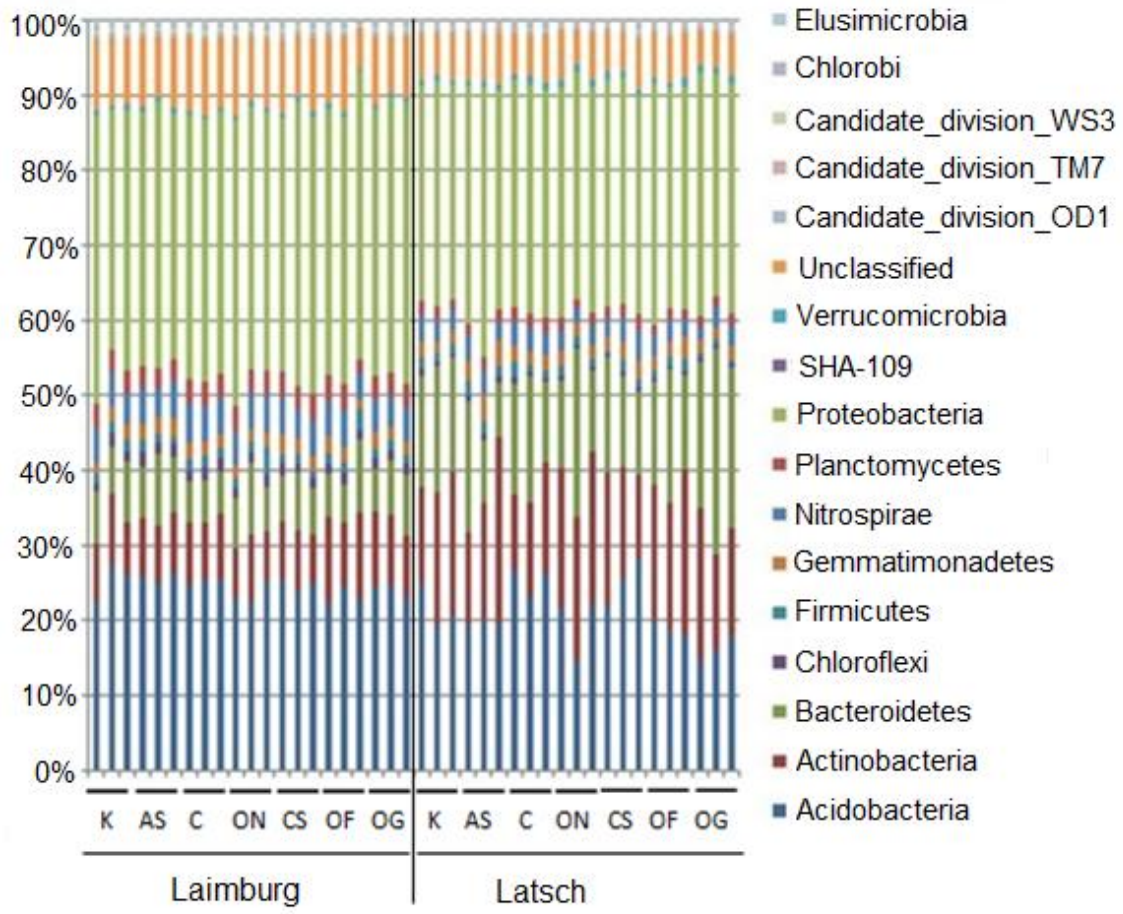


Because of the significant differences in terms of the chemical and ecophysiological properties between the two sites, multivariate analysis was conducted according to the factor treatment. Treatments were first considered separately, and then grouped according to three different categories of fertilizer type: untreated control, mineral fertilizer (ammonium sulfate) and organic fertilizer (compost, organic N fertilizer, compost + fermented slurry, organic fertilizer from fungal biomass). Regarding the Laimburg samples, no grouping according to treatment was recognized at low stress values by NM-DS analysis ($stress_{treatments}$= 0.2072; $stress_{min\_vs\_org}$= 0.2073). Analysing fertilizers by category revealed that mineral and organic fertilizer affected OTU structure differently. These findings were confirmed by subsequent ANOSIM and SIMPER analysis ($p<0.5$ and an overall average dissimilarity of 24.4). In contrast, according to NM-

DS, Latsch samples differed both between treatments ($stress_{treatments}$= 0.1836; $stress_{min\_vs\_org}$= 0.1836) and between treatment type (organically fertilized grouped separately from the others). Subsequent ANOSIM and SIMPER analysis confirmed the results ($p<0.05$; overall average dissimilarity of 36.4). NM-DS plots of mineral *versus* organic treatments are not shown.

In total, 39 bacterial phyla were detected in all soils, and 27,256 OTUs were classified across all taxonomic groups. The phyla *Proteobacteria*, *Actinobacteria*, *Acidobacteria* and *Bacteroidetes* accounted for a minimum of 0.5 % in each of the samples. These phyla comprised between 24% and 71% of all sequences (Figure 10) and in total, 197 genera were found. NP-MANOVA was performed to detect differences among treatments at the two sites, separately. NP-MANOVA performed with Bray-Curtis distance and grouping the two sites separately showed a highly significant difference ($p<0.001$) between Laimburg and Latsch. For Laimburg soils, there was no effect of treatment on the bacterial composition, while Latsch soils were significantly different at the phylum level ($p<0.05$). Furthermore, DNA replicates from each soil type did not always group together. This indicates a heterogenous bacterial community compostion in the soils under investigation.

