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CHEMICAL, BIOCHEMICAL AND MICROBIOLOGICAL INDICATORS TO ASSESS SOIL QUALITY IN TEMPERATE AGRO-ECOSYSTEMS

Presentata da: Dr.ssa Caterina Giacometti

Coordinatore Dottorato:

Prof. Giovanni Dinelli

Relatore:

Prof. Claudio Marzadori

Correlatore:

Dott. Luciano Cavani

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CFE	Chloroform-fumigation extraction
C_{mic}	Microbial biomass carbon
CV	Coefficient of variance
DMSO	Dimethyl sulfoxide
DRIFTS	Diffuse reflectance Fourier transform spectroscopy
EL	Electical conductivity
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
FID	Flame ionization detector
GC	Gas cromatography
GLASOD	Global Assessment of Soil Degradation
INFT	2-*p*-iodo-nitrophenyl formazan
INT	2-*p*-iodo-nitrophenyl tetrazolium chloride
ISO	International Organization for Standardization
IUBMB	International Union of Biochemistry and Molecular Biology
LSD	Least significant difference
LTE	Long-term experiment
MIR	Mid-infrared
MOM	Mobile organic matter
MUF	4-methylumbelliferone
N_{mic}	Microbial biomass nitrogen
N_{min}	Mineral nitrogen
PCA	Principal component analysis
PLFA	Phospholipid fatty acids
PLS	Partial least square regression analysis
PNG	*p*-nitrophenyl-β-glucoside
PN	*p*-nitrophenol
qCO_2	Metabolic quotient
r	Pearson's product moment correlation coefficient
R^2	Coefficient of determination
$RMSE_{CV}$	Root mean square error of cross validation

LIST OF ABBREVIATIONS

RMSEP	Root mean square error of prediction
RPD	Ratio of standard deviation to standard error of prediction
RPD_{CV}	Ratio of standard deviation to standard error of cross validation
RUF	Relative units of fluorescence
SOM	Soil organic matter
TOC	Total organic carbon
TN	Total nitrogen
tyr	Tyrosine
USDA	United States Department of Agriculture
WEOC	Water extractable organic carbon
WEOM	Water extractable organic matter
WHC	Water holding capacity

ENZYMES ABBREVIATIONS

β-GLU	β-1,4-glucosidase
α-GLU	α-1,4-glucosidase
N-AG	*N*-acetyl-β-glucosaminidase
β-XYL	β-1,4-xylosidase
β-CEL	β-1,4-cellobiosidase
SULF	Arylsulfatase
PME	Phosphomonoesterase
PDE	Phosphodiesterase

ABSTRACT

Soil is a critically important component of the earth's biosphere. Developing agricultural production systems able to conserve soil quality is essential to guarantee the current and future capacity of soil to provide goods and services.

This study investigates the potential of microbial and biochemical parameters to be used as early and sensitive soil quality indicators. Their ability to differentiate plots under contrasting fertilization regimes is evaluated based also on their sensitivity to seasonal fluctuations of environmental conditions and on their relationship with soil chemical parameters. Further, the study addresses some of the critical methodological aspects of microplate-based fluorimetric enzyme assays, in order to optimize assay conditions and evaluate their suitability to be used as a toll to asses soil quality.

The study was based on a long-term field experiment established in 1966 in the Po valley (Italy). The soil was cropped with maize (*Z. mays* L.) and winter wheat (*T. aestivum* L.) and received no organic fertilization, crop residue or manure, in combination with increasing levels of mineral N fertilizer.

The soil microbiota responded to manure amendment increasing it biomass and activity and changing its community composition. Crop residue effect was much more limited. Mineral N fertilization stimulated crop residue mineralization, shifted microbial community composition and influenced N and P cycling enzyme activities. Seasonal fluctuations of environmental factors affected the soil microbiota. However microbial and biochemical parameters seasonality did not hamper the identification of fertilization-induced effects. Soil microbial community abundance, function and composition appeared to be strongly related to soil organic matter content and composition, confirming the close link existing between these soil quality indicators. Microplate-based fluorimetric enzyme assays showed potential to be used as fast and throughput toll to asses soil quality, but required proper optimization of the assay conditions for a precise estimation of enzymes maximum potential activity.

Keywords: Long-term experiment; Fertilization; Soil quality indicators; Mid-infrared spectroscopy; Microplate-based fluorimetric enzyme assays.

GENERAL INTRODUCTION

Soil quality has been defined as the continued capacity of a specific kind of soil to function within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance air and water quality and support human health and habitation (Karlen et al., 1997). The interest in evaluating soil quality has been stimulated by the increasing awareness that soil is a critically important component of the earth's biosphere, representing not only a resource base for food and fiber production, but also playing a fundamental role in ecosystem functioning and in environmental quality maintenance (Dick, 1992). Soil acts as an environmental buffer, protecting the surrounding ecosystems such as watersheds and grounwaters from contamination, and sequestering carbon that would otherwise go to the atmosphere and contribute to the global warming (Reeves, 1997).

Soil quality degradation can be defined as a process that lower the current or future capacity of soils to produce goods or services (Olderman, 1992). According to the Global Assessment of Soil Degradation (GLASOD), more than one third of all the agricultural land has undergone degradation. On the other hand, according to the United Nations Food and Agriculture Organization (FAO), world population will grow from 6 billion people today to 8.3 billion people in 2030, and as a consequence an extra billion tons of cereals will be needed. Therefore, while soil productive capacity is decreasing as a result of soil degradation, food demand is increasing due to the constant growth of human population. Based on these considerations it appears clear that maintaining or improving soil quality is crucial if agricultural productivity and environmental quality are to be preserved for future generations (Reeves, 1997). Thus the necessity of developing agricultural production systems that conserve or enhance soil quality is nowadays a priority.

A wide range of soil properties have been proposed for useful indicators of soil quality (Reeves, 1997; Arshad and Martin, 2002; Anderson, 2003; Schloter et al., 2003; Winding et al., 2005). Among these properties soil organic matter (SOM) has been suggested as the most important single indicator of soil quality since it affects other physical, chemical and biological properties related to soil quality (Reeves, 1997). However, SOM modifications occur slowly and therefore require long periods of time before being experimentally detectable (Rasmussen and Collins, 1991; Bending et al., 2004; Körschens, 2006).

On the other hand the soil microbiota quickly adapts to changes in environmental conditions by adjusting its biomass, community composition and activity (Schloter et al., 2003). Therefore, microbial and biochemical parameters may represent early and sensitive indicators of soil quality changes (Dick, 1992; Bending et al., 2004; Winding et al., 2005).

Moreover, since most of the processes that occur in soil are microbial mediated, microbial and biochemical parameters can offer insights into the processes involved in SOM turnover and nutrient cycling, improving our understanding of the mechanisms behind soil quality modifications (Dick, 1992; Winding et al., 2005).

The soil microbiota is strongly affected by environmental conditions such as air and soil temperature, precipitations and soil moisture. As a consequence soil microbial and biochemical parameters are subject to wide seasonal fluctuations (Debosz et al., 1999; Marinari et al., 2006). Under field conditions the interaction between environmental and anthropogenic factors could hamper the discrimination between causes and effects of the observed variations. Therefore, it should be clarified whether microbial and biochemical parameters are affected by too many factors to be considered useful indicators of human-induced changes in soil quality.

The assay of soil enzyme activities has been proposed as a useful tool to monitor the changes in soil quality (Ekenler and Tabatabai, 2003a, Sinsabaugh et al., 2008). Soil enzymes catalyze a complex web of reactions necessary for life processes of microorganisms, depolymerization of macromolecular organics, cycling of nutrients and formation of organic matter and soil structure, and therefore play a fundamental role in soil functioning (Dick, 1994). Since the organic materials entering the soil are both structurally complex and highly diverse, their breakdown and subsequent mineralization requires the combined activity of many different microorganisms and enzymes (Burns et al., 2013). Therefore, involving in the study a large number of enzyme activities should provide a better picture of the multiple biochemical processes occurring in soil. Microplate-based fluorimetric assays appear to offer a fast and throughput approach for studying multiple enzyme activities involved in main soil biochemical processes of organic substrate degradation and nutrient elements cycling. At date the use of high-throughput microplate methods is becoming prevalent in studies concerning soil enzymes (German et al., 2011). However, currently available protocols are still not thoroughly evaluated (Popova and Deng, 2010) and require proper optimization of the assay conditions for each study site (German et al., 2011).

Every single farming practice may influence soil quality either in a positive or negative manner (Emmerling et al., 2002) and long-term agricultural experiments (LTE) are essential for providing the empirical data necessary to establish the critical cause-effect relationship between soil management and soil quality. LTE offer the possibility to observe SOM changes and to study the relationships existing between this fundamental but slowly changing quality indicator and the early

changing microbial and biochemical indicators. Therefore LTE still represent the most cost-effective research method for agricultural and environmental studies concerning soil quality (Körschnes, 2006).

In the context of the Po valley (Italy), the LTE located at the University of Bologna Experimental Farm of Cadriano (44°33′ N, 11° 24′ E), represent a typical succession of the region, in which maize (*Zea mays* L.) and winter wheat (*Triticum aestivum* L.) are cropped in a 2-year rain fed rotation. This LTE may serve as a simplified example of the evolution that agriculture has undergone in the last decades in terms of fertilizers management. In some of the plots of the LTE cattle manure is used to maintain soil fertility and to improve crop yield. The use of animal manure has been a common practice for centuries and its beneficial effects on soil chemical, physical and biological status as well as on crop productivity have been well documented (Dick, 1992; Kandeler et al., 1999; Marinari et al., 2000, 2006; Eivazi et al., 2003; Böhme et al., 2005). Other plots of the LTE receive only mineral fertilizers (NPK). This practice has though to be sufficient to maintain soil fertility and to ensure high levels of crop yields in the decades following the massive introduction of chemically synthesized fertilizers. However, is nowadays increasing the awareness that the use of mineral fertilizers without significant inputs of organic matter of animal or plant origin may prove insufficient to maintain soil quality and productivity in the long-term. Even though increased amounts of agricultural inputs such as mineral fertilizers can mask losses in productivity due to soil quality degradation, this strategy could prove to be economically and environmentally unsustainable (Reeves, 1997). Finally, other plots of the LTE are amended with crop residue derived from maize and wheat cultivation, representing a good example of the present tendency of recycling agricultural by-products with the aim to improve resource use efficiency and possibly to increase soil carbon stocks (Triberti et al., 2008). Even though animal manure's positive effects on soil quality has been reported to exceed that of plant residue (Rasmussen and Collins, 1991; Reeves, 1997; Rasmussen et al., 1998; Triberti et al., 2008) the incorporation of straw may represent a more feasible management strategy in cereal based production systems nowadays. In fact, animal manure is becoming scarce in modern agricultural production systems (Triberti et al., 2008).

The LTE includes also plots in which both organic and mineral N fertilizers are applied. Apart from increasing plant biomass production, mineral N availability may also affect the decomposition of the organic inputs entering the soil, through changes in the chemical, physical and microbiological

properties of the environment under which the decomposition processes take place (Green et al., 1995; Mary et al., 1996; Chantigny et al. 1999).Therefore, for a more comprehensive understanding of organic and mineral N fertilization impact on soil quality, their combined effect should also be studied.

The LTE of Cadriano could hence be considered a precious case study for the evaluation of the impact of organic and mineral N fertilization on the present state and the future trends of soil quality in cereal-based agricultural lands of the Po valley.

OBJECTIVES

The overall objective of this research was to study the impact of organic and mineral N fertilization on soil quality in the context of a long-term agricultural field experiment of the Po valley, Italy.

The specific objectives were:

(i) Evaluate the potential of microbial and biochemical parameters to be used as early indicators of soil quality changes, based also on their relation with slowly changing resilient parameters such as SOM content and composition and on their sensitivity to seasonal fluctuations of environmental factors;

(ii) Define the optimal conditions under which soil enzymes should be assayed in order to obtain repeatable and reproducible estimations of maximum potential activities using microplate-based fluorimetry;

(iii) Asses the suitability of microplate-based fluorimetric multiple enzyme assays as a fast and throughput approach for studying soil functional diversity and monitor soil quality;

These objectives will be addressed in turn in the following chapters.

CHAPTER 1

Chemical and microbiological soil quality indicators and their potential to differentiate fertilization regimes in temperate agro-ecosystems

CHAPTER 1

1.1. Introduction

Maintaining or improving soil quality is crucial if agricultural productivity and environmental safety are to be preserved for future generations (Reeves, 1997). Every farming practice may influence soil quality either in a positive or negative manner (Emmerling et al., 2002).

Among the different farming practices, the management of organic amendments and mineral fertilizers could have a major impact on soil fertility and quality status, influencing the quantity and quality of organic residues and nutrient inputs entering the soil and the rate at which the residues and organic matter decompose (Gerzabek et al., 2006). The use of animal manure has been a common practice for centuries in traditional agriculture and its beneficial effects on soil chemical, physical and biological status have been well documented (Dick, 1992; Kandeler et al., 1999; Marinari et al., 2000, 2006; Eivazi et al., 2003; Böhme et al., 2005). However, animal manure is becoming scarce in modern agricultural production systems (Triberti et al., 2008). Although animal manure's positive effects on soil quality has been reported to exceed that of plant residues (Rasmussen and Collins, 1991; Reeves, 1997; Rasmussen et al., 1998; Triberti et al., 2008), the incorporation of cereal straw could represent a more feasible management strategy in cereal based production systems nowadays. Mineral fertilizers are commonly applied to cereal crops mainly to maximize their yield. However, increasing plant biomass production and hence the amount of plant residue returned to the soil, mineral fertilizers may also affect soil properties (Dick, 1992; Green et al., 1995; Rasmussen et al., 1998).

Assessing the impact that organic and mineral fertilizers and their combined application could have on soil quality status would contribute to the development of more suitable agricultural production systems, able to prevent soil degradation.

A wide range of soil physical, chemical and biological properties have been proposed for useful indicators of soil quality (Reeves, 1997; Arshad and Martin, 2002; Anderson, 2003; Schloter et al., 2003; Winding et al., 2005). At present, the key indicators to take into account when evaluating the impact of different management practices on soil quality is still the subject of debate (Schloter et al., 2003; Winding et al., 2005). Soil organic matter (SOM) has been suggested as the most important single indicator of soil quality and agricultural sustainability since it affects other physical, chemical and biological soil properties (Reeves, 1997). However, the measure of SOM content does not give any insight into the biogeochemical mechanisms that influence SOM turnover and are responsible for the observed modifications.

Moreover, SOM changes slowly with time and management-induced effects require a long time before being experimentally detectable (Rasmussen and Collins, 1991; Bending et al., 2004; Körschens, 2006). Long-term experiments are then essential to providing the empirical data necessary to establish the cause-effect relationship between management practices and SOM content changes (Rasmussen and Collins, 1991; Reeves, 1997; Eivazi et al., 2003).

Besides affecting SOM content, the repeated incorporation of organic and mineral fertilizers may also influence the composition of the organic matter accumulating in soils. Recent advances in infrared spectroscopy allow the application of the technique directly on bulk soil samples without any specific pre-treatment or fractionation. This new approach represents a simple and powerful means for the chemical characterization of SOM (Chapman et al., 2001). Its usefulness in assessing qualitative changes in SOM due to contrasting management regimes have been reported in previously published work (Capriel, 1997; Gerzabek et al. 2006; Lima et al., 2009; Demyan et al., 2012). Moreover, the infrared spectra of bulk soil samples may relate to more general soil chemical properties (Chapman et al., 2001; Du and Zhou, 2009). By means of multivariate calibration these relationships can be quantified and used to create predictive models. In particular, predictive models based on soil infrared spectra could be used as a rapid soil testing technique for the future monitoring of SOM content (Chang et al., 2001; Chodak et al, 2001; Coûteaux et al., 2003; Vasques et al., 2008; Du and Zhou, 2009).

The water extractable fraction of soil organic matter (WEOM) could also represent a useful indicator of shifts in soil quality status (Zsolnay, 1996, 2003). Compared to SOM the concentrations of WEOM are very small (Embacher et al., 2007). Nevertheless, WEOM is linked to many important soil functions involving substrate supply to microorganisms, C and N cycling, and improvement of soil physical properties (Zsolnay, 1996), and thus plays an important ecological role in the unsaturated zone of soil (Zsolnay, 1996; Chantigny, 2003). Soil management is one of the main anthropogenic factors affecting WEOM (Embacher et al., 2007). Generally, positive effects of organic amendments on WEOM concentrations are observed (Chantigny et al., 1999, 2002; Embacher et al., 2008) while conflicting results are reported on the effect of N fertilization under field conditions (Liang et al., 1998; Chantigny et al., 1999; Embacher et al., 2008).

The soil microbiota quickly adapts to environmental constrains by adjusting its biomass, activity rates and community composition (Schloter et al., 2003). Moreover, since most of the soil processes are microbial mediated, soil microorganisms are central to the ecological functions of soil (Dick, 1992; Winding et al., 2005).

Microbial and biochemical properties may then represent early and sensitive indicators of management-induced modifications of soil quality because they manifest themselves over shorter time scales compared to SOM (Dick, 1992; Bending et al., 2004; Winding et al., 2005). Due to the multitude of microbiological components and biochemical pathways subsisting in soil it is unlikely that a single measure can adequately depict soil quality status (Schloter et al, 2003). Soil quality indicators should describe the major ecological processes in soil (Doran and Safley, 1997), therefore a variety of biochemical and microbial analysis should be used when considering the impact of management on soil quality (Bending et al., 2004).

Microbial biomass and soil enzyme activities had been successfully applied to evaluate the effects of organic and inorganic fertilization on the microbiological status of different soil types and climates (Debosz et al., 1999; Kandeler et al., 1999; Marinari et al., 2000; Pajares et al., 2009). Eco-physiological indicators such as microbial and metabolic quotients have been found to reflect soil management history and be closely associated with soil organic matter content (Trinchera et al., 1999, 2001; Anderson, 2003; Böhme et al., 2005; Marinari et al., 2006). Soil microbial community profiling based on phospholipid fatty acids has been reported to discriminate between soils with different fertilization regimes (Bossio et al., 1998; Bardgett and McAlister, 1999; Peacock et al., 2001; Marschner et al., 2003; Böhme et al., 2005).

Besides being used for predicting soil chemical properties, attempts have been made to relate the infrared spectra of bulk soils to microbiological parameters. Since both poor (Chang et al., 2001; Chapman et al., 2001) and high correlations (Chodak et al, 2001; Coûteaux et al., 2003) have been observed, we believe that further efforts in investigating the quantitative relationship between soil infrared spectra and microbiological parameters are needed.

Management practices are not the sole factors affecting the soil microbiota in field-based experiments: environmental conditions such as air and soil temperature, precipitations, soil moisture and substrate availability fluctuate widely over time and can have large effects on microbial and biochemical parameters. Debosz et al. (1999) reported pronounced temporal variations in soil microbial biomass and enzymatic activities estimated at monthly intervals over 2 years in a field experiment comparing plots under low and high organic inputs. Most interestingly, while for the enzymatic activities the treatment effect was constant over the sampling period, for microbial biomass carbon the treatment effect varied over time. Marinari et al. (2006) reported that despite variations among sampling dates, both microbial biomass content and activity of an organically managed field were always higher than conventionally managed field.

These contrasting observations cast doubts on the ability of microbial and biochemical parameters to clarify the effects of different farming practices on soil quality because these parameters are influenced by too many factors at the same time and subjected to strong seasonal fluctuations. A marked seasonality has been reported also for the WEOM (Zsolnay, 2003). According to Chantigny (2003) and Embacher et al. (2007), under field conditions the interaction between environmental factors and soil management could hamper the discrimination between causes and effects of WEOM variations. Repeated samplings over time could help assessing the magnitude of seasonal variability (Forster, 1995) and clarify whether microbial and biochemical parameters as well as WEOM are affected by too many factors to be considered useful indicators of changes in soil quality.

The aim of the present study was to combine microbial and biochemical parameters regarded as early indicators of soil quality changes but also subjected to strong seasonal fluctuations, with slowly changing and resilient chemical parameters, such as soil organic matter content and composition, in order to compare their ability to differentiate long-term plots under contrasting fertilization regimes, and to investigate their possible relationships.

A field experiment established in 1966 in which cattle manure and crop residue are factorially combined with increasing levels of mineral N fertilizer was selected and soil samples were collected four times during the maize growing season to test the following hypotheses: (i) soil microorganisms respond to differences in organic and mineral N fertilization by varying their biomass, activity and community composition, (ii) soil microorganisms are also affected by environmental factors, resulting in a large temporal variability of microbial biomass, activity and composition, (iii) the repeated application of manure and crop residue and their combination with N fertilization have also modified soil organic matter quantity and quality, and (iv) the microorganisms’ response to organic and mineral N fertilization is linked to the quantity and quality of the soil organic matter that have accumulated under the different treatments.

1. 2. Materials and methods

1.2.1. Study site and experimental design

The study site was a long-term field experiment started in 1966, located at the Experimental Farm of the University of Bologna, in the South-east of the Po valley (Italy, 44°33′ N, 11° 24′ E; 23 m.a.s.l.). The climate of the region is classified as sub-humid. In 2010 total annual rain fall and mean air temperature registered by the meteorological station of the Experimental Farm were 840 m and 13°C, respectively. Their monthly trends are reported in Fig. 1.1.

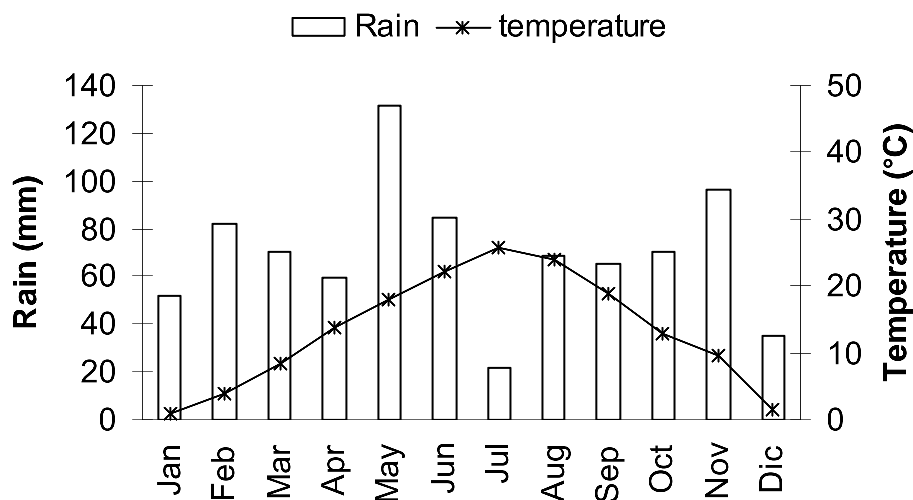

Fig. 1.1. Monthly trends of temperature and rainfalls registered at the experimental site over 2010.

The soil was classified as a fine silty, mixed, mesic Udic Ustochrepts (USDA Soil Taxonomy). According to Triberti et el. (2008), when the trial was established in 1966, the soil had the following characteristics: 56 % sand, 16 % silt, 28 % clay, traces of carbonates, 6.9 pH in water, 1.33 % organic matter (Lotti and Galoppini, 1967), 0.11 % total Kjeldal N and 19 mg kg^{-1} available P (Olsen method). The experimental design was a split-plot with four replicates. Maize (*Zea mays* L.) and winter wheat (*Triticum aestivum* L.) were cropped in a 2-year rain fed rotation. Wheat straw or maize stalk (*crop residue*) and cattle manure (*manure*) were applied every year to the same plots and compared to unamended plots (*control*). Each plot was then split to receive 0 (N_0) or 200 (N_{200}) kg ha^{-1} of mineral N, supplied as urea. Manure and crop residue were added every year in October at the same dry matter rate, corresponding to 6.0 t dry matter ha^{-1} after wheat and 7.5 t dry matter ha^{-1}

after maize crop, and incorporated into the soil by mouldboard ploughing to 0.40 cm depth. Expressed as a percentage of their dry weight the organic materials had the following composition (Triberti et al., 2008): (i) wheat straw: organic C 32.8 %, total N 0.47 %, C:N ratio 69.8; (ii) maize stalks: organic C 32.6 %, total N 0.70 %, C:N ratio 46.6; (iii) cattle manure: organic C 33.1 %, total N 2.64 %, C:N ratio 12.3. Urea was supplied in two applications: 30 % at the end of tillering and 70 % at the beginning of stem elongation in wheat; 50 % at seeding and 50 % at the 4th leaf stage in maize. For a more detailed description of the experimental site see Triberti et al. (2008).

1.2.2. Soil sampling and storage

Soil samples from the top 20 cm were taken four times over the maize growing season, between March and September 2010. Sampling dates were selected in order to minimize disturbance effects due to N fertilization and chemical weed control.

From each subplot of 33 m^2 nine soil samples were collected with an auger and combined to form one composite sample. Freshly sampled soil was kept in a cooler for transportation back to the laboratory, sieved through a 2 mm sieve, and thoroughly mixed. Roots and visible plant residue were removed by hand-picking. The soil was stored in plastic bags at 4 °C and analyzed within two weeks of collection. Fifty grams aliquots of soil were frozen (approximately -20 °C) for later determination of the microbial community structure.

1.2.3 Soil water content and soil pH

Soil pH was measured using a glass electrode in 1:5 (v:v) suspensions of air dried soil in 10 mM $CaCl_2$ (ISO 10390, 2005). Soil water content was determined gravimetrically by drying soil samples to constant mass at 105 °C.

1.2.4. Total organic carbon and total nitrogen

Total organic carbon (TOC) and total nitrogen (TN) content were determined on air dried, finely ground soil aliquots. Subsamples of 10 mg were weighted into tin caps and analyzed by an elemental analyzer (CHNS-O Elemental Analyzer 1110, Thermo Scientific GmbH, Dreieich, DE).

1.2.5. Water extractable organic carbon

Field moist soil, equivalent to 150 g of oven dried soil, was weighted into 500 ml Nalgene bottles. Milli-Q® analytical grade water was used as an extractant at a ratio of 1:1 (volume to oven dry mass equivalent). The extracts were shaken on a horizontal shaker for 1 h at room temperature, followed

by centrifugation at 9000 rpm for 30 min and filtration through 0.40 µm pore polycarbonate filters. Centrifugation was done to expedite filtration. Filters pore size was selected in order to exclude microbial cells and particulate organic matter (Zsolnay, 1996), while polycarbonate was chosen since it neither adsorbs nor releases WEOM during usage (Zsolnay, 2003). The organic C content of the extracts (WEOC) was then determined with an elemental analyzer (TOC-VCPH/CPN, Shimadzu, Kyoto, JP). Gentle extraction conditions were used to extract as much as possible only the organic material which could readily enter the dissolved state in situ (Zsolnay, 2003). Conceptually WEOM extracted under these conditions can be considered as the potential mobile organic matter (MOM) (Zsolnay, 1996).

1.2.6. DRIFT-MIR spectroscopy

Soil samples were dried (35 °C over night) and ball milled prior to analysis. Neat powdered soil samples were analyzed with a Bruker Tensor 27 (BRUKER OPTIK GmbH, Ettlingen, DE) mid-infrared (MIR) spectrometer equipped with a Praying Mantis diffuse reflectance Fourier transform (DRIFT) accessory (Harrick Scientific Products, New York, USA). The absorbance spectra were in the range of 4000-400 cm^{-1}, with a resolution of 4 cm^{-1}, for a total of 1763 data points. Three scans per sample were recorded, averaged and corrected against a background of potassium bromide (KBr). Spectral pre-processing included atmospheric correction for carbon dioxide (CO_2) and water, baseline correction, and vector normalization in order to compensate for slight variations in operating conditions (e.g. air humidity, temperature, and CO_2 concentration) at the time of measurement. For the chemical characterization of soil organic matter four bands, representing different organic functional groups were selected (Demyan et al., 2012): (i) the band at 2930 cm^{-1} (2995 to 2810 cm^{-1}), representing aliphatic C-H vibrations of aliphatic methyl and methylene groups; (ii) the band at 1620 cm^{-1} (1660 to 1580 cm^{-1}), assigned to aromatic C=C stretching and COO^- symmetric stretching; (iii) the band at 1520 cm^{-1} (1540 to 1503 cm^{-1}), attributed to aromatic C=C stretching vibrations and N-H (amide II) bending vibrations; (iv) the band at 1160 cm^{-1} (1172 to 1140 cm^{-1}), corresponding to the C-OH stretching of aliphatic alcoholic groups. Wavenumber assignment to functional groups was based on Stevenson (1982) and Baes and Bloom (1989). Peak area integration on the corrected spectra was performed using OPUS software package version 6.5 (BRUKER OPTIK GmbH, Ettlingen, DE) using the above defined peak extents.

In order to quantify relative changes in the DRIFTS spectra and for spectral comparison, relative peak areas (rA) were calculated by dividing the correct peak area of a distinct peak (e.g. 2930 cm^{-1}, 1620 cm^{-1}, 1520 cm^{-1}, and 1160 cm^{-1}) by the sum of all the peak areas at 2930 cm^{-1}, 1620 cm^{-1}, 1520 cm^{-1}, and 1160 cm^{-1} and multiplying it by 100.

1.2.7. Basal respiration

Microbial respiration was estimated according to Isermeyer (1952). Field moist soil, equivalent to 20 g of oven dried soil, was weighted into airtight glass jars. Water content was adjusted to 70 % of water holding capacity (WHC) and samples were incubated at 25 °C for 3 weeks. 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 21st day was used as a measurement of the basal respiration.

1.2.8. Microbial biomass carbon and metabolic quotient

Microbial biomass carbon (C_{mic}) was determined using the chloroform-fumigation extraction (CFE) method (Brookes et al., 1985). The equivalent of 4 g of oven dried soil was fumigated with ethanol-free chloroform for 24 h at room temperature in a desiccator. Fumigated and unfumigated samples were dispersed in 16 mL of 0.5 M K_2SO_4 and extracted on an horizontal shaker at 250 rev min^{-1} for 1 h. Extracts were filtered through Whatman no. 42 filter paper and analyzed for the organic C content with an elemental analyzer (TOC-VCPH/CPN, Shimadzu, Kyoto, JP). C_{mic} was calculated as organic C in the fumigated minus organic C in the unfumigated soil extracts. The eco-physiological parameter metabolic quotient (qCO_2) was calculated by dividing the basal respiration by the C_{mic}.

1.2.9. Enzymatic assays

Enzyme activities were assayed on duplicate field-moist samples using colorimetric techniques. Briefly, dehydrogenase activity was measured according to von Mersi and Schinner (1991). Field moist soil (1 g oven-dry equivalent) was incubated with 1.5 mL of 0.1 M tris(hydroximethyl)aminomethane buffer (TRIS, pH 7.0) and 2 mL of 10 mM 2-*p*-iodo-nitrophenyl tetrazolium chloride (INT) at 40 °C for 2 h. The 2-*p*-iodo-nitrophenyl formazan (INFT) released was measured colorimetrically at 464 nm, and the activity was expressed as µg INFT g^{-1} h^{-1}.
Arginine deaminase activity was determined using a modified protocol based on Alef and Kleiner

(1986) and Lin and Brookes (1999). Field moist soil (2 g oven-dry equivalent) was amended with 1.5 mL of 3 mM arginine solution (0.37 mg arginine g^{-1} dry soil). Soil water content was adjusted to 2.0-fold WHC and incubated at 25 °C for 5 h. At the end of the incubation samples were stored at -20 °C for at least 4 h. The initial soil NH_4-N and NO_3-N was determined adding only water and freezing the samples immediately. These samples were used both as controls for the determination of arginine deaminase activity and as a measure of the mineral N content of the soil (N_{min}). The soil samples were than thawed in hot water and extracted with 8 mL of 2 M KCl for 1h. The amounts of NH_4-N and NO_3-N were determined by an automatic NH_4^+ and NO_3^- analyzer (AACE 5.46, Bran Luebbe GmbH, Norderstedt, DE) using the ISO 14256-2 method. The activity was expressed as total arginine mineralization rate (μg N_{min}-N g^{-1} h^{-1}). The activity of β-glucosidase was measured following the procedure of Eivazi and Tabatabai (1988). Field moist soil (1 g oven-dry equivalent) was incubated with 4 mL of modified universal buffer (MUB, pH 6.0) and 1 mL of 25 mM *p*-nitrophenyl-β-glucoside (PNG) at 37°C for 1 h. The *p*-nitophenol (PN) released was measured colorimetrically at 400 nm and the activity was expressed as μg PN g^{-1} h^{-1}. Urease activity was assayed according to Kandeler and Gerber (1988). Field moist soil (1 g oven-dry equivalent) was incubated with 1.5 mL of 0.8 M urea solution at 37 °C for 2 h. Released ammonium was extracted with a mixture of 1 M KCl and 0.01 M HCl and determined colorimetrically at 660 nm by a modified Berthelot reaction. The activity was expressed as μg NH_4^+-N g^{-1} h^{-1}.The assay of protease activity was based on Ladd and Butler (1972). Field moist soil (1 g oven-dry equivalent) was incubated with 5 mL of 0.05 M TRIS buffer (pH 8.1) and 5 mL of 2 % Na-caseinate at 50 °C for 2 h; enzyme activity was stopped by the addition of trichloroacetic acid (15 % w:v).The aromatic amino acids released were measured colorimetrically using Folin-Ciocalteau reagent, at 700 nm, with the aminoacid tyrosine (tyr) as standard. The activity was expressed as μg tyr g^{-1} h^{-1}.

1.2.10. PLFA profiles

The total phospholipid fatty acids (PLFA) content was used as an index of the viable microbial biomass (Frostegård et al., 1991). Total lipids were extracted from soil samples (6 g oven-dry equivalent) with a modified single phase mixture of chloroform-methanol-citrate buffer (1:2:0.8, v:v:v), according to Bligh and Dyer (1959). The resulting material was fractionated using silica-bonded phase columns into neutral lipids, glycolipids and phospholipids by elution with 5 mL of chloroform, 20 mL of acetone and 5 mL of methanol, respectively. Phospholipids were trans-esterified to the fatty acid methyl esters (FAME) by a mild alkaline methanolysis.

Samples were analyzed by a gas chromatograph (GC/FID, AutoSystem XL Gas Chromatograph, Perkin-Elmer GmbH, Rodgau-Jügesheim, DE) fitted with a HP-5 column (50 m length, 0.20 mm internal diameter, coated with cross-linked 5 % phenyl-methyl phase with a film thickness of 0.33 µm) interfaced to a flame ionization detector (FID). The injection temperature was 260 °C, the FID temperature was 280 °C and the initial temperature was 70 °C. Temperature was subsequently increased to 160 °C at 30 °C min^{-1} and then to 280 °C at 3 °C min^{-1}. Fatty acid methyl esters were quantified using nonadecanoate (fatty acid methyl ester 19:0) as the internal standard. A Bacterial acid methyl ester mix (BACmix) and a 37-component FAME mix were used for the identification of the measured fatty acids methyl esters, based on their retention times. PLFA were designated using the nomenclature described in Palojärvi (2006) and their content was expressed as nmol g^{-1} soil. PLFA bioindicators were selected according to Frostegård et al. (1993) and Zelles (1999), using only the PLFAs which were clearly identified by GC-FID: terminal branched PLFA i15:0, a15:0, i16:0 and i17:0 were selected as indicators of Gram-positive bacteria, cyclopropil PLFA cy17:0 and cy19:0 as indicators of Gram-negative bacteria, and the polyunsaturated PLFA 18:2ω6 as an indicator of fungi. The bacteria-to-fungi ratio was calculated according to Frostegård and Bååth (1996): for the estimation of the bacterial contribution, the linear unsaturated PLFA 15:0 and 17:0 were added to the Gram-positive and Gram-negative bacteria bioindicators, while for the estimation of fungal biomass only the PLFA 18:2ω6 was used.

1.2.11. Data handling and statistics

All results are expressed on an oven-dried basis. Results represent the arithmetic means of the field replications (e.g. subplots), which in turn are the mean of two analytical replications.

The experimental data was analyzed as a split-plot arrangement of treatments with organic fertilization as the main factor and mineral N fertilization as a subfactor using the aov procedure of R (R software version 2.13.1). Data from different sampling dates were analyzed both separately and together. When data from all sampling dates were grouped together for statistical testing of the sampling date effect, sampling date was considered as a subfactor and Greenhouse-Geisser test was used to account for the correlation of the repeated measurements and correct the degrees of freedom of the ANOVA. The assumptions of the statistical models were tested for every data set. Normality of the residuals was evaluated graphically and with the Shapiro-Wilk test. Homogeneity of variance was tested with the Levene's test. If necessary the data were transformed following the Box-Cox procedure.

Means were separated calculating least significant difference (LSD) at the significant level P = 0.05. For simplicity results reported in the tables correspond to the original untransformed data. Association between soil properties was estimated using linear regression. Normality of the residuals was evaluated graphically and with the Shapiro-Wilk test. Pearson's product moment correlation coefficients (r) and significance of the associations (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$) are reported.