Figure 10. Comparison of bacterial communities at the phylum level in all soil communities (considering phyla occurring for >0.5% in any sample). Bars represent relative abundance of phyla in each replicate.

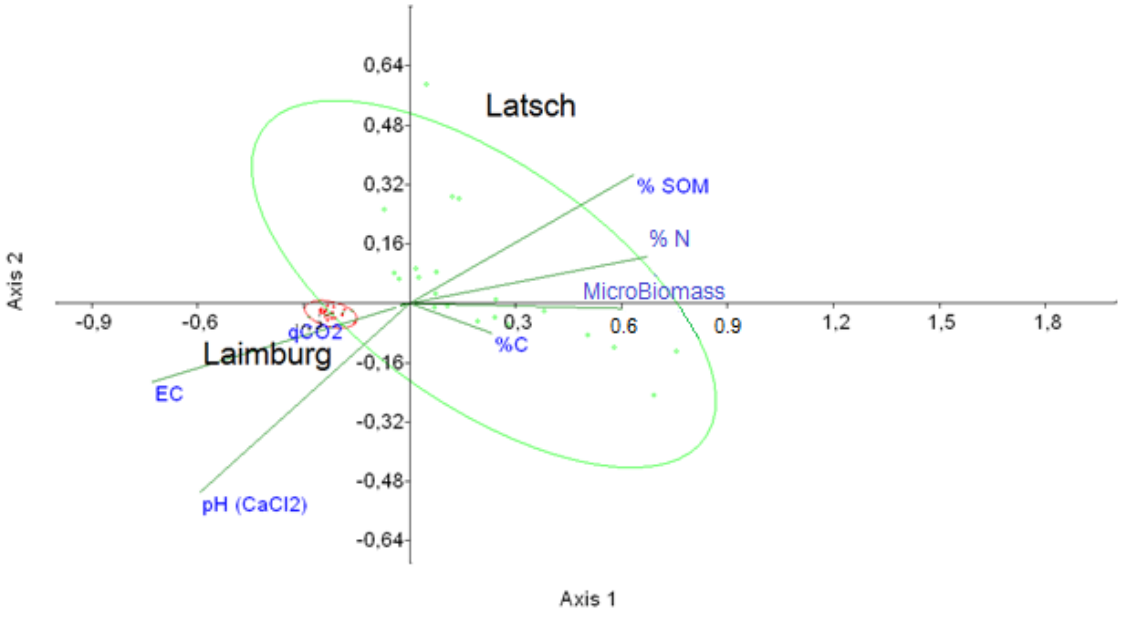


9.3.3. Relationships between abiotic variables, bacterial genera and plant growth parameters

CCA was performed in order to graphically describe the relationships between abiotic variables and bacterial genera along an environmental gradient. Samples included in 95% level concentration ellipses are positioned according to

the maximized relationship between detected genera and abiotic factors. Figure 11 shows the CCA triplot whereby the bacterial communities of Laimburg soils cluster together and tightly in the third quadrant (P< 95%), indicating high similarity; conversely, Latsch samples fell into three different quadrants, revealing greater dissimilarity of the samples. The vectors for EC and pH are oriented in the same direction as the Laimburg samples, indicating that the parameters strongly affected bacterial composition in these samples. On the other hand, vectors representing SOM and N_{tot} point in the direction of the Latsch samples, suggesting that in these soils, amendments and natural supply of organic matter were influential on the bacterial community composition. CCA performed separately on Laimburg and Latsch samples did not show any grouping of samples according to treatment (data not shown).

Figure 11. Triplot of canonical correspondence analysis performed on most frequent genera and physico-chemical and ecophysiological parameters for all soils. Axis 1 explains 77.64% of the variability (eigenvalue 0.05), and axis 2 explains 11.21% (eigenvalue 0.001).



The finding that site exerted a different effect on plant growth parameters according to ANOVA ($p<0.001$) resulted in further investigation being conducted in order to determine any correlations between genus abundance and plant growth. This was done so to try to elucidate bacterial genera in the rhizosphere that were involved in plant growth reduction. Since CCA revealed a relationship between biotic and abiotic variables at the two sites, a comprehensive Pearson correlation matrix was computed. In order to gain more information about the effects exerted by environmental variables on bacterial communities, more detailed analysis was performed at the genus level. Table 7 shows the Pearson correlation coefficients between chemical and ecophysiological parameters with

the genera occurring at a frequency of >1% in each of the samples. Table 8 shows the correlation coefficients between chemical and ecophysiological parameters with the genera having the highest correlations to plant growth.

Table 7. Correlation Pearson coefficients between physico-chemical and ecophysiological parameters with the most abundant genera in all soils (genera occurring at a frequency of >1% in any sample).