The suitability of partial least square (PLS) regression analysis of DRIFTS measurements was tested for predicting TOC, C_{mic}, and PLFA contents. Both a cross validation was performed on the whole data set using a leave one out procedure and a test-set validation where the dataset was split into 48 samples for calibration (model development and optimization) and 48 samples for validation. Every first sample was assigned to the "calibration set", and every second sample was assigned to the "validation set". In this way, two field replicates of each treatment would be in the calibration set and two replicates in the validation set. Finally, a reverse validation was performed using the former validation set as a calibration set and likewise, the former calibration set as a validation set to examine the robustness and stability of the test-set validations.

Wavenumbers > 3900 and < 700 cm^{-1} (edges of the measurement range) and the main CO_2 absorbance range, 2400 to 2200 cm^{-1}, were excluded from the PLS procedure. During the PLS model building step, both for cross-validation and test-set validation, the optimize feature in the QUANT2 procedure in the OPUS© software program (BRUKER OPTIK GmbH, Ettlingen, DE) was used. The optimize feature tries different spectral pre-processing methods (e.g. derivatives, normalization, constant offset) and combinations of wavenumbers in order to minimize the root mean square error of cross validation ($RMSE_{CV}$) in the case of cross-validation or the root mean square error of prediction (RMSEP) in the case of test-set validation. The best three or four optimization combinations were then inspected to ensure an optimum number of latent roots was chosen to minimize $RMSE_{CV}$ or RMSEP (to avoid underfitting) with the lowest possible rank or number of factors (to avoid overfitting).

Following this step, a cross-validation model was selected based on the highest coefficient of determination (R^2), lowest $RMSE_{CV}$, the ratio of the standard deviation to the standard error of cross validation (RPD_{cv}) and the mean value of deviation (bias). Test-set validation models were evaluated by the R^2, the RMSEP, the ratio of standard deviation to standard error of prediction (RPD), and bias.

To test the importance of the peaks utilized in the rA specific peak investigation, in addition to using the entire spectra for the OPUS QUANT2 optimize procedure, a peak pre-selection was used

to try to reduce noise or eliminate peaks which are most likely not related to organic functional group vibrations. In addition to the previously used peaks, the wavenumbers of 3660 to 3517 and 3517 to 3252 cm^{-1}, 1928 to 1830 cm^{-1}, 1830 to 1754 cm^{-1}, 1428 to 1273 cm^{-1}, 1084 to 921 cm^{-1}, and 854 to 736 cm^{-1} were used for the PLS analysis.

1.3. Results

1.3.1. Organic carbon and total nitrogen

Long-term application of manure increased TOC and TN, while no significant differences between control and crop residue treatments were observed, independently of the sampling date (Table 1.1). The subplot factor N fertilization did not affect TOC content, and significantly increased TN only in the samples taken in April, after the first application of urea. In April, the interaction between organic and N fertilization was significant for both parameters. Mineral N fertilization had an organic treatment-dependent influence on TOC and TN. Namely, urea application increased TOC in the control plots, where organic fertilizers were not applied, slightly decreased TOC in the crop residue treatment, and left TOC unchanged in the manure treatment (data not shown). Similarly, urea application strongly increased TN in the control plots, while it did not cause a significant change of TN in crop residue and manure treatments (data not shown). With regard to their seasonal trends, the average TOC and TN content fluctuated little over time. Lower values were observed at planting, followed by an increase in April. TOC content remained stable thereafter while TN decreased again between June and September (Table 1.1).

As observed for TOC content, WEOC was higher in treatments amended with manure, throughout the cropping season (Table 1.1). However, none of these differences were statistically significant. The subplot factor N fertilization significantly affected WEOC only in September, with lower values observed in N_{200} compared to N_0. With regard to its seasonal trends, WEOC level remained stable from March to June, while between June and September a significant increase occurred (Table 1.1).

1.3.2. Mineral nitrogen, soil moisture and soil pH

In March significantly lower values of N_{min} were observed in crop residue treatment as compared to the control and manure treatments, while no differences between N_0 and N_{200} were observed (Table 1.1). In the samples taken in April, 35 days after the first application of urea, the concentration of N_{min} had reached significantly higher values in the N_{200} plots, as compared to the concentration observed in the N_0 plots, while no differences between the organic fertilization treatments were observed. Similar results were obtained in June, 20 days after the second application of urea. By September the average concentration of N_{min} in the N_{200} plots had decreased, but was still significantly higher than the concentration of the N_0 plots. As observed in March, averaged over the N fertilization levels, N_{min}

values were significantly lower in crop residue treatment as compared to the control and the manure treatments.

A significant reduction of soil pH was observed in the N_{200} plots as compared to the N_0 plots in the samplings following urea application (Table 1.1).

The gravimetric soil moisture content was not affected by the fertilization treatments throughout the sampling period. Maximum values were observed in March when soil moisture averaged 15 %. At the end of April soil moisture had slightly decreased to an average value of 14 %, while by June it reached the minimum value of 10 %. In September, after maize harvest, soil moisture was close to the values observed in early spring and averaged 13 %.

Table 1.1. Marginal means of TOC, WEOC, TN, N_{min}, pH, C_{mic}, qCO_2, and total PLFA for the effects of organic fertilization, mineral N fertilization and sampling date*.

	Organic fertilization						N fertilization				Sampling	
	Control		Crop residue		Manure		N_0		N_{200}		date	
18-March												
TOC	6.74	b	6.72	b	8.36	a	7.17	a	7.37	a	7.27	B
WEOC	2.78	a	3.64	a	4.23	a	3.47	a	3.63	a	3.55	B
TN	0.87	b	0.82	b	1.00	a	0.89	a	0.91	a	0.90	C
N_{min}	5.11	a	3.68	b	5.28	a	4.52	a	4.99	a	4.76	C
pH	6.25	a	6.21	a	6.51	a	6.33	a	6.32	a	6.32	B
C_{mic}	19.80	a	29.01	a	38.61	a	28.61	a	29.72	a	29.24	C
qCO_2	5.46	a	3.23	a	2.57	a	3.52	a	3.99	a	3.75	A
PLFA	15.20	b	15.40	b	20.10	a	16.80	a	17.01	a	16.91	B
30-April												
TOC	6.89	b	6.81	b	8.97	a	7.56	a	7.55	a	7.56	A
WEOC	3.92	a	3.74	a	4.69	a	4.29	a	4.13	a	4.21	B
TN	0.94	b	0.96	b	1.17	a	1.00	b	1.05	a	1.02	A
N_{min}	37.54	a	33.31	a	28.14	a	15.0	b	63.73	a	34.42	A
pH	6.34	a	6.29	a	6.60	a	6.50	a	6.33	b	6.41	A
C_{mic}	58.82	b	58.82	b	77.61	a	64.41	a	65.82	a	65.14	B
qCO_2	2.02	a	1.83	a	1.72	a	1.85	a	1.86	a	1.86	B
PLFA	12.74	b	13.03	b	18.14	a	14.42	a	14.81	a	14.62	C
10-June												
TOC	6.78	b	6.93	b	8.67	a	7.48	a	7.44	a	7.45	A
WEOC	3.04	a	3.23	a	4.13	a	3.98	a	2.96	a	3.47	B
TN	0.95	b	0.97	b	1.16	a	1.01	a	1.04	a	1.03	A
N_{min}	29.44	a	41.52	a	37.03	a	9.80	b	61.24	a	35.52	A
pH	6.19	a	6.19	a	6.48	a	6.42	a	6.15	b	6.29	B
C_{mic}	77.01	b	56.71	b	93.04	a	74.42	a	76.71	a	75.64	B
qCO_2	1.48	a	2.15	a	1.02	a	1.69	a	1.41	a	1.55	B
PLFA	12.92	b	13.01	b	16.51	a	14.94	a	13.41	a	14.11	D
17-Sept												
TOC	6.91	b	7.10	b	8.78	a	7.69	a	7.52	a	7.60	A
WEOC	7.89	a	8.20	a	9.19	a	9.24	a	7.61	b	8.43	A
TN	0.91	b	0.94	b	1.10	a	0.97	a	1.00	a	0.98	B
N_{min}	9.17	b	7.80	a	10.3	b	5.86	a	14.04	b	9.93	B
pH	6.33	a	6.32	a	6.62	a	6.50	a	6.35	b	6.42	A
C_{mic}	82.04	c	90.82	b	120.03	a	85.04	b	111.05	a	97.44	A
qCO_2	1.88	a	1.74	a	1.03	b	1.87	a	1.22	b	1.55	B
PLFA	17.81	b	17.2	b	23.13	a	19.61	a	19.14	a	19.32	A

*Marginal means for two and three-way interactions are not shown because they were not significant except for TOC, TN and soil pH interaction between organic and N fertilization in April. Values followed by different lowercase letters indicate significant (P < 0.05) differences between organic or mineral N fertilization treatments within the same sampling date as determined by LSD. Capital letters indicate significant differences between sampling dates as determined by LSD. See text for abbreviations and units of measurements.

1.3.3. Spectroscopic characterization of soil organic matter

Soil samples spectra showed the same general peak pattern in all fertilization treatments. An example is reported in Fig. 1.2. The relative peak area (rA) of the band at 2930 cm^{-1} was higher in manure treatment as compared to control and crop residue treatments, independent of the sampling date (Table 1.2). The opposite trend was observed for the peak at 1620 cm^{-1} where the relative area was lower in manure treatment as compared to control and crop residue treatments. Similar results were observed for the peak at 1520 cm^{-1} in March, June and September, while in April both crop residue and manure treatments showed a significant reduction of the rA at 1520 cm^{-1} rA compared to the control. No significant differences between organic fertilization treatments were observed for the relative peak area at 1160 cm^{-1}. None of the studied bands were affected by the subplot factor mineral N fertilization. In April and June, a weak significant ($0.10 < P > 0.06$) interaction between organic and N fertilization was observed for the peak at 2930 cm^{-1}. The cause of this interaction was similar to that observed for TOC. Namely, mineral N fertilization increased the 2930 cm^{-1} rA in the control plots, where organic fertilizers were not applied, decreased the 2930 cm^{-1} rA in the crop residue treatments, and left it unchanged in the manure treatment. The 2930 cm^{-1} rA showed maximum values at planting, followed by a drop in April and an increase thereafter (Table 1.2). Opposite temporal patterns were observed for the others rAs.

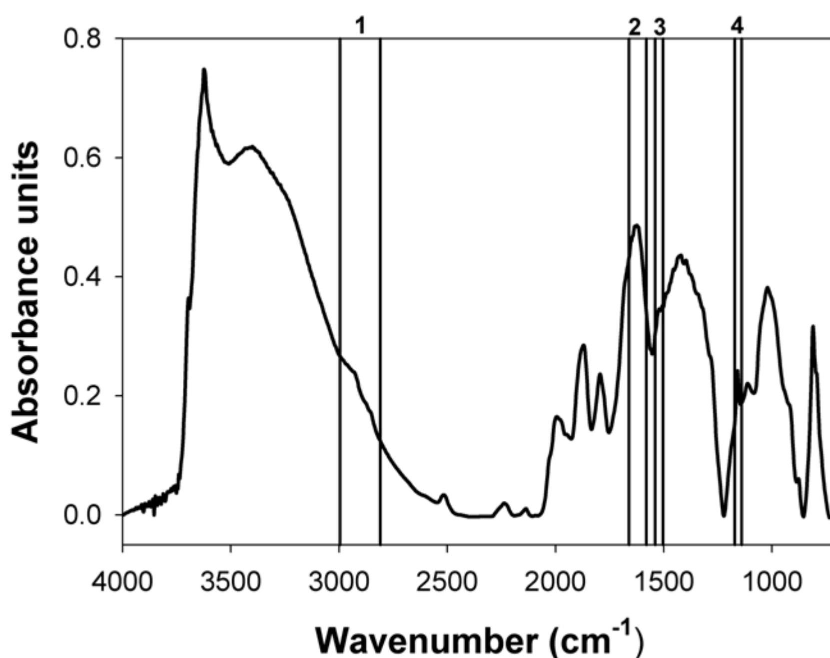

Fig. 1.2. Example of a baseline corrected DRIFT spectra of a bulk soil sample. The indicated regions were assigned to the following molecular vibrations for peak area integration: (1): 2930 cm^{-1} (C-H), (2): 1620 cm^{-1} (C=C and COO^{-}), (3): 1520 cm^{-1} (C=C and N-H), (4): 1160 cm^{-1} (C-OH).

Table 1.2. Marginal means of the relative peak areas in percentage of the sum of all selected peak areas of the DRIFTS spectra (rA; %) for the effects of organic fertilization, mineral N fertilization and sampling date*.

	Organic fertilization						N fertilization				Sampling date	
	Control		Crop residue		Manure		N_0		N_{200}			
18-March												
2930 cm^{-1}	9.38	b	9.90	b	17.72	a	11.95	a	12.71	a	12.33	A
1620 cm^{-1}	71.91	a	71.13	a	64.55	b	69.45	a	68.93	a	69.19	D
1520 cm^{-1}	7.80	a	7.80	a	6.83	b	7.51	a	7.44	a	7.48	D
1160 cm^{-1}	10.91	a	11.18	a	10.91	a	11.08	a	10.92	a	11.00	C
30-April												
2930 cm^{-1}	6.61	b	7.70	b	14.48	a	9.58	a	9.62	a	9.60	D
1620 cm^{-1}	73.57	a	73.02	a	67.05	b	71.27	a	71.16	a	71.21	A
1520 cm^{-1}	8.34	a	7.89	b	7.43	b	7.88	a	7.89	b	7.89	A
1160 cm^{-1}	11.47	a	11.39	a	11.04	a	11.28	a	11.32	a	11.30	B
10-June												
2930 cm^{-1}	7.60	b	8.12	b	15.16	a	10.29	a	10.29	a	10.29	C
1620 cm^{-1}	72.74	a	72.6	a	66.46	b	70.36	a	70.84	a	70.60	B
1520 cm^{-1}	8.12	a	8.07	a	7.27	b	7.82	a	7.83	a	7.82	B
1160 cm^{-1}	11.54	a	11.21	a	11.11	a	11.53	a	11.04	a	11.29	B
17-Sept												
2930 cm^{-1}	8.41	b	8.57	b	15.80	a	10.71	a	11.14	a	10.92	B
1620 cm^{-1}	72.2	a	72.13	a	66.28	b	70.37	a	70.02	a	70.19	C
1520 cm^{-1}	8.01	a	7.64	a	7.05	b	7.49	a	7.65	a	7.57	C
1160 cm^{-1}	11.66	a	11.66	a	10.87	a	11.43	a	11.37	a	11.40	A

*Marginal means for two and three-way interactions are not shown because they were not significant. Values followed by different lowercase letters indicate significant (P < 0.05) differences between organic or mineral N fertilization treatments within the same sampling date as determined by LSD. Capital letters indicate significant differences between sampling dates as determined by LSD.

1.3.4 Microbial biomass carbon and metabolic quotient

Treatments amended with manure showed higher values of C_{mic} as compared to crop residue and control treatments throughout the cropping season (Table 1.1), even though at planting the treatment effect was only weakly significant (P = 0.08). Crop residue treatment showed C_{mic} values higher than the control only in September. Similarly, the subplot factor mineral N fertilization affected C_{mic} only in the samples taken in September, with treatments receiving urea showing higher values. The average C_{mic} content changed significantly over time, increasing steadily form March to September (Table 1.1). In March, April and June C_{mic} was significantly correlated with TOC. Correlation coefficients (r) were 0.74***, 0.82***, 0.61**, respectively. No correlation between C_{mic} and TOC was found in September (r = 0.28; P = 0.187).

Organic fertilization affected the metabolic quotient (qCO_2), with manure treatment showing lower values as compared to control and crop residue treatments (Table 1.1). However these differences were significant only for the samples taken in September. In this sampling date, significantly lower qCO_2 values were also observed in N_{200} as compared to N_0.

1.3.5. Enzyme activities

In general, enzyme activities were significantly higher in manure treatment compared to crop residue and control treatments, independent of the sampling date (Table 1.3). However, in the case of arginine deaminase, differences were statistically significant only in two out of four sampling dates. The response to mineral N fertilization varied depending both on the activity and the sampling date: arginine deaminase and β-glucosidase were not influenced by N fertilization, throughout the maize growing season. In June a significant reduction of dehydrogenase activity was observed in N_{200} compared to N_0 plots. Similarly, an overall negative effect of N fertilization was observed for protease activity for the samples taken in September. In April N fertilization had an organic fertilization-dependent effect, inhibiting dehydrogenase and protease activity only in the plots amended with organic materials. Similar though weaker ($0.10 < P > 0.06$) negative interactions between organic and N fertilization were observed for urease activity in June and September.

In general, the average level of enzymatic activities fluctuated over the cropping season (Table 1.3). Arginine deaminase increased between April and June and remained stable thereafter. β-glucosidase, protease and urease activities had similar temporal patterns declining between March and April and increasing again to reach maximum values by the end of the cropping season. As compared to β-glucosidase and urease, protease activity rise started earlier in the season, between April and June, and was followed by a further increase between June and September. Dehydrogenase activity fluctuated little from March to September and no significant differences were observed between the sampling dates.

With the exception of arginine deaminase, enzymatic activities showed statistically significant correlation with TOC and total PLFA at all sampling dates (Table 1.4). In March and April, similar correlations among enzymatic activities and C_{mic} were observed (Table 1.4), while in June the correlations were significant only for extracellular activities. In September, the correlations between enzyme activities and C_{mic} were either weak or not significant.

Table 1.3. Marginal means of arginine deaminase, dehydrogenase, β-glucosidase, protease and urease for the effects of organic fertilization, mineral N fertilization and sampling date*.

	Organic fertilization						N fertilization				Sampling date	
	Control		Crop residue		Manure		N_0		N_{200}			
18-March												
Arginine	1.56	a	1.61	a	1.66	a	1.71	a	1.50	a	1.61	B
Dehydrogenase	20.36	b	22.25	b	30.54	a	24.95	a	28.81	a	24.38	A
β-glucosidase	51.73	b	52.07	b	67.52	a	55.32	a	58.89	a	57.10	B
Protease	20.06	b	18.31	b	34.79	a	23.1	a	24.86	a	23.97	C
Urease	8.77	b	9.79	b	14.64	a	10.95	a	11.17	a	11.06	B
30-April												
Arginine	1.41	b	1.44	b	2.00	a	1.61	a	1.63	a	1.62	B
Dehydrogenase	21.76	b	21.35	b	27.79	a	24.15	a	23.16	a	23.64	A
β-glucosidase	43.37	b	44.38	b	60.61	a	48.66	a	50.25	a	49.45	C
Protease	19.97	b	17.14	b	24.87	a	20.97	a	19.02	a	19.99	C
Urease	9.38	b	9.89	b	13.63	a	10.67	a	11.27	a	10.97	B
10-June												
Arginine	1.74	a	1.93	a	2.45	a	2.11	a	1.97	a	2.04	A
Dehydrogenase	21.16	b	20.19	b	25.95	a	24.19	a	20.67	b	22.43	A
β-glucosidase	44.18	b	44.03	b	59.10	a	48.04	a	50.25	a	49.1	C
Protease	26.64	b	23.85	b	37.89	a	31.29	a	27.63	a	29.46	B
Urease	7.25	b	8.33	b	11.70	a	9.43	a	8.75	a	9.09	C
17-Sept												
Arginine	1.81	b	1.77	b	2.16	a	1.93	a	1.90	a	1.91	A
Dehydrogenase	24.78	b	22.30	b	31.36	a	27.80	a	24.50	a	26.15	A
β-glucosidase	59.56	b	61.64	b	77.95	a	65.56	a	67.21	a	66.38	A
Protease	35.41	b	33.85	b	57.36	a	46.79	a	37.62	b	42.21	A
Urease	12.70	b	13.56	b	20.60	a	16.43	a	14.81	a	15.62	A

*Marginal means for two and three-way interactions are not shown because they were not significant except for dehydrogenase and protease activities interaction between organic and N fertilization in April. Values followed by different lowercase letters indicate significant ($P < 0.05$) differences between organic or mineral N fertilization treatments within the same sampling date as determined by LSD. Capital letters indicate significant differences between sampling dates as determined by LSD. See text for abbreviations and units of measurements.

Table 1.4. Pearson's product-moment correlation coefficients between total organic carbon (TOC), microbial biomass carbon (C_{mic}), total phospholipids (PLFA) and enzymatic activities*.

	TOC	C_{mic}	PLFA
18-March			
Arginine	0.79 ***	0.72 ***	0.43 *
Dehydrogenase	0.80 ***	0.78 ***	0.90 ***
β-glucosidase	0.83 ***	0.66 ***	0.74 ***
Protease	0.68 ***	0.63 ***	0.62 **
Urease	0.85 ***	0.76 ***	0.90 ***
30-April			
Arginine	0.57 **	0.34	0.40
Dehydrogenase	0.83 ***	0.85 ***	0.82 ***
β-glucosidase	0.84 ***	0.73 ***	0.84 ***
Protease	0.79 ***	0.75 ***	0.73 ***
Urease	0.80 ***	0.73 ***	0.88 ***
10-June			
Arginine	0.19	0.28	0.13
Dehydrogenase	0.68 ***	0.29	0.55 **
β-glucosidase	0.83 ***	0.69 ***	0.76 ***
Protease	0.72 ***	0.72 ***	0.71 ***
Urease	0.74 ***	0.57 **	0.82 ***
17-Sept			
Arginine	0.86 ***	0.34	0.77 ***
Dehydrogenase	0.80 ***	0.03	0.71 ***
β-glucosidase	0.67 ***	0.18	0.88 ***
Protease	0.58 **	0.55 **	0.59 **
Urease	0.93 ***	0.36	0.91 ***

*Significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

1.3.6. Total PLFA and microbial community structure

As observed for C_{mic}, at all sampling dates the total PLFA content (Table 1.1) was significantly higher in the manure treatment while the differences between control and crop residue treatments were small and not significant. Nitrogen fertilization did not affect total PLFA content, independently of the considered sampling date. The total content of PLFA changed significantly over time, with a pattern similar to that observed for extracellular enzyme activities: a first decline occurred between March and April, followed by a further decrease between April and June.

A strong increase was observed from June to September with total PLFA reaching maximum values by the end of the cropping season. Besides being correlated with most of the enzymatic activities (Table 1.4) total PLFA content was also significantly correlated with TOC: in March, April, June and September the correlation coefficients (r) were 0.92***, 0.88***, 0.77*** and 0.86***, respectively. Similar significant correlations were observed between total PLFA and C_{mic} with the exception of the last sampling date, when no correlation was found (r = 0.69***, 0.84***, 0.60** and 0.19 in March, April, June and September, respectively). Signature PLFA were used to characterize the microbial community structure of the samples taken in March and September (Table 1.5). For the samples taken in April and June the short chained PLFA were to variable to obtain an accurate analytical estimation. These sampling dates were thus excluded from the microbial community structure study. The PLFA profiles of March and September revealed that, as observed for total PLFA, single bacterial PLFA bioindicators were also enriched under manure, whereas the difference between control and crop residue treatments were small and not significant. In September the subplot factor mineral N fertilization showed significant effects on Gram-negative PLFA cy17:0, which was higher in N_{200} compared to N_0. At both sampling dates the fungal PLFA 18:2ω6 increased under manure treatment compared to control and crop residue treatments and decreased under N_{200} compared to N_0. However, none of these differences were significant.
With respect to their relative contribution, at both sampling dates the percentage of Gram-positive PLFA bioindicators increased under manure treatment, while the percentage of Gram-negative PLFA bioindicators decreased as compared to the control and crop residue treatments (data not shown). As a consequence the Gram-positive to Gram-negative ratio was higher under manure (Table 1.5). In March the subplot factor mineral N fertilization did not show any influence on the bacterial community composition. On the contrary, in the samples taken in September the percentage of Gram-negative bacteria increased in the N fertilized treatments. This led to a significantly lower Gram-positive to Gram-negative ratio in N_{200} compared to N_0. No significant differences between the bacteria to fungi ratios of the different treatments were observed at both sampling dates.

Table 1.5. Marginal means of Gram negative-bacteria, Gram-positive bacteria, fungal PLFA and ratios of Gram-positive to Gram-negative bacteria and bacteria to fungi PLFA for the effects of organic and mineral N fertilization of the samples taken in March and September*.

		Organic fertilization						N fertilization			
		Control		Crop residue		Manure		N_0		N_{200}	
18-March											
Gram -	cy17:0	0.80	b	0.84	b	0.98	a	0.85	a	0.89	a
	cy19:0	0.14	b	0.15	b	0.18	a	0.16	a	0.16	a
Gram +	i15:0	2.51	b	2.66	b	3.36	a	2.81	a	2.88	a
	a15:0	1.50	b	1.59	b	2.02	a	1.68	a	1.72	a
	i16:0	1.04	b	1.08	b	1.36	a	1.14	a	1.17	a
	i17:0	0.65	b	0.68	b	0.87	a	0.73	a	0.74	a
Fungi	18:2ω6	0.55	a	0.52	a	0.67	a	0.60	a	0.56	a
Gram + / Gram -		6.02	b	6.10	b	6.54	a	6.27	a	6.17	a
Bacteria / fungi		13.10	a	15.32	a	14.73	a	13.72	a	15.02	a
17-September											
Gram -	cy17:0	0.78	b	0.74	b	0.95	a	0.77	b	0.87	a
	cy19:0	0.16	b	0.17	b	0.20	a	0.17	a	0.18	a
Gram +	i15:0	1.49	b	1.49	b	2.10	a	1.73	a	1.66	a
	a15:0	0.92	b	0.94	b	1.32	a	1.07	a	1.05	a
	i16:0	0.87	b	0.85	b	1.14	a	0.94	a	0.97	a
	i17:0	0.60	b	0.61	b	0.81	a	0.67	a	0.68	a
Fungi	18:2ω6	0.68	a	0.63	a	0.69	a	0.69	a	0.65	a
Gram + / gram -		4.15	b	4.29	b	4.70	a	4.63	a	4.13	b
Bacteria / fungi		8.07	a	8.87	a	10.20	a	8.76	a	9.34	a

*Marginal means for two-way interactions are not shown because they were not significant. Values followed by different lowercase letters indicate significant ($P < 0.05$) differences between organic or mineral N fertilization treatments within the same sampling date as determined by LSD. See text for units of measurements.

Separate principal component analysis (PCA) for March and September samplings were carried out to reveal differences among the treatments (Figs. 1.3 a and 1.3 b). An additional PCA including the PLFA profiles of both March and September was carried out to evidence variations in microbial community composition due to sampling date (Fig. 1.3 c). Only 21 of the 27 identified PLFA were detectable in all the field replicates of all the treatments and were thereby included in the analysis. Soil pH, TOC and N_{min} were also included in the analysis. Pearson's product moment correlation coefficients (r) between the first two principal components variates and the original variates were used to obtain detailed information about the variables that were responsible for the separation of the treatments (Table 1.6).

In March the first two principal components accounted for the 74 % and the 8 % of the total variance, respectively (Fig. 1.3 a and Table 1.6). High correlations ($-0.95 < r > -0.99$, $P < 0.05$) between PC1 and the original variates were observed for the Gram-positive PLFA bioindicators i15:0, a15:0, i16:0, i17:0, for the unsaturated PLFA 14:0, 15:0, 16:0 and 17:0, and for some of the polyunsaturated C18 PLFA, indicating that these variables contributed more to the separation of the samples along this component. Soil samples of plots receiving manure were separated from the other ones along PC1. Other treatments showed no obvious differences. Along PC2, which was dominated by the polyunsaturated PLFA 20:4ω6 and 20:3ω6, and by N_{min}, samples of plots receiving crop residue and N were separated from the others. In September the first two principal components accounted for the 64 % and the 12 % of the total variability, respectively (Fig. 1.3 b and Table 1.6). The correlation coefficients between PC1 and the original variates revealed that terminal branched PLFA i15:0, a15:0, and i16:0, Gram-positive bacteria bioindicators, and unsaturated PLFA 15:0, 16:0 and 17:0, contributed most to the separation of the samples along this component ($-0.95 < r > -0.99$, $P < 0.05$). As observed in March, soil samples of plots receiving manure were clearly separated from the other ones along PC1. PC2 was dominated by the PLFA 20:3ω6 and 20:0 and by soil pH. Along PC2 samples of plots receiving N fertilizer were separated from the unfertilized ones.

In the PCA including both sampling dates (Fig. 1.3 c and Table 1.6) samples collected in March were clearly separated from the ones collected in September along PC2. The variables contributing more to the separation along this component were N_{min}, the Gram-positive PLFA bioindicators i15:0 and a15:0, the unsaturated PLFA 14:0 and 15:0, the Gram-negative bioindicator cy 19:0 and the polyunsaturated fatty acid 20:3ω6. Samples of plots receiving manure collected in March were separated from the other samples along PC1, while for the samples of plots receiving manure collected in September overlapping with other treatments of the March sampling was observed.

Table 1.6. Results of principal component analysis*.

	Sampling date					
	18-March		**17-Sept**		**March and Sept**	
	PC		PC		PC	
	1	2	1	2	1	2
% of variance	74	8	64	12	58	14
14:0	**-0.98**	0.06	**-0.94**	-0.11	**-0.73**	**-0.67**
i15:0	**-0.98**	0.18	**-0.96**	0.16	**-0.83**	**-0.53**
a15:0	**-0.95**	0.08	**-0.97**	0.04	**-0.82**	**-0.56**
15:0	**-0.98**	0.05	**-0.99**	0.00	**-0.85**	**-0.50**
i16:0	**-0.98**	0.11	**-0.99**	-0.04	**-0.97**	-0.19
16:1ω7	**-0.96**	0.01	**-0.80**	0.10	**-0.88**	**-0.35**
16:0	**-0.99**	0.10	**-0.98**	0.16	**-0.97**	-0.15
i17:0	**-0.98**	0.14	**-0.93**	0.08	**-0.95**	0.09
cy17:0	**-0.87**	0.12	**-0.80**	**-0.45**	**-0.83**	0.05
17:0	**-0.95**	-0.15	**-0.97**	-0.09	**-0.93**	0.20
18:2ω6	**-0.77**	-0.13	**-0.55**	0.26	**-0.55**	**0.46**
18:2nω6t/18:1ω9/18:3ω3	**-0.97**	0.01	**-0.82**	0.36	**-0.92**	0.22
X18:1ω7/18:1ω9t	**-0.96**	0.08	**-0.91**	0.32	**-0.93**	0.13
18:0	**-0.92**	-0.30	**-0.81**	-0.16	**-0.83**	0.19
cy19:0	**-0.93**	0.27	**-0.90**	-0.06	**-0.69**	**0.60**
20:4ω6	**-0.60**	**-0.70**	**-0.59**	0.08	**-0.59**	0.16
20:5ω3	**-0.54**	**-0.78**	**-0.48**	-0.18	**-0.58**	-0.21
20:3ω6	**-0.74**	-0.28	-0.16	**0.70**	-0.13	**0.52**
20:0	**-0.59**	0.15	**-0.52**	**-0.72**	**-0.42**	**0.41**
22:0	**-0.85**	0.07	**-0.73**	**-0.57**	**-0.69**	**0.42**
24:0	**-0.86**	0.18	**-0.82**	**-0.42**	**-0.85**	-0.02
pH	**-0.63**	0.23	-0.43	**0.71**	**-0.48**	0.24
TOC	**-0.93**	0.09	**-0.84**	0.25	**-0.79**	**0.39**
Nmin	-0.25	**-0.57**	**-0.46**	-0.31	-0.11	**0.63**

*Pearson's product moment correlation between the original variates and the principal component variates. Correlation is significant at the $P \leq 0.05$ level (values marked in bold).

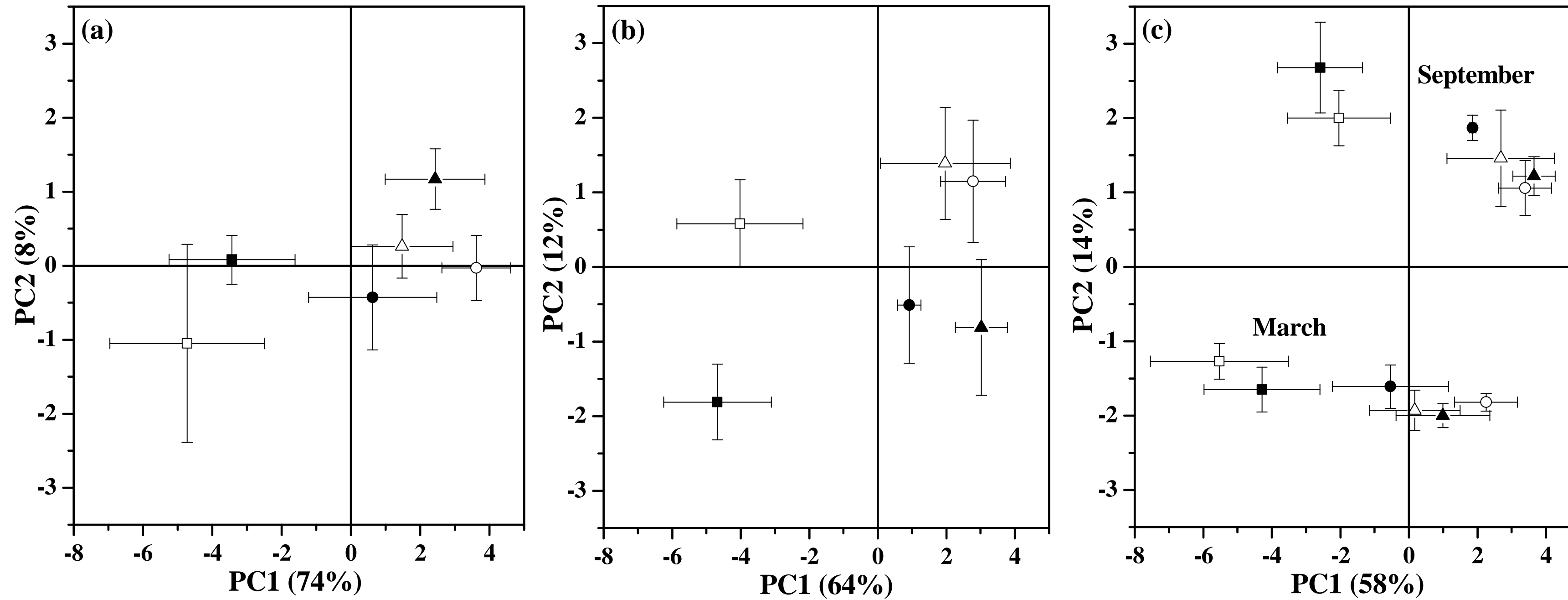

Fig. 1.3. Plots of PC1 and PC2 means (± standard error) produced from the principal component analysis of PLFA profiles obtained from (○) control, (●) control + N, (Δ) crop residue, (▲) crop residue + N, ( ) manure and (■) manure + N soils of (a) March, (b) September and (c) March and September samplings.

1.3.7. Correlations between DRIFTS spectra and soil properties

With the exception of C_{mic} and arginine deaminase whose correlation with the relative peak area (rA) of the selected bands was not always significant, positive correlations were found between the rA at 2930 cm^{-1} and soil chemical, microbial and biochemical properties. Conversely, negative correlations were observed between the rAs at 1620 cm^{-1} and 1520 cm^{-1} and the same soil properties (Table 1.7).

DRIFTS spectra were related to TOC, C_{mic} and total PLFAs using partial least square (PLS) analysis. Results presented in Table 1.8 a refer to PLS models that include the whole DRIFTS spectra, while results presented in 1.8 b refer to PLS models that include only the peaks utilized in the rA investigation and the wavenumbers of 3660 to 3517 and 3517 to 3252 cm^{-1}, 1928 to 1830 cm^{-1}, 1830 to 1754 cm^{-1}, 1428 to 1273 cm^{-1}, 1084 to 921 cm^{-1}, and 854 to 736 cm^{-1}.

As compared to the whole DRIFTS spectra, peak pre-selection generally produced PLS models that performed slightly, but consistently better in terms of percentage of variance explained (R^2), error of prediction ($RMSE_{CV}$ and RMSEP), bias and robustness (RPD). As a consequence only the results from the peak pre-selection will be considered in this section.

The best PLS models were the ones relating the first derivate of the selected DRIFTS peaks and TOC content (Table 1.8 b). The calibration, validation and reverse validation models accounted for the 93, the 91 and the 86 % of the total variance, respectively. In the scatter plot (Fig. 1.4) the slope of the regression line between predicted and measured TOC values was lower than 1 (0.884), indicating that the PLS model tended to underestimate this soil property to some extent.

PLS models relating the second derivate of the selected DRIFTS peaks and total PLFA content produced slightly lower R^2 with the calibration, validation and reverse validation models accounting for the 93, the 83 and the 82 % of the total variance, respectively (Table 1.8 b). The ratio of standard deviation to standard error of prediction changed little from the validation model (RDP = 2.54) to the reverse validation model (RPD = 2.45), indicating a high stability of the model. In the scatter plots of predicted versus observed values (Fig. 1.5) the slope of the regression line was close to 1 (0.917), indicating that PLS model contained little bias in estimating this soil property.