Genus	Correlation coefficient									
	pH	**EC**	**WHC**	**% SOM**	**%C**	**%N**	**SBR**	**C_{mic}**	**qCO_2**	**SL**
Arenimonas	-0.52	-0.55	0.3	0.58	0.29	0.63	0.05	0.66	-0.35	0.59
Arthrobacter	0.1	0.02	0.04	-0.08	-0.08	-0.03	0.21	0.02	0.12	-0.05
Bacillus	0.08	0.04	-0.384	-0.39	-0.33	-0.26	-0.09	-0.45	0.14	-0.21
Blastocatella	-0.18	-0.12	0.34	0.44	0.3	0.39	0.06	0.52	-0.26	0.41
Candidatus Entotheonella	0.03	-0.05	0.03	0.11	0.14	0.18	0.08	0.15	-0.01	0.03
Duganella	-0.33	-0.5	0.11	0.26	0.09	0.39	-0.06	0.27	-0.26	0.42
Ferruginibacter	-0.63	-0.59	0.46	**0.78**	0.43	**0.77**	0.03	**0.8**	-0.46	**0.74**
Flavobacterium	-0.43	-0.54	0.15	0.38	0.22	0.56	0.07	0.45	-0.26	0.52
Friedmanniella	-0.61	-0.68	0.5	0.66	0.16	0.63	-0.03	0.53	-0.38	**0.7**
Gaiella	-0.16	-0.21	0.28	0.33	0.27	0.44	0.14	0.53	-0.21	0.37
Gemmatimonas	-0.58	-0.3	0.21	0.43	0.13	0.38	-0.12	0.33	-0.32	0.57
Haliangium	-0.14	0.04	0.05	0.15	0.27	0.28	0.12	0.24	-0.04	0.22
Lysobacter	0.38	0.22	-0.2	-0.38	-0.13	-0.25	0.34	-0.30	0.50	-0.41
Nitrospira	-0.6	-0.44	0.43	0.5	0.03	0.46	-0.07	0.31	-0.27	0.64
Ohtaekwangia	0.2	0.19	0.01	-0.02	0.25	0.14	0.24	0.11	0.16	-0.04
Pedobacter	-0.34	-0.41	0.21	0.38	0.27	0.49	-0.02	0.40	-0.29	0.42
Pir2_lineage	0.67	0.68	-0.37	-0.65	-0.18	-0.48	0.09	-0.55	0.40	-0.60
Polaromonas	0.61	0.68	-0.3	-0.67	-0.23	-0.53	0.16	-0.51	0.43	-0.66
Rhodanobacter	**-0.7**	-0.46	0.13	0.39	-0.03	0.29	-0.09	0.07	-0.10	0.51
Rivibacter	0.14	0.17	-0.04	0	0.09	0.1	0.23	0.19	0.13	0.01
Variovorax	-0.61	-0.54	0.38	0.65	0.4	0.71	0.1	**0.74**	-0.36	0.68

Note: Correlations (r) with plant growth > 0.7 or < -0.7 shown in bold type. Abbreviations: EC- electrical conductivity (µS cm^{-1}); WHC- water holding capacity (ml H_2O g^{-1}); SOM- soil organic matter; C- total carbon; N- total nitrogen; SBR- soil basal respiration (µg CO_2-C g DM^{-1} h^{-1}); C_{mic}- microbial biomass (µg C_{mic} g DM^{-1}); qCO_2- metabolic quotient (mg CO_2-C g^{-1} h^{-1}); SL- shoot length (cm).

Table 8. Correlation Pearson coefficients between physico-chemical and ecophysiological parameters with the genera most highly correlated with plant growth.

Genus	Correlation coefficient									
	pH	**EC**	**WHC**	**% SOM**	**%C**	**%N**	**BR**	**C_{mic}**	**qCO_2**	**SL**
Acidiferrobacter	0.66	**0.77**	-0.47	**-0.71**	-0.21	-0.61	-0.05	-0.65	0.34	**-0.70**
Candidatus Methylomirabilis	0.63	**0.83**	-0.42	**-0.70**	-0.23	-0.59	0.02	-0.59	0.35	**-0.73**
Candidatus Microthrix	-0.54	-0.56	0.57	**0.77**	0.44	**0.78**	0.13	**0.79**	-0.37	**0.75**
Conexibacter	-0.66	-0.58	0.46	**0.72**	0.23	0.60	0.06	0.68	-0.39	**0.71**
Cupriavidus	**0.73**	0.85	-0.42	**-0.76**	-0.27	-0.64	0.05	-0.64	0.44	**-0.77**
Demequina	**-0.70**	-0.59	0.42	0.69	0.37	**0.71**	0.09	**0.70**	-0.34	**0.72**
Dokdonella	**-0.76**	-0.51	0.44	**0.75**	0.34	0.66	0.06	**0.70**	-0.38	**0.74**
Fictibacillus	0.65	**0.76**	-0.51	-0.73	-0.21	-0.59	-0.07	-0.66	0.33	**-0.75**
Kribbella	**-0.76**	-0.57	0.40	**0.71**	0.16	0.55	0.02	0.64	-0.41	**0.72**
Microlunatus	**0.74**	-0.55	0.42	**0.72**	0.24	0.62	0.11	**0.70**	-0.35	**0.71**
Mycobacterium	-0.76	-0.66	0.46	0.83	0.29	0.69	-0.01	**0.81**	-0.50	**0.78**
Nakamurella	**-0.71**	-0.61	0.47	**0.74**	0.27	0.67	0.14	**0.76**	-0.36	**0.75**
Ornatilinea	0.66	0.67	-0.44	**-0.73**	-0.22	-0.65	0.09	-0.61	0.42	**-0.74**
Patulibacter	-0.66	-0.55	0.51	**0.73**	0.20	0.65	0.14	**0.71**	-0.33	**0.72**
Phycicoccus	0.69	**0.76**	-0.43	**-0.77**	-0.28	-0.66	0.04	-0.66	0.42	**-0.73**
Pirellula	-0.58	-0.64	0.56	**0.71**	0.12	0.54	0.02	0.65	-0.38	**0.71**
Pseudonocardia	**-0.70**	**-0.73**	0.47	**0.73**	0.25	**0.70**	-0.04	0.62	-0.42	**0.73**
Schlesneria	-0.69	-0.63	0.37	**0.73**	0.27	0.67	-0.02	**0.70**	-0.41	**0.75**
Singulisphaera	**-0.70**	-0.65	0.41	0.65	0.11	0.59	0.08	0.60	-0.33	**0.74**
Solirubrobacter	-0.63	-0.60	0.53	**0.72**	0.22	0.59	0.02	0.68	-0.43	**0.72**
Streptoalloteichus	-0.67	-0.66	0.50	**0.75**	0.18	0.59	-0.04	0.67	-0.46	**0.75**