PLS models relating the first derivate of the selected DRIFTS peaks and C_{mic} resulted in the poorest correlations (Table 1.8 b). The calibration, validation and reverse validation models accounted for the 78, the 64 and the 51 % of the total variance, respectively. The ratio of standard deviation to standard error of prediction for the validation model was lower as compared to the former

validation models (RDP = 1.68) and further decreased in the reverse validation model (RPD = 1.44). In the scatter plots of predicted versus observed C_{mic} values (Fig. 1.6) the slope of the regression line was only 0.568, indicating a considerable underestimation of C_{mic} by the PLS model. Five points, all referring to C_{mic} values measured on the samples taken in September, formed a clearly separated cluster and could be the cause of the poor fitting.

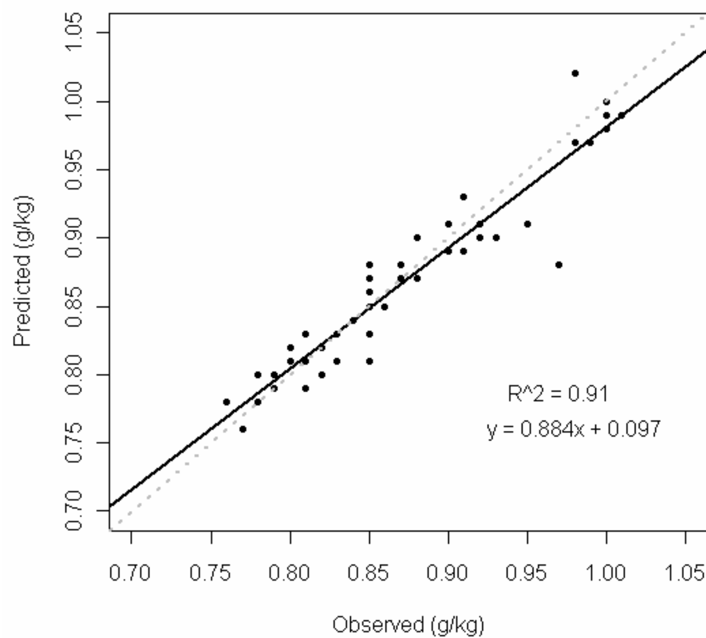

Fig. 1.4. Predicted versus observed TOC values for the validation set.

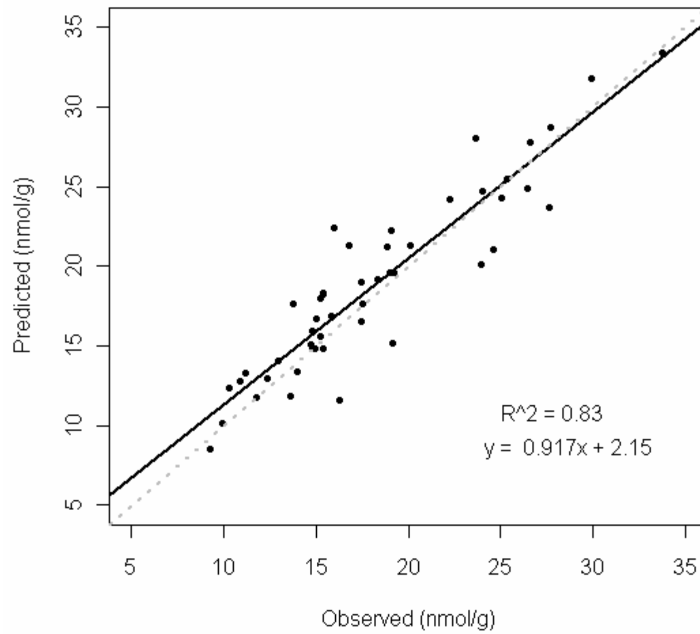

Fig. 1.5. Predicted versus observed total PLFA values for the validation set.

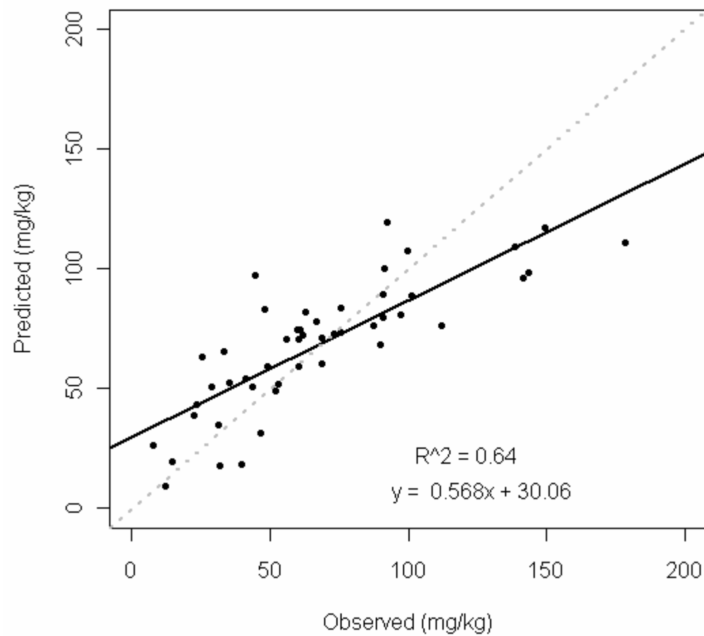

Fig. 1.6. Predicted versus observed C_{mic} for the validation set.

Table 1.7. Pearson's product-moment correlation coefficients between DRIFTS relative peak areas (rA; %) and soil properties*.

	rA at 2930 cm^{-1}				rA at 1620 cm^{-1}				rA at 1520 cm^{-1}			
	Sampling date				Sampling date				Sampling date			
	18-March	30-April	10-June	17-Sept	18-March	30-April	10-June	17-Sept	18-March	30-April	10-June	17-Sept
TOC	0.94 ***	0.96 ***	0.94 ***	0.96 ***	- 0.92 ***	- 0.93 ***	- 0.89 ***	- 0.94 ***	- 0.83 ***	- 0.72 ***	- 0.76 ***	- 0.77 ***
TN	0.92 ***	0.87 ***	0.88 ***	0.92 ***	- 0.90 ***	- 0.84 ***	- 0.88 ***	- 0.91 ***	- 0.82 ***	- 0.68 ***	- 0.61 **	- 0.70 ***
C_{mic}	0.64 ***	0.81 ***	0.66 ***	0.24	- 0.64 ***	- 0.75 ***	- 0.66 ***	- 0.30	- 0.56 **	- 0.78 ***	- 0.39	0.18
PLFA	0.84 ***	0.85 ***	0.77 ***	0.87 ***	- 0.79 ***	- 0.79 ***	- 0.73 **	- 0.84 ***	- 0.83 ***	- 0.81 ***	- 0.62 **	- 0.61 **
Arginine	0.56 **	0.62 **	0.17	0.81 ***	- 0.52 *	- 0.72 ***	- 0.26	- 0.76 ***	- 0.61 **	- 0.27	0.14	- 0.64 ***
Dehydrogenase	0.58 **	0.81 ***	0.64 ***	0.78 ***	- 0.51 *	- 0.74 ***	- 0.59 **	- 0.69 ***	- 0.76 ***	- 0.86 ***	- 0.62 **	- 0.84 ***
β-glucosidase	0.86 ***	0.78 ***	0.85 ***	0.67 ***	- 0.85 ***	- 0.77 ***	- 0.78 ***	- 0.65 ***	- 0.67 **	- 0.52 **	- 0.65 ***	- 0.50 *
Protease	0.81 ***	0.73 ***	0.70 ***	0.52 **	- 0.84 ***	- 0.70 ***	- 0.66 ***	- 0.59 **	- 0.58 **	- 0.58 **	- 0.50 *	- 0.09
Urease	0.80 ***	0.76 ***	0.75 ***	0.88 ***	- 0.75 ***	- 0.69 ***	- 0.62 **	- 0.85 ***	- 0.73 ***	- 0.73 ***	- 0.82 ***	- 0.66 ***

*Significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Table 1.8. Summary statistics for the DRIFTS models of TOC, total PLFA and C_{mic} produced by partial least-square analysis using: (a) the whole spectra or (b) pre-selected peaks*.

Predicted Property	Pre-processing	data points	Calibration			Validation					Reverse validation				
			Rank	R^2	$RMSE_{CV}$	Rank	R^2	RMSEP	RPD	Bias	Rank	R^2	RMSEP	RPD	Bias
(a) Whole spectra															
TOC	Min-max normalization	960	7	0.90	0.0219	7	0.89	0.0237	3.17	0.00310	8	0.82	0.0270	2.38	- 0.00436
PLFA	Second derivate	886	6	0.92	1.63	6	0.82	2.39	2.38	- 0.126	3	0.73	2.76	1.93	0.0866
C_{mic}	Vector normalization	608	6	0.66	20.5	6	0.63	23.2	1.66	- 3.22	6	0.48	23.3	1.40	3.49
(b) Peak pre-selection															
TOC	First derivate + vector normalization	555	7	0.93	0.0179	7	0.91	0.0216	3.43	0.00374	4	0.86	0.0236	2.70	- 0.00228
PLFA	Second derivate	332	7	0.93	1.58	7	0.83	2.33	2.54	- 0.644	6	0.82	2.28	2.45	0.685
C_{mic}	First derivate	293	8	0.78	16.9	8	0.64	22.8	1.68	- 0.931	5	0.51	22.6	1.44	- 1.74

*Pre-processing: mathematical transformation applied to the spectral data; Data points: number of data points employed to develop the validation model; abbreviations are explained in the text.

1.4. Discussion

1.4.1. Temporal patterns

Total soil organic C (Table 1.1) changed little over the growing season, indicating that factors other than fertilization (i.e. environmental conditions) had only small effects on this parameter.

In contrast to TOC, TN and N_{min} fluctuated more over the season: the increase observed from March to April is likely the result of urea application, while the subsequent reduction observed from June to September could be ascribed to plant uptake and possibly also to losses from the soil system through leaching and denitrification.

With respect to the values observed in the first three sampling dates, WEOC content doubled in September, after maize harvest (Table 1.1). According to Zsolnay (1996) and Chantigny (2003), one of the main sources of dissolved organic matter in agricultural soils is surface and subsurface litter, including decaying roots. In our study plant-derived compounds coming from the decaying roots left in the field after harvest were probably responsible of the increase in WEOC.

The relative contribution of DRIFTS bands at wavenumbers corresponding to different organic functional groups showed small though significant variations during the cropping season (Table 1.2). The rA at 2930 cm^{-1} (aliphatic C) fluctuated more over time and was probably responsible for the small changes observed in the other rAs, since the relative peak area was calculated and a change in a single peak could affect also the others. Factors such as mineralization of labile organic compounds, microbial turnover and release of root exudates could have contributed to the shifts in SOM quality observed over the season.

Microbial biomass estimated as total PLFA and C_{mic} were subjected to seasonal fluctuations as well (Table 1.1). With regard to total PLFA, two factors, namely soil moisture and substrate availability, could be considered the main causes of these fluctuations. Soil moisture probably became a limiting factor during late spring when the lowest values of total PLFA were observed. These results are in accordance with Debosz et al. (1999) and Geisseler and Horwath (2009), who reported a decline of microbial biomass due to a reduction in soil moisture. C_{mic} was not affected by soil moisture fluctuations, probably due to the fact that it may include both active and inactive microbial forms. On the contrary, since active microorganism contain a higher amount of PLFA as compared to inactive microorganisms (Zelles, 1999), total PLFA concentration may lend evidence for changes in the active fraction of soil microbial biomass. In accordance with what was observed in our study, Jen in our study, Jensen et al. (1997) reported a limited effect of soil water content on microbial

biomass estimated with chloroform-fumigation extraction (CFE) method and attributed this result to the methodology used. Carbon input from crop roots, rhizosphere products and crop residue can have a large effect on soil microbial biomass and activity (Chantigny et al., 1999; Debosz et al., 1999). In our study maximum concentrations of C_{mic} and total PLFA were observed in September, when aboveground plant biomass had been harvested and crop roots were left into the soil. Similarly, Debosz et al. (1999) and Geisseler and Horwath (2009) observed an increase of microbial biomass following the incorporation of new crop residue and Granatstein et al. (1987) reported a significant increase in microbial biomass soon after harvest. The increase of WEOC observed in September supports the hypothesis that microbial proliferation was promoted by an increase in substrate availability. Moreover, in the post-harvest sampling, DRIFT spectra showed an increase in aliphatic C (rA at 2930 cm^{-1}), suggesting that a change in the availability of substrates for microorganisms had occurred at this time.

Urease and β-glucosidase activities followed temporal patterns similar to that observed for the total PLFA, showing minimum values in June and maximum values in September (Table 1.3). In contrast, protease activity seemed to be less affected by environmental factors and particularly by soil moisture. Distinct temporal variations in microbial biomass and activity of extracellular enzymes could be due to the presence of enzymes adsorbed on clay minerals or associated with humic colloids (Burns, 1982). These immobilized enzymes can retain their catalytic capacity independently of soil microorganism status (Burns, 1982). A similar decoupling of extracellular enzyme activities from microbial biomass has been reported to occur in analogues field experiments (Debosz et al., 1999; Geisseler and Horwath; 2009).

Since intra-cellular enzymes are strictly related to soil microbial activity, we expected them to be more sensitive to environmental conditions as compared to extra-cellular enzymes and thus to fluctuate more over the season. Surprisingly, arginine deaminase and dehydrogenase fluctuated little over the season as compared to extracellular activities (Table 1.3).

1.4.2. Soil organic matter content and characterization

The repeated application of cattle manure resulted in a considerable increase of TOC and TN, while soil amendment with crop residue did not produce any relevant change (Table 1.1). Although the two organic materials were applied at the same dry matter rate, which roughly correspond to the same quantity of organic C (see Section 1.2.1), their ability to affect SOM remarkably differed.

In other long-term experiments animal manure positive effects on SOM have been reported to exceed that of crop residue (Rasmussen and Collins, 1991; Reeves, 1997; Triberti et al., 2008), suggesting that the level of organic matter that accumulate in soil depends on the chemical composition of the material added (Rasmussen and Collins, 1991). Moreover, when organic materials with a high C:N ratio such as crop residue are applied to the soil, another factor affecting their decomposition and thus their ability to influence SOM content, is mineral N availability (Mary et al., 1996). According to Green et al. (1995) and Chantigny et al. (1999), in the case of single application of crop residue, N availability accelerates the initial stages of decomposition, dominated by mineralization of sugars, hemicellulose, and cellulose, but not the total amount of C retained in soil. On the contrary in long-term experiments where crop residue and mineral N fertilizers are added annually, the effect of mineral N availability on cumulative amounts of C mineralized become greater with each additional annual cycle and could result in lower concentrations of TOC (Green et al., 1995). In accordance with these observations we found a negative interaction of soil amendment with crop residue and mineral N fertilization for TOC. We hypothesize that the increased availability of mineral N in the soil system stimulated the decomposition of crop residue and that most of the straw-derived C was mineralized. On the contrary in the plots where N fertilizer was not applied, straw decomposition was limited by the lack of N, and a modest increase of TOC was observed as compared to the control soil. As a consequence the overall effect of crop residue amendment, taking both the N fertilization treatments into account, was nullified. Even though the negative interaction between crop residue amendment and mineral N fertilization was evident (i.e. statistically significant) only in April, we believe that over the years it was able to affect the cumulative amounts of straw-derived C retained into the soil. Plowing the residue under may have also contributed to accelerate their decomposition and reduced the conversion efficiency of straw-derived C into SOM (Duiker and Lal, 1999). In a study using the same long-term experiment, Triberti et al. (2008) found that crop residue incorporation was less effective than soil manuring in increasing TOC and TN, but anyway produced a significant increase of both the parameters as compared to the unamended control. The lack of correspondence with our results could be partially due to the wide annual variability of TOC data observed from 1972 to 2000 by Triberi at al. (2008). The authors attributed this variability to the erratic weather conditions that regulate the organic matter degradation rate and to the lack of accuracy of TOC measurements. However the lack of correspondence between our results and that obtained by Tribetri et al. (2008)

on the same experimental site are still not fully understood.

Although the pronounced variability of WEOC between blocks observed in our experiment hampered the identification of statistically significant differences, the results obtained suggest that WEOC followed the same pattern as TOC (Table 1.1). Amending the soil with manure induced an increase in the WEOC fraction in accordance with what was reported by Embacher et al. (2008) and Chantigny (2003). The fact that the positive effect of manure was observed throughout the season implied that repeated inputs of this organic material have induced a steady increase in the amount of WEOM (Zsolnay, 1996). On the contrary no changes in WEOC were observed in plots amended with crop residue, suggesting that under this treatment WEOC from crop residue was rapidly consumed by soil microorganisms (Jensen et al., 1997), and thus the increase in WEOC was short-lived. The type of amendment thus influenced the level of WEOM and its chemical composition (Chantigny, 2003), which in turn determined its fate in soil. Similarly, Jensen et al. (1997) reported that, as compared to an unamended control soil, K_2SO_4 extractable organic C was significantly increased only after amendment with high rates of oil-seed rape straw (8 t dry matter ha^{-1}), while no differences were observed at lower application rates (4 t dry matter ha^{-1}). Moreover, the authors reported that during the year following oil-seed rape straw incorporation an increase in K_2SO_4 extractable organic carbon was observed only soon after amendment but not later in the season.

In accordance with what observed by Liang et al. (1998) and Chantigny et al. (1999), in our experiment the levels of WEOC tended to decrease under N fertilization, suggesting that fertilizer application could have stimulated WEOC cycling by providing additional N to soil microbes.

The greatest and most consistent source of water extractable organic matter is the solid phase of soil organic matter (Zsolnay, 1996). The two pools tend to be in equilibrium (Chantigny, 2003) and a quantitative relationship is expected to exist between them. Thus, the low levels of WEOC observed in our study could be related to low levels of organic matter in the solid phase, as suggested by the relatively low content of TOC. Expressed as a percentage of TOC, WEOC ranged from 0.04 to 0.13, in accordance with results reported by Zsolnay (1996) for field fresh soil water extracts. Moreover, it is important to remember that comparing data from different studies is often delicate since large discrepancies pertaining to the methodological approaches used to characterize WEOM may exist (Chantigny, 2003).