Note: Correlations (r) with plant growth > 0.7 or < -0.7 shown in in bold type. Abbreviations: EC- electrical conductivity (µS cm^{-1}); WHC- water holding capacity (ml H_2O g^{-1}); SOM- soil organic matter; C- total carbon; N- total nitrogen; SBR- soil basal respiration (µg CO_2-C g DM^{-1} h^{-1}); C_{mic} - microbial biomass (µg C_{mic} g DM^{-1}); qCO_2- metabolic quotient (mg CO_2-C g^{-1} h^{-1}); SL- shoot length (cm).

No strong correlation between OTUs (considering a threshold of 1% relative abundance for each sample, OTUs accounted were 108,791) and basal respiration was observed (r= 0.23). Shoot length was significantly different among amendment types and it was found to be negatively correlated to pH (r= -0.73)

and to the metabolic quotient (r= -0.54). In contrast, shoot length was positively correlated with (r= 0.8), total nitrogen (r= 0.7) and microbial biomass (r= 0.7). In Table 9, correlation coefficients between shoot length and OTUs, with the most relevant chemical and ecophysiological parameters are reported.

Table 8. Correlation Pearson coefficients between physico-chemical and ecophysiological parameters with shoot length and OTU.

	Correlation coefficient										
	pH	**EC**	**WHC**	**% SOM**	**%C**	**%N**	**SBR**	**C_{mic}**	**qCO_2**	**SL**	**OTU**
SL	-0.75	-0.73	0.56	0.86	0.48	0.79	-0.21	0.77	-0.75	-	-0.47
OTU	0.55	0.55	-0.45	-0.52	-0.04	-0.33	0.23	-0.35	0.48	-0.47	-

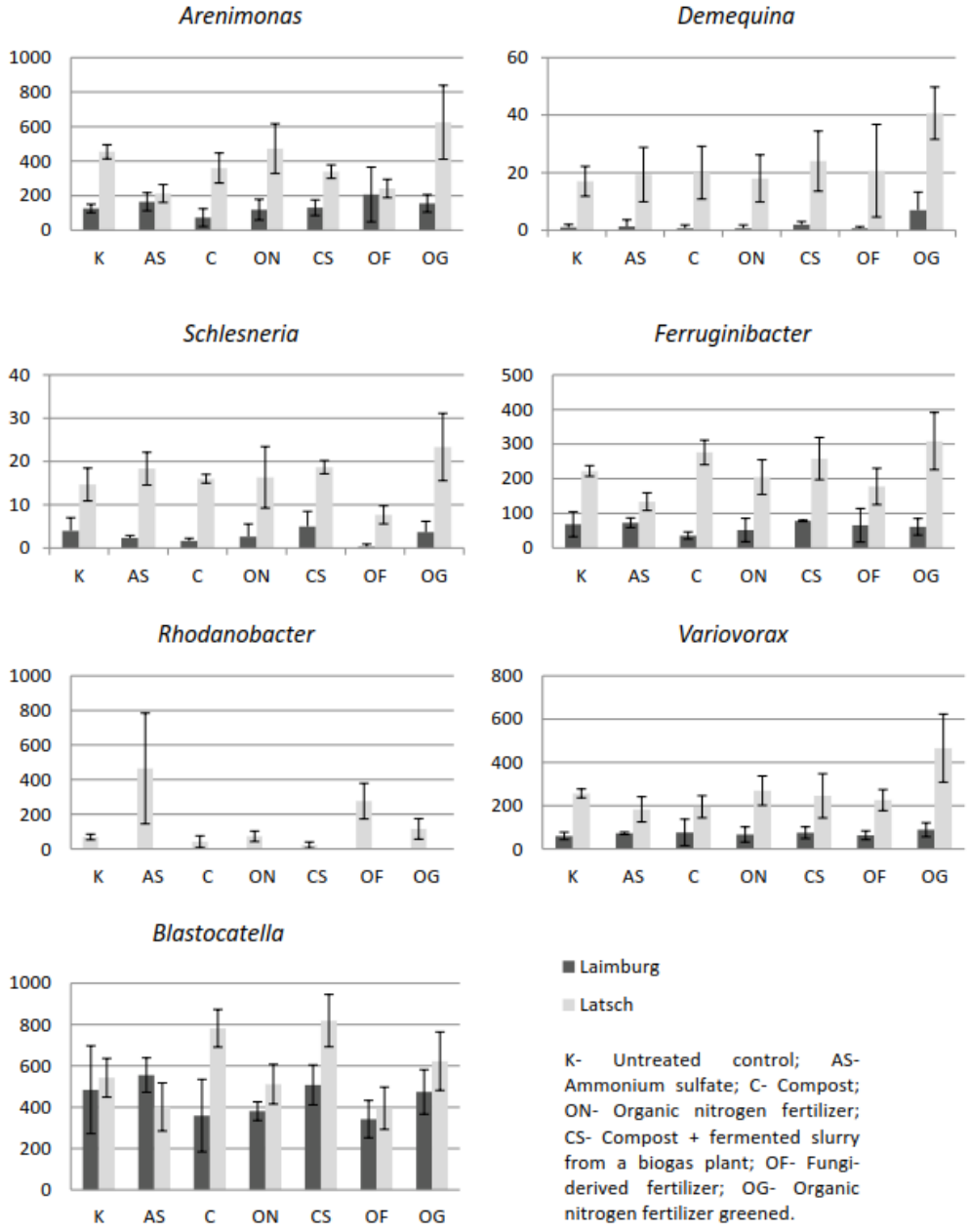
Abbreviations: EC- electrical conductivity (µS cm^{-1}); WHC- water holding capacity (ml H_2O g^{-1}); SOM- soil organic matter; C- total carbon; N- total nitrogen; SBR- soil basal respiration (µg CO_2-C g DM^{-1} h^{-1}); C_{mic}- microbial biomass (µg C_{mic} g DM^{-1}); qCO_2- metabolic quotient (mg CO_2-C g^{-1} h^{-1}); SL- shoot length (cm).