The spectroscopic characterization of soil organic matter with DRIFTS revealed that the long-term amendment of soil with manure had affected not only the quantity but also the quality of SOM. Changes in the relative absorption of the selected bands as a response to organic fertilization were

evident only for manure, indicating that under this treatment alone soil organic matter chemical composition was appreciably modified (Table 1.2). The increase of the relative area of the band at 2930 cm^{-1} observed in manure fertilized plots indicated that a relative enrichment of aliphatic C had occurred under this treatment (Table 1.2). These results are in accordance with Gerzabek et al. (2006) and Demyan et al. (2012), who found a relative enrichment of aliphatic C in the spectra of soils under long-term manure fertilization with respect to soils under mineral fertilization and to an unfertilized control soil. The enrichment in aliphatic C observed in our study was also accompanied by a higher organic matter content: in accordance with Gerzabek et al. (2006) a strong positive correlation between the relative area of the band at 2930 cm^{-1} and TOC and TN (Table 1.7) was observed. Similar positive correlations with TOC were reported by Demyan et al. (2012) using the corrected 2930 cm^{-1} peak area instead of the relative 2930 cm^{-1} peak area. Demyan et al. (2012) postulated that the aliphatic C peak at 2930 cm^{-1} may correspond to the more labile C compounds. The hypothesis was based on the positive correlation found between the corrected 2930 cm^{-1} peak area and the organic C of fractions dominated by labile organic matter, such as hot water extractable organic C and the light fraction of soil organic matter. The tendency of WEOC to increase under manure observed in our study seems to corroborate this hypothesis. Conversely, in a previous work Capriel (1997) attributed the enrichment in aliphatic C observed in the spectra of soil receiving high amounts of plant residues and organic fertilizers to a selective degradation of the organic inputs entering the soil, in which the hydrophilic fraction (i.e. cellulose, hemicellulose, proteins) was metabolized easily, while the hydrophobic fraction (i.e. lipids containing aliphatic C-H units) was more recalcitrant to microbial degradation and accumulated in the soil.

The rA at 1620 cm^{-1} and rA at 1520 cm^{-1} showed opposite trends (Table 1.2): a relative enrichment in aromatic C and N-H (amide II) groups was observed in the control soil and in the soil amended with crop residue as compared to the soil under manure. The correlations of the rA of these bands with TOC and TN (Table 1.7) were negative, indicating a decrease of the relative contribution of aromatic C and N-H (amide II) groups with increasing levels of organic matter. Similarly, Demyan et al. (2012) reported that the relative peak areas at 1620 cm^{-1} and 1520 cm^{-1} were higher in the spectra of an unfertilized control soil with respect to soil under long-term manure fertilization, and observed negative correlations between the corrected peak areas at 1620 cm^{-1} and 1520 cm^{-1} and TOC. According to Demyan et al. (2012) soils receiving small amount of organic inputs are depleted in labile organic compounds and as a consequence their spectra show a relative enrichment

in the peaks at 1620 cm^{-1} and 1520 cm^{-1} which may be related to more stable components of SOM. In contrast to our results, Gerzabek et al. (2006) reported a relative enrichment of carboxylic, aromatic and N-H (amide II) groups in the spectra of soil under long-term manure fertilization as compared to an unfertilized control soil. In our study the decline of the rA at 1620 cm^{-1} (aromatic C) and of the rA at 1520 cm^{-1} (aromatic C and N-H amide II groups) observed under manure could be partially due to the large quantity of aliphatic compounds that characterize this treatment, which leads to an increase of the band at 2930 cm^{-1} and influenced the other relative absorbance values since the relative peak area is calculated as the sum of all selected peak areas. In terms of absolute values, in fact, the areas of the peak at 2930 cm^{-1} of soil under manure were two to three times greater than the areas of the other treatments. On the contrary the changes observed in the absolute areas at 1620 cm^{-1} and at 1520 cm^{-1} were in the order of the 10 to 20 %. Differences in the peak area extents selection could have also contributed to the contrasting results obtained by Gerzabek et al. (2006).

1.4.3. Microbial and biochemical responses to fertilization

Any management practice that increases the incorporation of organic materials typically promotes soil biological activity (Dick, 1992). Under the conditions of our study however, only manure significantly stimulated the soil microbiota. As observed for SOM, the contrasting effects of manure and crop residue could be related to their different chemical composition. Manure increased soil organic matter and its more labile fraction and provided the microorganisms simultaneously with C and N, satisfying both the C and N requiring components of the microbial biomass and sustaining the microbial proliferation by slowly releasing nutrients (Liang et al., 1998). The soil microbiota responded to soil manuring with greater microbial biomass (Table 1.1) as well as with higher levels of intracellular and extracellular enzymatic activities (Table 1.3). Other investigations (Kandeler et al., 1999; Marinari et al., 2000, 2006; Böhme et al., 2005) have also reported positive effects of manure on microbial activity and biomass. The higher levels of enzyme activities observed under manure treatment could be mainly related to an increased production of enzymes promoted by the enlarged microbial biomass. The positive correlations observed between the enzymatic activities and the soil microbial biomass estimated as total PLFA (Table 1.4) support this hypothesis and confirm that soil enzymes are mainly produced by soil microorganisms to degrade available substrates (Winding et al., 2005; Geisseler and Horwath, 2009).

The tendency of the metabolic quotient (qCO_2) to decrease under manure treatment (Table 1.1) may indicate that besides stimulating microorganisms growth, soil manuring favored the development of an energetically more efficient microbial community, able to promote organic C accumulation (Trinchera et al., 2001; Dilly, 2005). In accordance with our results, Marinari et al. (2006) observed lower qCO_2 values in organic based farming systems as compared to conventional farming systems, and Böhme et al. (2005) found lower qCO_2 under manure than under mineral NPK fertilization.
On the contrary crop residue amendment had little impact on soil microbial biomass and activity (Table 1.1 and 1.3). Similarly, Marschner et al. (2003) observed that the long-term amendment of soil with manure significantly increased soil microbial biomass while the use of straw left it unchanged as compared to a control soil receiving only mineral fertilizers. In our experiment it is likely that the easily degradable compounds added with crop residue in October 2009 had already been depleted by the time we sampled the soil, while part of the more recalcitrant compounds as cellulose and lignin were still present in the system. In the absence of N fertilization their decomposition was probably limited since soil microorganisms were N limited. Moreover it is possible that the production and secretion of the depolymerising enzymes required for cellulose and lignin degradation implied an additional energy cost for the decomposer organisms and further decreased the amount of C available for biosynthesis (Recous et al., 1995). Fertilizer application stimulated crop residue cycling by providing additional N to soil microbes. Most of the straw derived C was probably lost as CO_2 since no increase in microbial biomass was observed. This hypothesis is in accordance with Recous et al. (1995) who observed that in soil amended with maize straw the cumulative amount of C mineralized increased with increasing rates of N fertilization while the C assimilated by microorganisms decreased.
The poor or not significant correlations between microbial biomass C estimated with the fumigation-extraction method and enzymatic activities observed in September (Table 1.4), could be attributed to a bias in the measure of C_{mic} in this sampling date. The origin of this bias could be that in this sampling date, when a large quantity of plant residues were in the field, small fragments of plant material were still present in some of the samples after sieving and affected the microbial biomass measurements.
The use of mineral fertilizers can increase plant biomass production which in turn increase the amount of residue returned to the soil with positive effects on soil biological activity (Dick, 1992). Even when aboveground plant biomass is removed with harvest, as it occurred in our experiment, an indirect positive effect of mineral fertilization on microbial processes could be observed.

According to Lynch and Panting (1980) plants respond to mineral fertilization with an increased root growth and release of exudates which would not only provide more root mass for decomposition but also a more favorable environment for microbial activity. In our study such a positive indirect effect of mineral N fertilization on microbial processes was not clearly observed. Even though N fertilization slightly increased microbial biomass and activity in the absence of organic amendments, the overall effect, taking into account all the treatments, was significant only for the C_{mic} of the samples taken in September (Table 1.1). Moreover N fertilization had transitory negative effects on protease and urease activities, particularly in the plots where N fertilization was combined with organic fertilization. High levels of mineral N which is the end-product of these two enzymes could have inhibited their activity (Allison and Vitousek, 2005). Similar selective inhibition of biochemical processes due to long-term mineral fertilization was reported by Dick et al. (1988) and Eivazi et al. (2003). In plots receiving only mineral N the positive indirect effect of urea application on microbial biomass and activity could have dwarfed the inhibition mechanism due to the presence of high concentrations of end-products of these enzymes.

The changes in microbial biomass and functionality were also accompanied by changes in the community structure (Table 1.5; Fig. 1.3), confirming that fertilization history strongly affect the composition of the soil microbial community (Zelles, 1999). The concentration of bacterial bioindicators was higher in manure treatment as compared to crop residue and control treatments. The increase was more pronounced for Gram-positive bacteria than for Gram-negative bacteria, and as a consequence Gram-positive to Gram-negative ratio was higher in manure plots. Principal component analysis clearly separated manure treatments from the other ones at both sampling dates. Gram positive bioindicators were within the most important variables contributing to the separation (Table 1.6). As indicated by total and water extractable organic carbon as well as by DRIFT-MIR spectroscopy, the organic matter that had accumulated under manure treatment strongly differs in terms of quantity, quality and availability as compared to crop residue and control treatments. These differences were probably the causes of the shift in the bacterial community structure observed under manure. Bacteria added with manure could as well have contributed to the observed changes (Marschner et al., 2003). Our results are in accordance with Marschner et al. (2003), who observed a shift toward Gram-positive bacteria under manure and sewage sludge amendment as compared to straw amendment and mineral fertilization.

The bacterial community responded to N fertilization with a shift toward Gram-negative bacteria, as indicated by the increase of the cyclopropil fatty acid cy17:0 and by the consequent reduction of the

Gram-positive to Gram-negative bacteria ratio. Similarly, Marschner et al. (2003) observed lower ratios of Gram-positive to Gram-negative bacteria under mineral fertilization than under organic fertilization. However, in our experiment the effect of N fertilization on bacterial community became evident only in September, after urea distribution. As compared to organic fertilization, N fertilization had a seasonal-dependant effect on the soil bacterial community. Principal component analysis of PLFAs profiles of September clearly separated treatments receiving mineral N from the unfertilized ones (Fig. 1.3 b). This separation was to a large extent determined by soil pH. This finding confirms that soil reaction strongly affect microbial community composition (Bääth and Anderson, 2003) and that mineral N fertilization may affect it through acidification mechanisms (Clegg, 2006). In the same sampling date a significant reduction of qCO_2 was observed, suggesting that the shift in the community composition could possibly be related to improved substrate use efficiency.

Although the high variability between blocks observed for the fungal bioindicator 18:2ω6 hampered the identification of statistically significant differences, the results obtained suggest that fungal contribution decreased as a consequence of mineral N fertilization (Table 1.5). In accordance with what observed in our study, N fertilization has been reported to reduce fungal biomass and relative contribution (Bardgett and McAlister, 1999; Frey et al, 2004; Six et al., 2006).

In the PCA including both sampling dates (Fig. 1.3 c), samples collected in March were clearly separated from the ones collected in September along PC2. The increase of soil mineral N from March to September was one of the main factors contributing to the separation of the treatments (Table 1.6), and was clearly related to urea distribution. However, PLFA bioindicators also contributed to the separation, indicating that a shift toward Gram-negative bacteria and an increase in the fungal contribution had occurred over the season. The reasons for the observed shift in the microbial community composition could be related to the fact that while in March the soil was bare, in September, maize had just been harvested. Over the growing season maize plants released litter material and root exudates modifying the amount and quality of available nutrients and influencing soil microbial community composition (Ngosong et al., 2010).

1.4.4. Relationship between DRIFTS spectra and soil microbial and biochemical properties

Besides being correlated with TOC and TN, the rA at 2930 cm^{-1}, 1620 cm^{-1} and 1520 cm^{-1} were generally correlated with microbial biomass and enzymatic activities (Table 1.7). As observed for

TOC and TN, correlations were positive for the relative area of the band at 2930 cm^{-1}, assigned to aliphatic C vibrations, and negative for the band at 1620 cm^{-1}, attributed to aromatic C=C stretching and COO^- vibrations, as well as for the band at 1520 cm^{-1}, representing aromatic C=C stretching vibrations and N-H (amide II) bending vibrations.

This findings further supports the hypothesis that the aliphatic C peak at 2930 cm^{-1} may correspond to the more labile C compounds (Demyan et al., 2012), able to promote microbial proliferation and activity. Moreover, the negative correlation observed between the rA at 1620 cm^{-1} and 1520 cm^{-1} and microbial biomass and activity confirms that these peaks represent more stable components (Demyan et al., 2012) that do not directly affect soil microorganisms, and that their relative contribution increase in soils depleted in labile organic compounds.

Chang et al. (2001) observed that soil properties that do not directly respond to near-infrared light (secondary properties) can be related to DRIFTS spectra due to their covariance with other soil properties (primary properties) exhibiting a direct response in the near-infrared region. Similar indirect relationships could probably be observed also when DRIFTS spectra are recorded in the mid-infrared region as occurred in our study. This is probably the case of microbial biomass and enzymatic activities, secondary properties that have shown covariance with primary properties such as TOC and TN (Table 1.4).

1.4.5. Prediction of soil properties based on DRIFTS-PLS models

The results of the PLS analysis confirmed that besides giving specific information on the broad chemical groups contained in the soil, mid-infrared spectra may relate to more general soil chemical and biological properties (Chapman et al., 2001), and by means of multivariate calibration these relationships can be quantified and used to create predictive models.

Chemometric models such as PLS are generally expected to improve with an increasing number of spectral data points. However, in some cases spectral noise or additional components in the samples may cause the PLS algorithm to interpret these features, which can degrade the model. In our study PLS models based on pre-selected peaks (Table 1.8 b) performed consistently better as compared to PLS models based on the whole DRIFTS spectra (Table 1.8 a). These findings suggest that the peaks pre-selection eliminated spectral noise that did not add information about the parameters we wanted to estimate. The pre-selection of the peaks generally increased the percentage of variance explained by the models and reduce the error of prediction and the bias.

Moreover it is noteworthy that the model robustness was improved (RPD), suggesting that also for the estimation of new unknown samples the pre-selection method would be preferred.
According to Chang et al. (2001), the ability of infrared spectra to predict soil properties can be grouped into different categories. Category A includes properties with RPD values higher than 2 and with measured versus predicted R^2 values between 0.80 and 1.00. According to the authors, properties belonging to category A can readily and accurately be estimated using infrared spectroscopy. In our study both TOC and total PLFA are classified as category A soil properties, and can thus be successfully predicted using DRIFT spectra. TOC measured versus predicted R^2 values found in our study (0.86-0.93) were comparable with results obtained using near infrared spectroscopy reported by Chang et al. (2001) ($R^2 = 0.87$) and Chodak et al. (2001) ($R^2 = 0.97$). With respect to total PLFA the R^2 found in our study (0.82-0.93) was higher than the one reported by Chapman et al. (2001) in a study regarding a peat soil ($R^2 = 0.65$). The prediction of C_{mic} was moderately accurate (RPD = 1.4 - 2, $R^2 > 0.50$). According to the classification proposed by Chang et al. (2001) this property belongs to category B, and its prediction could be improved by using different calibration strategies, eliminating artifacts related to sample handling and analysis, and by including more samples. As compared to models with $R^2 > 0.80$, which allows a quantitative prediction of the soil properties of interest, models with R^2 between 0.50 and 0.70 allow only a rough screening (Coûteaux et al., 2003). The values obtained in our study are in accordance with results obtained by Chang et al. (2001), who reported RPD values of 1.5 and R^2 of 0.60. A higher prediction ability was reported by Coûteaux et al. (2003) who found $R^2 > 0.95$ and RPD > 4.00.
According to Chapman et al. (2001) microbiological parameters generally show a poorer correlation with infrared spectra as compared to chemical parameters mainly due to the fact that microbial biomass is a too small component to have a direct effect on infrared spectra. Nevertheless, the microbial biomass is strictly related to soil chemical parameters such as organic C content, for which a theoretical basis for DRIFTS prediction is apparent (Chang et al., 2001; Chapman et al., 2001). In our study, the contrasting results obtained for total PLFAs and C_{mic} seems to indicate that the unsatisfying ability of DRIFTS-PLS models to predict C_{mic} was due to a lack of accuracy in the analytical determination of the latter and not to a scarce ability of DRIFTS spectra to directly or indirectly respond to changes in the soil microbiological component.

1.5. Conclusions

The soil microbiota responded to soil manuring by increasing its biomass, activity and substrate use efficiency. The changes in microbial biomass and functionality observed under manure were also accompanied by a change in the microbial community structure. Moreover the long-term application of manure had also increased SOM content and shifted its relative composition toward aliphatic compounds as determined by DRIFT spectroscopy. In contrast, crop residue amendment had limited effects on soil microbial processes as well as on content and quality of soil organic matter. Compared to organic fertilization, the impact of mineral N fertilization was less obvious and depended both on the measured parameter and sampling date. Where crop residue were applied N fertilization stimulated their mineralization and further reduced the amount of straw-derived C retained into the soil.

Microbial-related parameters were generally more affected by environmental factors as compared to content and quality of soil organic matter. However, even though their average levels fluctuated over the cropping season, their response to organic fertilization remained stable. On the contrary, the effects of mineral N fertilization varied over time. These variations appeared to be related to changes in mineral N availability due to urea distribution and not to factors other than soil management (i.e. environmental conditions). We can thus infer that seasonal fluctuations of environmental factors that occurred over the considered sampling period did not hamper the identification of fertilization-induced effects on soil quality.

PLS regression models relating the DRIFTS spectra of bulk soil with soil chemical and microbiological properties confirms that DRIFT spectroscopy has the potential to be used as a rapid soil testing technique for the future monitoring of soil quality. However, the accuracy of the prediction of microbial biomass C as measured by chloroform-fumigation extraction was moderate and needs improvements.

In conclusion, the strong relationship between microbiological parameters and SOM content and composition observed in this study indicates that microorganisms response to organic and mineral N fertilization is linked to the quantity and quality of the SOM that have accumulated under the different treatments.

CHAPTER 2

Microplate-based fluorimetric enzyme assays: optimization, precision estimation and application to soils under contrasting managements and environmental conditions

CHAPTER 2

2.1. Introduction

Enzyme assays have to be simple and rapid but above all sensitive and accurate (Nannipieri et al., 2002). Many factors have to be carefully evaluated and optimized so that the only limitation to the measured reaction rate is the enzyme concentration (Dick, 2011 a). Appropriate substrate concentration and incubation conditions should be found and used for the assay of each soil enzyme (Nannipieri et al., 2002). Measuring the activity of a specific enzyme-catalyzed reaction in one soil relative to another is the aim of soil enzymologists (Dick, 2011 a; ISO/TS 22939, 2010). However, comparisons are meaningful only if the activities are measured using standard assay procedures providing reproducible results (Dick, 2011 a; Burns et al., 2013; Deng et al., 2013).