A two-way ANOVA was performed in order to detect any effect exerted by treatments on the most frequently occurring genera and on genera highly correlated with plant growth (Table 9). The genera *Rhodanobacter* ($p<0.01$), *Blastocatella* ($p<0.01$), *Schlesneria* ($p<0.01$), *Arenimonas* ($p<0.05$), *Variovorax* ($p<0.01$), Demequina ($p<0.05$), *Ferruginobacter* ($p<0.05$) were the genera significantly affected by the treatments (Figure 12). The others differed significantly between sites, except for *Ohtaekwangia*, *Bacillus*, *Rivibacter*, *Arthrobacter* and *Haliangium*, which were ubiquitous in the investigated soils.

Table 9. Two-way ANOVA (LSD post-hoc test; p ≤ 0.05) performed on most abundant and on genera most highly correlated with plant growth. Abundance values reported for the two sites. Note: ns- not significant, *P > 0.05, **P < 0.01; ***P < 0.001.

Genus	Abundance		Two way ANOVA		
	Laimburg	Latsch	P-value SITE	P-value TREATMENT	P-value INTERACTION
Acidiferrobacter	206	6	**	ns	ns
Arenimonas	976	2705	**	*	**
Arthrobacter	1199	1190	ns	ns	ns
Bacillus	1452	1197	ns	ns	ns
Blastocatella	3104	4075	**	**	**
Candidatus_Entotheonella	1147	1234	ns	ns	ns
Candidatus_Methylomirabilis	826	3	**	ns	ns
Candidatus_Microthrix	103	564	**	ns	ns
Conexibacter	1521	3694	**	ns	ns
Cupriavidus	410	7	**	ns	ns
Demequina	40	479	**	*	ns
Dokdonella	321	2866	**	ns	ns
Duganella	667	3199	**	ns	ns
Ferruginibacter	435	1582	**	*	**
Fictibacillus	125	3	*	ns	ns
Flavobacterium	3271	17286	**	ns	ns
Friedmanniella	606	4281	**	ns	ns
Gaiella	1014	1415	**	ns	ns
Gemmatimonas	1820	2546	**	ns	ns
Haliangium	1022	1100	ns	ns	ns
Kribbella	23	270	**	ns	ns
Lysobacter	994	551	*	ns	ns
Microlunatus	269	1341	**	ns	ns
Mycobacterium	953	4911	**	ns	ns
Nakamurella	626	4187	**	ns	ns
Nitrospira	6353	4296	**	**	ns
Ohtaekwangia	3351	3204	ns	ns	ns
Ornatilinea	1313	78	**	ns	ns
Patulibacter	261	874	**	ns	ns
Pedobacter	231	2012	**	ns	ns
Phycicoccus	775	89	**	ns	ns
Pir2_lineage	1618	917	**	ns	ns
Pirellula	182	545	**	ns	ns
Polaromonas	1052	287	**	ns	ns
Pseudonocardia	57	400	**	ns	ns
Rhodanobacter	0.70	1065	**	**	**
Rivibacter	1325	1299	ns	ns	ns
Schlesneria	59	345	**	**	ns
Singulisphaera	42	209	**	ns	ns
Solirubrobacter	202	1041	**	ns	ns
Streptoalloteichus	80	512	**	ns	ns
Variovorax	503	1841	**	**	*

Figure 12. Mean abundances of the genera showing both the highest correlations with plant growth and significantly different according to treatment, as detected by two-way ANOVA (LSD post-hoc test; $p \leq 0.05$) in all soils. Bars indicate standard error.



9.4. Discussion

The differences in the physico-chemical and ecophysiological parameters of Laimburg and Latsch soils resulted in the two sites being incomparable for an assessment of soil health based on microbiological indicators. First of all, in order to obtain more information regarding stress levels in the investigated soils, the metabolic quotient was calculated. Organically fertilized soils from Laimburg were found to have a higher qCO_2 than those from Latsch. This finding suggests the presence of dormant microorganisms in Latsch soils that could potentially become active after the addition of a carbon source to the soil. Conversely, treatments seemed not to exert any change in metabolic quotient, except for the high value reported in the Laimburg untreated plot. This finding is in line with what has been previously reported by Insam and Merschak (1995): organically fertilized soils show a lower qCO_2 than mineral fertilized soils, indicating a stress relief by organic fertilization. Furthermore, there was also higher variation within replicate treatments in Laimburg.

Bacterial and fungal communities in the soils were more affected by site than by treatment. This finding is consistent with other studies which revealed that at a single sampling date, rootstock genotype and sampling site are important factors affecting microbial rhizosphere community composition (Rumberger *et al*., 2004; Spath *et al*., 2015; Yao *et al*., 2006a). The investigated soils were sampled in orchards affected by ARD, for which the fungal component of the disease complex has been well documented (Rumberger *et al*., 2007; Wilson *et al*., 2004; Franke-Whittle *et al*., 2015; Caputo *et al*., 2015; Mazzola, 2007; Tewoldemedhin *et al*., 2011). In contrast, the scarcity of literature

concerning the function of many bacterial genera in soil, and in particular in ARD, justified the focus on bacterial communities. Taking these considerations into account, high-throughput sequencing targeting the 16S rRNA gene of bacteria was performed in order to more closely investigate the bacterial communities in the soils resulting from different amendments.

Rarefaction curves showed on the one hand the huge diversity of the microbiota, and on the other hand they also indicated a comprehensive sequence coverage. Other studies applying high-throughput sequencing report coverage rates ranging from 0.63-0.68 (Franke-Whittle *et al*., 2015), to 0.72 and 0.90 (Sun *et al*., 2014b; Uhlik *et al*., 2013; Zhang *et al*., 2013), supporting the good data quality in this study. Shannon diversity values were also consistent to those obtained in similar studies on apple orchard soils (Franke-Whittle *et al*., 2015; Sun *et al*. 2014b; Zhang *et al*. 2013).

As pointed out by Miller and Miller (2000), organic matter application to croplands can affect soil properties, but the effects generally may not be detectable in the short term. Thus, different soils can react differently shortly after fertilizer application. Organic amendments release nutrients slowly, causing an increase in crop yields in subsequent years (Diacono & Montemurro, 2010). Thus, the slow responses of soils to organic fertilization make it difficult to value shifts in ecophysiological and microbiological parameters over a short period.

Correlation of bacterial genus abundance in soils with plant growth was computed in order to determine which bacterial genera were potentially involved in ARD. Considering the differences between the two sites (in terms of geographic position, climatic and morphologic features) and among the treatments (which exerted contradictory effects on bacterial communities and on

ecophysiological parameters), conclusions on relationships between these data cannot be drawn definitively. Findings revealed that, in this study, *Rhodanobacter*, *Blastocatella*, *Arenimonas*, *Variovorax*, *Ferruginobacter, Demequina* and *Schlesneria* are the genera most affected by treatment type in terms of abundance. Furthermore, only *Ferruginobacter*, *Demequina* and *Schlesneria* were strongly correlated with plant growth. *Blastocatella*, *Arenimonas, Demequina* are genera that have been rarely reported in soils. *Demequina* was isolated from marine organisms and it was demonstrated to produce α-amylase (Al-naamani *et al*., 2015). This enzyme is involved in starch degradation to glucose and dextrine; *Demequina* abundance in Latsch soils suggests a higher content of plant residues compared to Laimburg. *Blastocatella* was predominant in Latsch plots, and notably in C and CS treatments: this evidence seems to be confirmed by the findings of Gao *et al*. (2016), who recently detected this genus in sludge samples. *Arenimonas* is significantly higher in Latsch greened plot: this finding is consistent to its copiotrophic and fast-growth characteristics identified by Li *et al*. (2014), who reported its occurrence in early growth-stage of a maize cultivar. *Duganella* is a rarely described genus that has been reported in chitin-treated soils (Cretoiu *et al*., 2014). The same observations have been made for species of the genus *Ferruginibacter*, which are capable of hydrolyzing certain types of organic matter. Both genera belong to the family *Chitinophagaceae* (Lim *et al*., 2009). These findings were partly confirmed in this study, whereby Latsch plots amended with fertilizer from animal sources reported a higher abundance of *Duganella* and *Ferruginobacter* in comparison to other treatments. Chitinolytic microorganisms, however, are often found as gut symbionts in soil animals (Borkott and Insam, 1991) which makes their quantitative extraction difficult. The

detection of *Duganella* in high abundance in the investigated soils was unexpected. Li *et al*. (2004) detected the genus in Chinese forest soils, while Bajaj *et al*. (2012) isolated *Duganella spp*. from selenium-contaminated soils, suggesting a tolerance towards Se or an ability to reduce Se (IV) to Se (0). The selenium-tolerance of *Duganella* cannot be confirmed in this study (values of selenium content were not measured), although it is possible to suppose that its growth could be a response to a past selenium-fertilization typical for forage crops. *Rhodanobacter* was detected only in Latsch soils, where its numerical significance differed among treatments (AS>OF>OG>ON>K>C>CS). High abundance in plots treated with ammonium sulfate is in line with the denitrification capability of species of the genus, especially of *R. denitrificans* (Prakash *et al*., 2012). *Variovorax* is a genus known to contain commonly recognized plant growth–promoting bacteria (Bertrand *et al*., 2001; Sanguin *et al*., 2015). In the current study, this genus was more abundant in Latsch than in Laimburg soils and in OG treated soils (in Laimburg and in Latsch) than in other soil amendments. Nevertheless, it was not correlated to plant growth, suggesting that its beneficial role is dependent on various environmental and biotic factors. Correlation analysis revealed a positive relationship between *Schlesneria*, *Patulibacter*, *Streptoalloteichus* and *Pirellula* and plant growth, consistent again with a higher abundance in Latsch soils.