Traditional bench-scale assays in which the analysis of a single enzyme activity is performed generally follow this approach: substrate concentration is selected in order to guarantee that saturation is reached. To minimize the contribution of microbial growth assay duration is the shortest over which a measurable level of activity is reached (Burns, 1978; Nannipieri et al., 2002). Enzyme assays are carried out at or close to the optimum pH in buffers of strength ensuring that fluctuations in pH during the incubation do not affect the results (Burns, 1978). On the other hand bench-scale assays are time consuming and labor intensive. Enzyme activities can be assayed one at a time on a limited number of samples. These features represent a constrain when the analysis of multiple enzyme activities of a large number of samples is required and until recently had limited our understanding of soil catalytic activity.

Microplate-scale assays have advantages over traditional bench-scale assays in that the analysis capacity is larger and the quantity of soil sample required is smaller (Popova and Deng, 2010). In particular, microplate-scale assays using fluorescent-dye conjugated substrates of 4-methylumbelliferone (MUF) have been proposed as high-throughput assays for the simultaneous determination of multiple soil enzyme activities on the same soil sample (Marx et. al., 2001; Vepsäläinen et al., 2001). MUF is a highly fluorescent compound. Flurometric enzyme assays that use MUF conjugates as substrates have a higher sensitivity compared to spectroscopic methods at microplate-scale (Marx et a., 2001; Popova and Deng, 2010; Deng et al., 2013). In MUF-based assays the extraction and purification of the reaction product from the soil can be avoided, since fluorescence can be measured directly in the reaction mixture. This feature speed up the analytical procedure and allow the processing of a large number of soil samples with an appropriate number of

analytical replicates. Moreover it implies that the measurements are not destructive and that product formation can be monitored over time (German et al., 2011).
On the other hand soil particles and phenolics present in the reaction mixture exercise a quenching effect which lead to a reduction of the fluorescence signal (Freeman et al., 1995). The degree of quenching depends on slurry turbidity and phenolics content and varies among soils, requiring correction for each soil sample (Freeman et al., 1995; Marx et al., 2001; German et al., 2011). This not only add on to the number of controls included in the assay but may also increase the uncertainty of the measurements (Popova and Deng, 2010).
At date the use of high-throughput microplate methods is becoming prevalent in studies related to soil enzymes (German et al., 2011). A range of MUF conjugates are commercially available and can be used as model substrates to study enzyme activities related to nutrient elements cycling and organic matter degradation in soil (German et al., 2011). However, currently available protocols based on MUF conjugates are still not thoroughly evaluated (Popova and Deng, 2010). Moreover, reagents preparation and storage, substrates final concentration, buffer strength and pH, soil slurry preparation, controls and blanks included in the assay and enzyme activity calculation vary widely (Vepsäläinen et al., 2001; Saiya-Cork et al., 2002; Marx et al., 2005; DeForest, 2009; Trap et al., 2012). Further, information regarding the precision of these protocols is scarce. An accurate evaluation of the methods reproducibility by means of true independent replicates (i.e. soil slurries produced from different aliquots of the same soil sample) is still missing since in microplate-scale protocols analytical replicates of sample assays are in most cases produced by dispensing aliquots of the same soil slurry repeatedly. Likely, the methods repeatability is still unclear and could hamper a correct interpretation of the analytical results. For instance Vepsäläinen et al. (2001) and DeForest (2009) reported that soil cold storage (+ 4 °C and - 20 °C) affected enzyme activities estimated with microplate-based fluorimetric assays in a random manner. However, since these assays were still not thoroughly evaluated and standardized is not possible to understand whether the observed variations in the levels of enzymatic activities were due to soil properties changes occurring during storage or to a poor reproducibility of the assay method. Finally, it is noteworthy that the most cited published methods (Marx et al., 2001; Vepsäläinen et al., 2001; Saiya-Cork et al., 2002) were developed for specific experimental sites and not tested on a broad spectrum of soils.
According to German et al. (2011) knowledge gaps of details and pitfalls of modern enzyme methodology could affect the quality and utility of soil enzyme data and make comparison between

data from different studies impossible. Proper optimization of microplate-based fluorimetric assays is advisable for each study site (German et al., 2011), particularly if the considered soil samples have chemical, physical and microbiological characteristics which strongly differ from the ones of the soils used for the method development.

Even when using a microplate-scale approach for the assay of soil enzyme activities, the basic assumptions of soil enzymology (Burns, 1978) should still be carefully considered. Several simplification have to be made in order to preserve the high-throughput nature of the method. For example, for the simultaneous determination of multiple soil enzyme activities on the same soil sample a compromise between the optimal assay conditions of the single enzymes such as temperature, pH and reaction time has to be reach. However other important assay conditions such as substrates concentration and soil slurry dilution could be optimized without compromising the analytical capacity typical of the microplate-scale approach.

Final assay substrates concentration should be optimized since saturating concentrations vary by site and enzyme (German et al., 2011; Burns et al., 2013). The use of sub-saturating substrate concentrations could cause an underestimation of the enzymatic activity which in turn could reduce the power to detect differences in enzyme activities, and increase the likelihood of Type II errors (i.e. false negatives) (German et al., 2011). On the other hand, at over-saturating concentrations substrate inhibition may occur (Freeman et al., 1995; ISO/TS 22939, 2010; German et al., 2012; Steen and Ziervogel, 2012). When substrate inhibition is relevant, the use of a too large substrate concentrations may also lead to an underestimation of the maximum reaction rate (Steen and Ziervogel, 2012). It is therefore important to confirm that each of the measured enzyme is assayed under saturating conditions, determining substrate saturation curves in all soils prior to analysis (ISO/TS 22939, 2010; German et al., 2011, 2012; Burns et al., 2013).

Another detail that require optimization is soil slurry preparation. The optimal soil-to-buffer ratio depends on the soil sample and enzyme (ISO/TS 22939, 2010). Reducing the dilution level may increase the soil slurry turbidity and the quenching of fluorescence. On the other hand soil slurries that are too diluted may lead to undetectable fluorescence or increase the analytical variability due to fine scale heterogeneity and to the small volume of slurry used for the assay (German et al., 2011). Finally, independently of the dilution level, the use of a relatively large mass of soil for the slurry preparation may increase the sample representativeness and reduce the variability among repeated measures (DeForest, 2009).

CHAPTER 2

This study addressed some of the critical aspects of microplate-based fluorimetric assays with the aim to optimize assay conditions and evaluate the method precision (i.e. reproducibility and repeatability). Further, the study verified whether the optimization of the method could yield a significant improvement of the results precision and whether this improvement could also affect the magnitude and statistical significance of differences observed between soil samples under contrasting conditions.

Soils coming from agricultural, grassland and pinewood environments were involved in the study. Within each environment, contrasting fertilization, land uses or constrains were considered. Eight soil enzyme activities were chosen for this study due to their relevance in organic matter degradation and nutrient elements cycling.

Herein we refer to assay conditions aiming to determining maximum potential enzymatic activity. Estimation of *in situ* activity, or realized activity, is out of interest of this work.

2.2. Materials and methods

2.2.1. Sampling sites

Soils coming from agricultural, grassland and pinewood environments were involved in the study. Within each of these three environments, contrasting fertilization, land uses or constrains were considered. The agricultural sites were located at the Experimental Farm of the University of Bologna, in the South-east of the Po valley, Italy. The sites were part of a long-term field experiment in which maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) were continuously cropped since 1966. The experimental design was a split-plot with four replicated blocks. The investigated sites consisted of unfertilized control plots (AG-C) and plots fertilized with cattle manure (AG-M). The grassland sites were located in the Schwäbische Alb, a limestone middle mountain range in the southeast of Germany and were part of the interdisciplinary project of the German Biodiversity Exploratories. The investigated grassland sites represented an unfertilized grassland used as sheep pasture and classified as low land-use intensity (GL-LOW), and a fertilized meadow, mown twice a year, classified as high land-use intensity (GL-HIGH) (Berner et al., 2011). The pinewood sites were located in the San Vitale pinewood, a dune and inter-dune system near the coastline of the Adriatic sea, Italy. The sites were characterized by soils developed on sand and calcareous deposits. The investigated pinewood sites consisted of a site located between the crest of the dune and a wide swale area covered by thermophilic submediterranean forest, occasionally affected by the water table but not saline (PW-NS), and a site located in the low-lying inter-dune area at sea level, covered by wood and swamp forests and periodically saturated with saline-sodic water (PW-S) (Marinari et al., 2012). An overview of the sites is presented in Table 2.1.

Table 2.1. Sites description.

Environment	Soil classification*	Site description	ID
Agricultural	Udic Ustocrepts	Maize-wheat rotation, unfertilized	AG-C
	Udic Ustocrepts	Maize-wheat rotation, cattle manure	AG-M
Grassland	Eutric Cambisol	Pasture, unfertilized	GL-LOW
	Eutric Cambisol	Mown meadow, fertilized	GL-HIGH
Pinewood	Aquic Ustipsamment	Submediterranean forest, not saline	PW-NS
	Sodic Psammaquent	Wood and swamp forests, saline-sodic	PW-S

*USDA Soil Taxonomy.

2.2.2. Soil sampling and characterization

Agricultural soil samples were collected in March 2012 before maize sowing. Soil samples were collected from four AG-C plots and four AG-M plots to a depth of 20 cm, in order to obtain a field replicate for each of the four blocks comprising the experimental design.

Grassland and pinewood soil samples were collected in September 2012. For each site a sampling area of approximately 50 m^2 was considered. Within this area six soil field replicates were collected to a depth of 10 cm using the sampling scheme described by Vittori et al. (2012).

Aboveground plant material and the organic top layer were discharged. Freshly sampled soil was kept in a cooler for transportation back to the laboratory, sieved through a 2 mm sieve and thoroughly mixed. Roots and visible plant residue were removed by tweezers. Each field replicate was then divided into three aliquots. One aliquot was used for the chemical and microbiological characterization. A second aliquot was used to form composite samples. Composite samples were prepared by pulling together field replicates of the same sampling site and used for the kinetic, reproducibility and repeatability experiments. A total of six composite samples were obtained. The third aliquot was used for the measurement of potential enzymatic activities with the optimized and bench mark methods.

Soil water content was determined gravimetrically by drying soil samples to constant mass at 105 °C. Soil pH was measured using a glass electrode in 1:5 (v:v) suspensions of air dried soil in10 mM $CaCl_2$ (ISO 10390, 2005). For the measurement of soil electrical conductivity (EC) a 1:5 (w:v) suspension of air dried soil in milliQ® analytical grade water was shaken on an horizontal shaker at 250 rev min^{-1} for 30 min. Extracts were filtered through Whatman no. 42 filter paper and EC was measured using a conductivity meter (ISO 11265, 2005). Total carbon (TC) and total nitrogen (TN) content were determined on air dried, finely ground soil aliquots. Soil subsamples of 10 mg were analyzed in triplicate by an elemental analyzer (CHNS-O Elemental Analyzer 1110, Thermo Scientific GmbH, Dreieich, DE). The agricultural and pinewood soil samples were pre-treated with a 1:1 HCl solution to eliminate the carbonates. No carbonates were detected in the grassland samples (Dietrich-Frühling method) and TC was taken as total organic carbon (TOC).

Microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) were determined within a week from soil collection on field moist soil samples stored at + 4 °C using the chloroform-fumigation extraction (CFE) method (Brookes et al., 1985). The equivalent of 4 grams of oven dried soil was fumigated with ethanol-free chloroform for 24 h at room temperature in a desiccator.

Fumigated and unfumigated samples were dispersed in 16 mL of 0.5 M K_2SO_4 and extracted on an horizontal shaker at 250 rev min^{-1} for 1 h. Extracts were filtered through Whatman no. 42 filter paper and analyzed for the organic carbon and total nitrogen content with an elemental analyzer (TOC-VCPH/CPN, Shimadzu, Kyoto, JP). C_{mic} was calculated as organic carbon in the fumigated minus organic carbon in the unfumigated soil extracts. Similarly, N_{mic} was calculated as total nitrogen in the fumigated minus total nitrogen in the unfumigated soil extracts.

2.2.3. Methods optimization and precision estimation

The potential activity of eight extracellular hydrolytic enzymes was studied (Table 2.2). All the assays were conducted on deep frozen soil samples (- 20 °C), within 4 months from the soil samples collection (ISO/TS 22939, 2010). Method optimization and precision estimation were conducted separately for the agricultural, grassland and pinewood sites under study. In the first step of method optimization (kinetic experiment) substrate saturating conditions were determined for each composite sample and enzyme activity using three different levels of soil slurry dilution, corresponding to 1:100, 2:100 and 4:100 (w:v).

The saturating substrate concentrations established in the kinetic experiment were then used as substrate concentrations for the repeatability and reproducibility experiments.

In the repeatability experiment the repeatability of methods using the three different levels of soil slurry dilution and three true independent replicates (i.e. prepared from different aliquots of the same sample) for each composite sample were estimated. The methods were then compared based on the repeatability variance showed by the true independent replicates. The soil slurry dilution level giving the poorer repeatability was excluded from the following reproducibility experiment.

In the reproducibility experiment the reproducibility of methods using two levels of soil slurry dilution and three true independent replicates for each composite sample was assessed by measuring maximum potential activity under standardized conditions on aliquots of the same soil sample for four successive days. The methods were then compared based on the variance showed by true independent replicates (mean repeatability variance over four days), on the variance of measurements made on the same soil samples on successive days (reproducibility variance), and on the overall precision (repeatability and reproducibility variances). The soil slurry dilution giving the higher precision for the majority of enzyme activities assayed for each of the sites under study was defined as the optimal. A total of three optimized methods were obtained.

Table 2.2. Enzymes included in the study, abbreviations, code numbers, catalyzed hydrolysis, and corresponding MUF model substrates*.

Enzyme	EC	Enzyme function	Substrate
β-1,4-glucosidase (β-GLU)	3.2.1.21	Cellulose oligomers into β-D-glucose	4-MUF-β-D-glucoside
α-1,4-glucosidase (α-GLU)	3.2.1.20	Starch into α-D-glucose	4-MUF-α-D-glucoside
N-acetyl-β-glucosaminidase (N-AG)	3.2.1.30	Chitooligosaccharides into chitin oligomers	4-MUF-*N*-acetyl-β-D-glucosamide
β-1,4-xylosidase (β-XYL)	3.2.1.37	Xylooligomers into xylan	4-MUF-β-D-xyloside
β-1,4-cellobiosidase (β-CEL)	3.2.1.91	Cellulose into cellobiose	4-MUF-β-D-cellobioside
Arylsulfatase (SULF)	3.1.6.1	Organic S into sulfates	4-MUF-sulfate
Phosphomonoesterase (PME)	3.1.3.2	Phospate monoesters into phosphate	4-MUF-phosphate
Phosphodiesterase (PDE)	3.1.4.1	Phosphate diesters into phosphate monoesters	bis-4-MUF-phosphate

*MUF = 4-methylumbelliferone. EC = enzyme code number defined by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB).

2.2.4. Comparison between optimized methods and benchmark method

The optimized methods obtained for the agricultural, grassland and pinewood environments were applied to all the collected field replicates in order to account for spatial variability occurring within each sampling site. A method using substrate final concentrations equal to 500 µM and a soil slurry dilution of 1:100 (w:v) was used as a benchmark for comparison with the optimized method.

2.2.5. Reagents

A 0.5 M sodium acetate buffer solution was made by mixing sodium acetate trihydrate (analytical grade, crystalline, Carlo Erba) with deionized water. The pH was adjusted to 5.5 using glacial acetic acid (99.9 % v:v, Carlo Erba) (ISO/TS 22939, 2010). This buffer solution was then used to dilute standard, substrates and soil samples.

To minimize variability due to reagents storage, substrates and standard solutions were prepared on the day of the assay. Freshly made solutions were kept away from light until use. To avoid microbial contamination glassware, buffers and deionised water were sterilized in autoclave (121 ± 3 °C for 20 min.) before usage (ISO/TS 22929, 2010).

Each substrate (Table 2.2) was pre-dissolved in DMSO (dimethyl sulfoxide; SIGMA). Sodium acetate buffer was then added to give the desired final concentration. For the kinetic experiment substrate stock solutions were prepared as described above and then further diluted in sterile buffer. Five mM 7-hydroxyl-4-methylcoumarin (MUF) standard solution was prepared in methanol and water (1:1, v:v). This stock solution was diluted to 100 or 250 µM in sodium acetate buffer. For the calibration curve used for the assays of agricultural samples the 100 µM MUF solution was further diluted to 1.00, 2.00, 4.00, 10.0, 20.0, 30.0, 40.0 µM in buffer. For the calibration curve used for the assays of grassland and pinewood soil samples the 250 µM MUF solution was further diluted to 2.50, 5.00, 10.0, 25.0, 50.0, 75.0, 100 µM in buffer. The calibration curves were designed to cover the range of fluorescence observed across the eight enzyme activities in each study sites (ISO/TS 22939, 2010).

2.2.6. Soil slurry

Depending on the desired soil slurry dilution (see section 2.2.3) moist soil samples corresponding to 1.00, 2.00, or 4.00 g of oven dried soil were weighted into sterilized Pyrex tall-form 150 mL becker. One hundred mL of 0.5 M acetate buffer was added and mixed using an Ultra Turrax ICA for 2 min at 9000 rpm (IKA-Werke, Staufen, DE). A magnetic stir bar was then added and soil was kept under continuous stirring. There was no delay between soil slurry preparation and dispensation in the micro-plate. The entire procedure of soil samples processing was staggered so that the time between soil slurry preparation and subsequent substrate addition never exceeded 40 min. Sufficient buffering capacity to maintain a stable pH over the incubation was verified in a bench-scale pre-experiment. Briefly, 5 mL of soil slurry produced by suspending the equivalent of 4 g of oven dried soil in 0.5 M acetate buffer were dispensed in sterile 50 mL Falcon tubes and mixed with 5 mL of

1000 μM substrate solution. To reproduce the conditions under which the microplate assay was conducted the Falcon tubes were incubated at 30°C for 3.5 h and shook by hand every 30 min. The pH was measured at the beginning and at the end of the incubation period using a glass electrode. Relevant variations of pH in the reaction mixture were never detected (pH 5.5 ± 0.2).