Lysobacter is a genus containing plant growth promoting bacteria which are able to produce antifungal compounds and control *Pythium* pathogenicity (Folman *et al*., 2004). According to Sun *et al*. (2014), *Lysobacter* is induced by high levels of pathogenic fungi in the soil, indicating that genus numbers increase in soils under biological attack. Members of this genus have been reported in

other ARD studies to exert a positive effect on plant growth (Franke-Whittle *et al*., 2014), however, in the present study, *Lysobacter* abundance was negatively correlated to plant growth (r= -0.41). *Nitrospira spp*. abundance was found to be affected both by site and treatment. Li *et al*. (2015) pinpointed that long-period cultivation changed microbial communities and structure, demonstrating that the genus *Nitrospira* (along with other genera) was significantly increased by agricultural management. This genus was not one of the genera most correlated to plant growth (r= 0.68), and these findings contradict previous studies where *Nitrospira* was found to be negatively correlated to plant growth (Franke-Whittle *et al*., 2015). The findings revealed that the genus *Mycobacterium* was strongly and positively correlated to plant growth, which was consistent with the high abundance reported in Latsch soils. *Nakamurella* and *Dokdonella*, like *Mycobacterium*, are psychrotolerant bacteria belonging to PAH-degrading bacterial consortia (Bacosa *et al*., 2015). Both genera were positively correlated to plant growth (r= 0.75 and r= 0.74, respectively), and occurred in higher numbers in Latsch soils.

The genera *Microlunatus*, *Blastococcus*, *Pseudonocardia*, *Solirubrobacter*, *Brevundimonas*, *Pseudomonas* and *Stenotrophomonas* have been reported to vary significantly in terms of relative abundance in soils subjected to organic and conventional farming systems (Li *et al*., 2012). *Microlunatus* was found to be positively correlated to plant growth in the present study (r= 0.71), consistently with a higher abundance in Latsch soils. *Pseudonocardia* is reported to have a mutual relationship with the fungus-growing ant (*Trachymyrmex septentrionalis*) and many other organisms. The organism is capable of producing antibiotics that specifically suppress diverse

pathogenic bacteria thus protecting fungal communities (Mueller U.G. *et al*., 2010; Apinya T. *et al*., 2015; Barke *et al*., 2010). *Pseudonocardia* was predominant in Latsch and it showed a positive correlation with plant growth.

Correlation analysis also revealed a positive relationship between *Schlesneria*, *Patulibacter*, *Streptoalloteichus* and *Pirellula* and plant growth, consistent again with a higher abundance in Latsch soils. *Schlesneria spp*. were significantly dominant in OG plots at both sites. *Kribbella* is reported to have antibiotic-producing abilities which have strong inhibitory activity toward *Botrytis spp*., *Rhizoctonia solani* and *Pyricularia oryzae* (Sun *et al*., 2014; Wang *et al*., 2003). These findings match with the positive correlations found with plant growth and dominance in Latsch soils. *Polaromonas* were found in higher numbers in Laimburg soils which were characterized by higher pH values, confirming a previous study which suggested the pH-dependence of this genus (Bartram, 2014). *Polaromonas* behavior is not well known, this genus is rarely found in soils.

Since *Pseudomonas* plays a controversial role in ARD, its occurrence in the investigated soils was analyzed. *Pseudomonas* was slightly and positively correlated to plant growth (r=0.34) and was not detected in high numbers, confirming what was observed by Catska *et al*. (1982). These authors reported that the densities of *Pseudomonas* species in the apple rhizosphere decreased over the years after replanting. *Pseudomonas* has been reported to both cause and suppress ARD in northwest USA (Mazzola *et al*. 2002), and soil suppressiveness supposedly depends on the capability of this genus to produce diverse antibiotics and hydrogen cyanide (Rumberger *et al*. 2007; Yang *et al*. 2012; Mazzola *et al*. 2002).

Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes were the dominating phyla of all soil communities, confirming the findings of previous studies (Franke-Whittle *et al*., 2015; St. Laurent *et al*. 2008; Sun *et al*. 2014). The soils under investigation contained significantly different microbial populations at the phylum and genus levels. Soil treatments in this study caused a shift in bacterial populations (based on OTUs analysis) only if treatments were grouped according to fertilizer typology (mineral *vs* organic). Focussing on phylum and genus levels, seven genera were found to be significantly influenced by treatments in terms of abundance. When analysing dominant genera (according both to site and to treatment), it was not possible to directly relate treatments to the growth of apple plants. Potentially, there were differences in the soils that were not able to be detected in this study, such as variations in minor populations, or in unclassified sequences. In support of this hypothesis, it seems to be pertinent to refer to a key-stone species concept, which implies that a less abundant species can exert an non-proportional effect on its environment (Paine, 1969; Rosier *et al*., 2014). This concept was recently applied in microbiology (Hajishengallis *et al*., 2012) by proposing the “Keystone-Pathogen Hypothesis” (Rosier *et al*., 2014). According to this hypothesis, a low-abundance species could trigger a shift in quantity and composition of the normal microbiota, making the host more responsive to other organisms. Along with this theory, Trosvik *et al*. (2010) suggested that in order to interpret community-wide interactions, it is advisable to take into account assemblages of organisms and to infer interactions based on combinations of these assemblages. The above-mentioned theory seems to be conducive to better interpretation of the results obtained. In this study, only a few genera of low abundance showed a positive correlation with

plant growth. Those genera are not commonly described as microorganisms involved in biocontrol. Furthermore, the numerous unclassified sequences precluded a complete description of the soils. As suggested by Stecher *et al*. (2013), lack in sequenced genomes of many species could make the ecological interpretations of these inter-relationships a challenging task. Considering the present study, there could have been a few species within minor populations or unclassified sequences, that may have been acting as key-stone species. According to this theory, and in line with previous findings, a site's microbiome could be altered by novel species (introduced via amendment) in different ways. Regarding this study, it is plausible to assert that many assemblages are unknown, and this lack of knowledge makes interpretations of interactions among bacterial species difficult. Since bacterial response to treatments was found to vary differently at the two sites, it is possible that undetermined environmental and biotic factors played an important role in ecological interactions.

10. Conclusions

Notwithstanding the importance of soil quality information, many models depicting the related issues, embody limited soil expertise. Assessment of soil quality deals with data artifacts and lack of data, absence of clear reference points or baseline values, model omissions and validation challenges (Andrews and Carroll, 2001; McBratney *et al*., 2014). These limitations were also found in the proposed case studies. This work confirms that simply measuring a single or specific soil property to infer soil quality is insufficient. Accurate, repeatable, systematic, and detailed measurements of soil parameters can enhance interpretation and comparability between sites.

In the case of Cu-polluted agrosystem under ecological restoration, soil resilience was effectively tested using biochemical and microbiological indicators (particular interest was paid for enzymatic activities). This level of detail and the focus on soil functionality, was revealed to provide consistent indication which was able to properly describe nutrients cycling and dynamics within a restoration process. Podere Pantaleone case study indicates, confirming previous studies, that biological responses are more importantly explained by other soil properties than soil heavy metal concentration (in particular carbon soil enrichment is shown as a leading factor). High level of total organic carbon in soil under re-naturalization, compared to that of cultivated soils, and microbial changes in response to heavy metal stress did not affect the ecological function of the site. Diversity increase and change in composition of bacterial communities in contaminated soil suggest high adaptability of bacteria to copper contamination stress. These shifts confirm the hypothesis that this mechanism is an attempt to

protect the bacterial community against functional decline, enhancing soil resilience. To the contrary, fungal communities seemed less reactive to copper than bacteria: a slower growth due to xenobiotic contamination is counteracted by the improvement of soil biochemical properties following long-term natural C enrichment. Findings of this study suggest that soil functioning is properly described by the physiological status of the microbial community. Soil disturbance can be reduced by applying conservation agriculture-based strategies which foster soil organic matter and therefore the many ecosystem services connected to it. The effectiveness of these strategies can be properly monitored by using microbiological indicators, in particular enzymatic activities and ecophysiological parameters.

As concerns the managed agrosystems affected by replant disease, soil health was assessed using biological indicators able to deeply investigate soil microbiota, focussing on soil diversity. Since the complex etiology of ARD, linking bacterial communities, amendment type and ARD symptoms was difficult. High-throughput sequencing confirmed the hypothesis that soil communities at the two sites were diverse and that, indeed, bacterial communities did not differ significantly according to treatment type, but they did according to site. Correlation of bacterial genus abundances with plant growth supplied novel information about the involvement of certain microorganisms in ARD. Since soil functions carried out by bacteria are still under debate and investigation, high-throughput sequencing can be proposed as a suitable method to deeply describe soil microbiological communities. The complexity in assessing soil health and soil suppressiveness justifies the need of interrelating all biotic and abiotic components. As well, a deeper investigation of the soil microbiota and

relationships among microbial assemblages in soils in order to gain a more complete knowledge about soil functioning, is required. The high detail level provided by next-generation sequencing technologies faces to the lack of information about the bacterial component in soils. The findings of this case study revealed that a few species can exert important shifts in soil microbiota composition which, in turn, could reflect on changes in soil functioning.

The two case studies corroborate the hypothesis that biochemical and biological properties are the more suitable indicators in assessing alterations in soil quality and soil degradation. Even though soil complexity makes the assessment of soil functionality difficult, biological indicators could give a deep insight to soil stability, due to biodiversity; moreover they could better identify specific groups accomplishing a given function. High-throughput DNA sequencing and multivariate statistical analysis are demonstrated to be effective tools in providing accurate description of microbial component of soils and in relating different soil features.

The outlook of this research project calls for a deeper investigation of soil microbial components (by means of metagenomics techniques and next-generation DNA sequencing) and for a comprehensive characterization of soil ecological networks (by applying detailed models and multivariate analysis). Microbiological indicators are demonstrated to be sensitive and accurate parameters suitable to describe soil functionality and dynamics along spatial and temporal gradients. This work demonstrates, more broadly, that diverse soil features related to quality assessment, could be properly investigated by applying methodologies which reach different levels of detail. Nevertheless, microbial-

derived features have necessarily to be integrated into a more complex ecological network, in order to give a reliable soil quality assessment.

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