2.2.7. Plate set-up

Flat-well black polystyrene 96-well microplates with a well capacity of 350 μL were used throughout the experiments (Greiner Bio-One, Frickenhausen, DE). The plate set-up varied slightly according to the aim of the investigation (e.g. estimation of kinetic parameters, estimation of method precision, simple measurement of enzyme activity rates). However the different components included in the assays as well as the number of their analytical replicates was consistent among all the trials (Table 2.3). Moreover, buffer, soil slurry, standard solutions and substrate solutions were dispensed in the microplates following always the same order: first 100 μL of sodium acetate buffer were dispensed in the wells that served as soil controls and substrate controls. Next 50 μL of sodium acetate were dispensed in the wells that served as quench controls. Then 150 μL of sodium acetate buffer was added in the wells that served as reference standards. Soil slurry aliquots of 100 μL were than withdrawn from the soil suspension under continuous stirring and dispensed in the wells that served as quench controls, soil controls and soil assays. After all the soil slurries included in the assay were processed and dispensed, 50 μL of MUF standard solutions were dispensed into wells that served as quench controls and reference standards. Lastly, 100 μL of substrate solutions were dispersed into wells that served as substrate controls and soil assays. The total volume of the reaction mixture was 200 μL. The addition of the substrates was considered the start of the incubation period. The microplates were covered and incubated in the dark at 30 °C.

Table 2.3. Different components included in the assay and their function.

ID	Soil slurry	Substrate solutions	Standard solutions	Analytical replicates	Purpose
Assay	yes	yes	no	8	Gross fluorescence produced by substrate hydrolysis
Reference standards	no	no	yes	4	Conversion from unit of fluorescence to unit of product
Quench controls	yes	no	yes	4	Calculation of the degree of quenching due to soil particles
Substrate controls	no	yes	no	8	Fluorescence emitted by the substrates due to autohydrolisis
Soil controls	yes	no	no	4	Estimation of naturally occurring fluorescence of soil

2.2.8. Fluorescence readings and activity calculation

The fluorescence intensity was measured using a microplate fluorometer (infinite200, TECAN, Männedorf, CH) with 365-nm excitation and 450-nm emission filters. Measurements were taken immediately after the plate set-up and from then on every 30 min over a 3 h incubation period. Before each reading the microplates were shaken for 5 s in order to homogenize the reaction mixture. Enzyme activities were expressed in nmol product h^{-1} g^{-1}. Rates of fluorescence increase rather than absolute amount of fluorescence at the end of the incubation period were used for the calculation. Rates of fluorescence increase were converted into enzyme activity according to the following equations (adapted from Marx et al. 2001 and German et al., 2011):

$$\text{Activity (nmol MUF g}^{-1}\text{ h}^{-1}\text{)} = \frac{\text{Net fluorescence (RUF min}^{-1}\text{) x 100 (mL) x 200 (}\mu\text{L) x 60 (min h}^{-1}\text{)}}{\text{Emission coefficient (RUF/ }\mu\text{mol L}^{-1}\text{) x 100 (}\mu\text{L) x Soil dry mass (g)}}$$

Where:

$$\text{Net fluorescence} = \left[\frac{\text{Assay slope} - \text{Soil control slope}}{\text{Quench coefficient}} \right] - \text{Substrate control slope}$$

Emission coefficient (RUF/ μmol L^{-1}) = Reference standard curve slope

$$\text{Quench coefficient} = \frac{\text{Quench controls curve slope (RUF /}\mu\text{mol L}^{-1}\text{)}}{\text{Reference standards curve slope (RUF /}\mu\text{mol L}^{-1}\text{)}}$$

RUF = relative units of fluorescence

2.2.9. Data handling and statistics

In the kinetic experiment significant differences among the activities observed at different substrate concentrations were determined using one-way ANOVA. Means separation was done with Holm adjusted Fisher's least significance difference (LSD) test at $P \leq 0.05$.

In the reproducibility experiments methods comparison was based on the ratio between the reproducibility variance showed by the different methods. The obtained F statistics were compared with critical value of F under the null hypothesis that the variance was equal at $P \leq 0.05$.

Similarly, in the repeatability experiments methods comparison was based on the ratio between the mean reproducibility variance, repeatability variance and overall precision variance showed by the different methods. The obtained F statistics were compared with critical value of F under the null hypothesis that the variances were equal at $P \leq 0.05$.

When the optimized methods and the benchmark method were applied to all field replicates significant differences among the treatments belonging to the same environment were determined using one-way analysis of variance. Means separation was done with the Fisher's least significance difference (LSD) test at $P \leq 0.05$. Association between soil properties and enzyme activities determined by the optimized methods and the benchmark method were estimated using linear regression. Normality of the residuals was evaluated graphically and with the Shapiro-Wilk test. Pearson's product moment correlation coefficients (r) and significance of the associations ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$) are reported.

2.3. Results

2.3.1. Main soil properties

Main properties of the soils under study are reported in Table 2.4. The pH values ranged form the sub-acidic of GL-HIGH to the moderately alkaline of PW-S. With regard to the electrical conductivity only the samples collected at the PW-S site showed EC values typical of saline soils. Based on data reported by Marinari et al. (2012) the soil of this site can be classified as saline-sodic. TOC and TN were maximum in the grassland soil samples and minimum in the agricultural soil samples. Higher values of TOC and TN were observed in GL-LOW as compared to GL-HIGH and in AG-M as compared to AG-C. The values found in the pinewood soils varied strongly depending on the considered sampling site, with PW-S showing roughly 4 times as TOC and TN as PW-NS.

With regard to soil microbial biomass, C_{mic} and N_{mic} were maximum in the grassland soil samples and minimum in the agricultural samples. As observed for TOC and TN, higher values of C_{mic} and N_{mic} were found in GL-LOW as compared to GL-HIGH and in AG-M as compared to AG-C. The values found in the pinewood soils varied strongly depending on the considered sampling site, with PW-S showing 10 times as C_{mic} and 6 times as N_{mic} as PW-NS.

Two of the six field replicates collected at the PW-S site showed anomalous values of C_{mic} and N_{mic} and anomalous values of calcium carbonate (data not shown), and were therefore excluded from the experiment. To keep the experimental design balanced two field replicates of the PW-NS were randomly selected and excluded from the experiment as well.

2.3.2. Substrate saturating concentrations

The kinetic experiments resulted in one substrate saturation curve for each of the enzyme activity, environment, sampling site, and soil slurry dilution level considered, for a total of 144 saturation curves. The substrate saturating concentrations estimated in the kinetic experiments are summarized in Table 2.5. For each of the environment under study it was possible to find a unique substrate concentration ensuring saturation for all the three levels of soil slurry dilutions and treatments considered. Of the eight tested enzyme activities β-cellobiohydrolase was the one for which saturation was reached at the lowest concentration, independent of the considered environment (Table 2.5). Concentrations of 4-MUF-β-D-cellobioside higher than 100 μM caused a reduction of the measured activity in most of the samples (Figs. 2.1, 2.2 and 2.3).

Table 2.4. Main soil properties*.

Environment	ID	pH		EC (μS cm^{-1})		TOC (g kg^{-1})		TN (g kg^{-1})		C_{mic} (mg kg^{-1})		N_{mic} (mg kg^{-1})	
Agricultural	AG-C	6.17	(0.49)	130	(16.1)	6.40	(0.238)	0.791	(0.0349)	50.7	(7.48)	6.84	(1.14)
	AG-M	6.59	(0.34)	145	(20.0)	9.11	(0.692)	1.09	(0.0732)	84.9	(13.20)	11.30	(1.23)
Grassland	GL-LOW	6.54	(0.07)	170	(24.6)	70.8	(1.26)	7.07	(0.136)	944	(18.7)	238	(12.40)
	GL-HIGH	5.71	(0.07)	61.5	(4.67)	62.2	(1.90)	6.09	(0.145)	812	(35.3)	113	(5.23)
Pinewood	PW-NS	7.79	(0.11)	67.5	(11.5)	16.6	(1.48)	1.34	(0.103)	103	(10.20)	13.5	(1.09)
	PW-S	7.97	(0.03)	9174	(1969)	61.6	(8.53)	4.86	(0.579)	459	(3.67)	81.5	(2.02)

*Means of field replicates (Agricultural sites n = 4; Grassland sites n = 6; Pinewood sites n = 4). Values reported in parenthesis are standard errors.

This reduction was more pronounced for the pinewood soils. In the case of PW-NS at the 1:100 soil slurry dilution, undetectable activity levels were observed at substrate concentrations higher than 200 μM (Fig. 2.3).

For α-glucosidase and β-xylosidase activities, substrate saturating concentrations were equal or close to 500 μM, independent of the considered environment. Similar results were observed for β-glucosidase substrate saturating concentration in the grassland soils (Fig. 2.5), while concentrations of 4-MUF-β-D-glucoside equal or higher than 200 μM caused a significant reduction of the measured activity in most of the agricultural and pinewood soil samples (Figs. 2.4 and 2.6).

The substrate saturating concentration estimated for *N*-acetil-β-glucosaminidase was equal to 200 μM, independent of the considered environment. Concentrations of 4-MUF-*N*-acetyl-β-D-glucosamide equal or higher than 200 μM caused a significant reduction of the measured activity in most of the soil samples (Figs. 2.7, 2.8, 2.9).

Likely, in the case of phosphomonoesterase activity, concentrations of 4-MUF-phosphate equal or higher than 200 μM caused a significant reduction of the measured activity in agricultural soil samples, while no reduction was observed in grassland and pinewood soils over the tested concentration range (Table 2.5).

In agricultural soil samples phosphodiesterase activity reached substrate saturating conditions at bis-4-MUF-phosphate concentrations equal to 500 μM, while for grassland and pinewood soil samples the concentration had to be increased to 1000 μM (Table 2.5).

In the case of arylsulfatase activity substrate saturating conditions were attained at concentrations of 4-MUF-sulfate equal to 1000, 2000 and 2500 μM in agricultural, pinewood and grassland samples, respectively. When the saturation curves of arylsulfatase activity were fitted with a Michaelis Menten model, the estimated constant K_m, corresponding to the substrate concentration at which ½ V_{max} is reached, ranged from 300 to 600 μM. An example for the soil slurry dilution of 4:100 is given in Fig. 2.10.

Table 2.5. Substrate saturating concentrations (μM) as determined by the kinetic experiments.

Activity	Environment		
	AG	GL	PW
β-GLU	100	400	200
α-GLU	400	500	300
N-AG	200	200	200
β-XYL	400	500	500
β-CEL	75	100	50
SULF	1000	2500	2000
PME	200	500	500
PDE	500	1000	1000

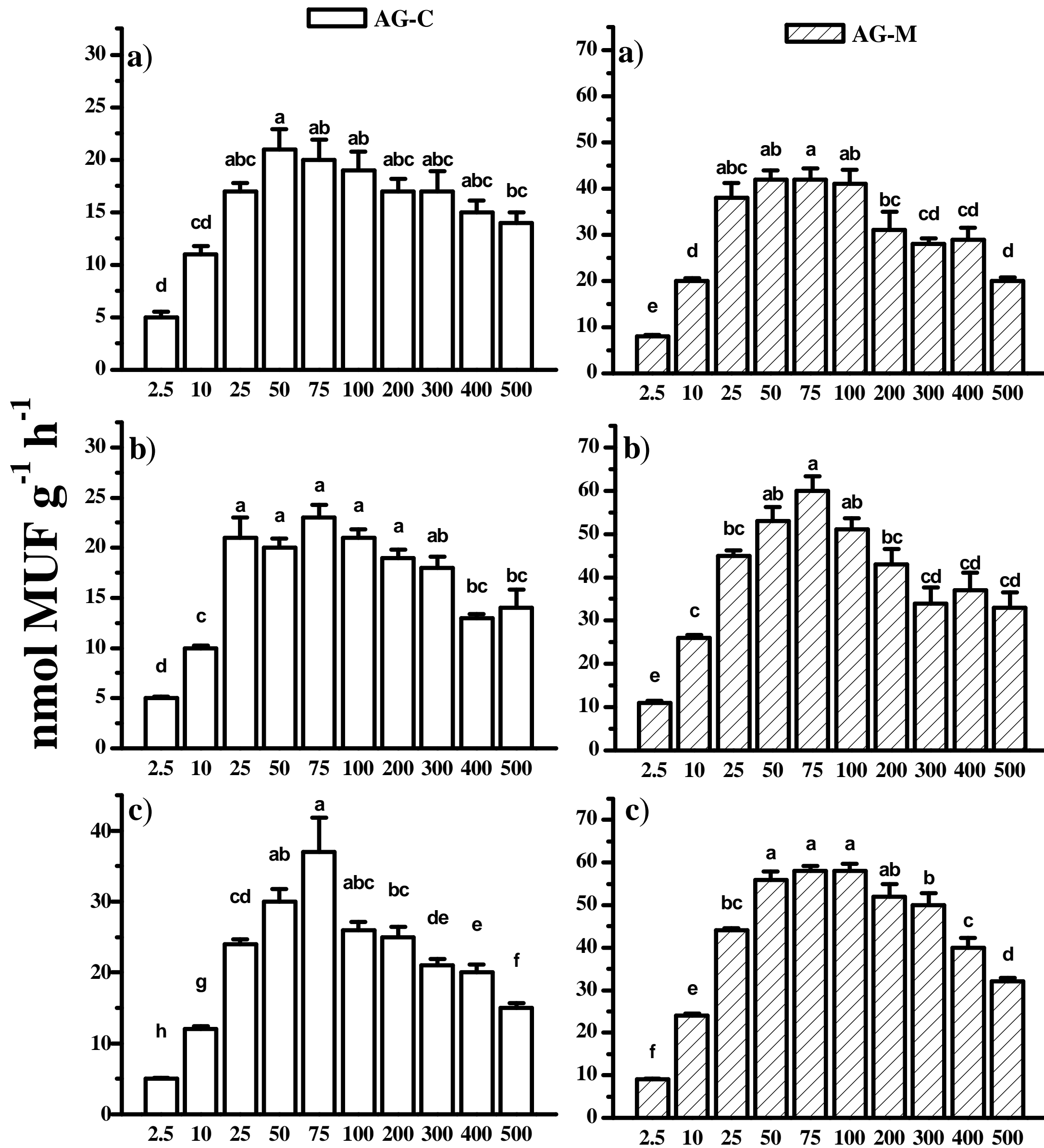

Fig. 2.1. β-cellobiohydrolase activity at increasing substrate concentrations (μM) measured in unfertilized control (AG-C) and manured (AG-M) agricultural composite soil samples at different levels of soil slurry dilution: a) 1:100; b) 2:100; c) 4:100. Means of eight analytical replicates. Error bars represent standard errors (n = 8). Different letters indicate significant differences ($P < 0.05$) as determined by LSD.

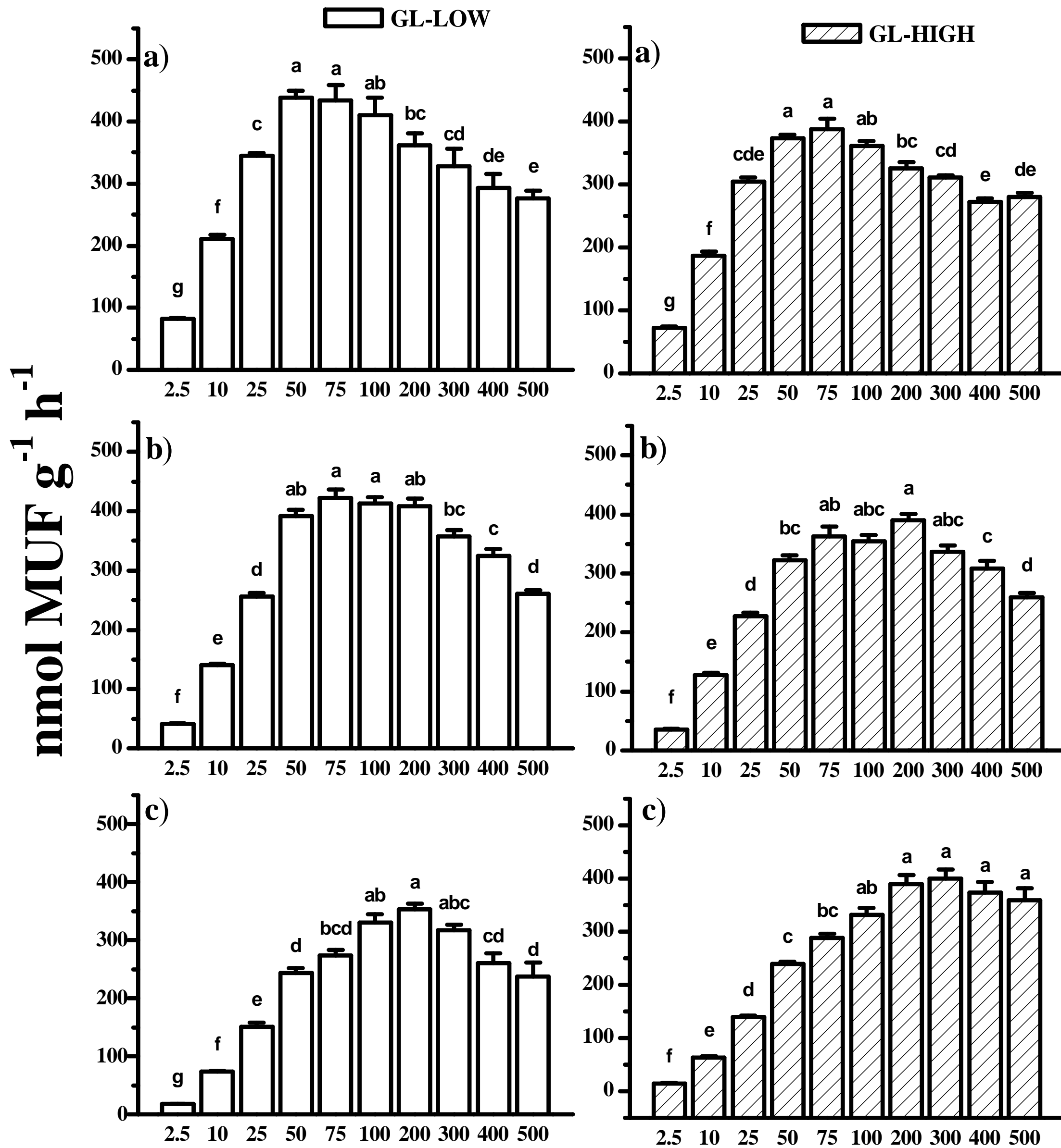

Fig. 2.2. β-cellobiohydrolase activity at increasing substrate concentrations (μM) measured in low (GL-LOW) and high (GL-HIGH) land-use intensity grassland composite soil samples at different levels of soil slurry dilution: a) 1:100; b) 2:100; c) 4:100. Means of eight analytical replicates. Error bars represent standard errors (n = 8). Different letters indicate significant differences (P <0.05) as determined by LSD.

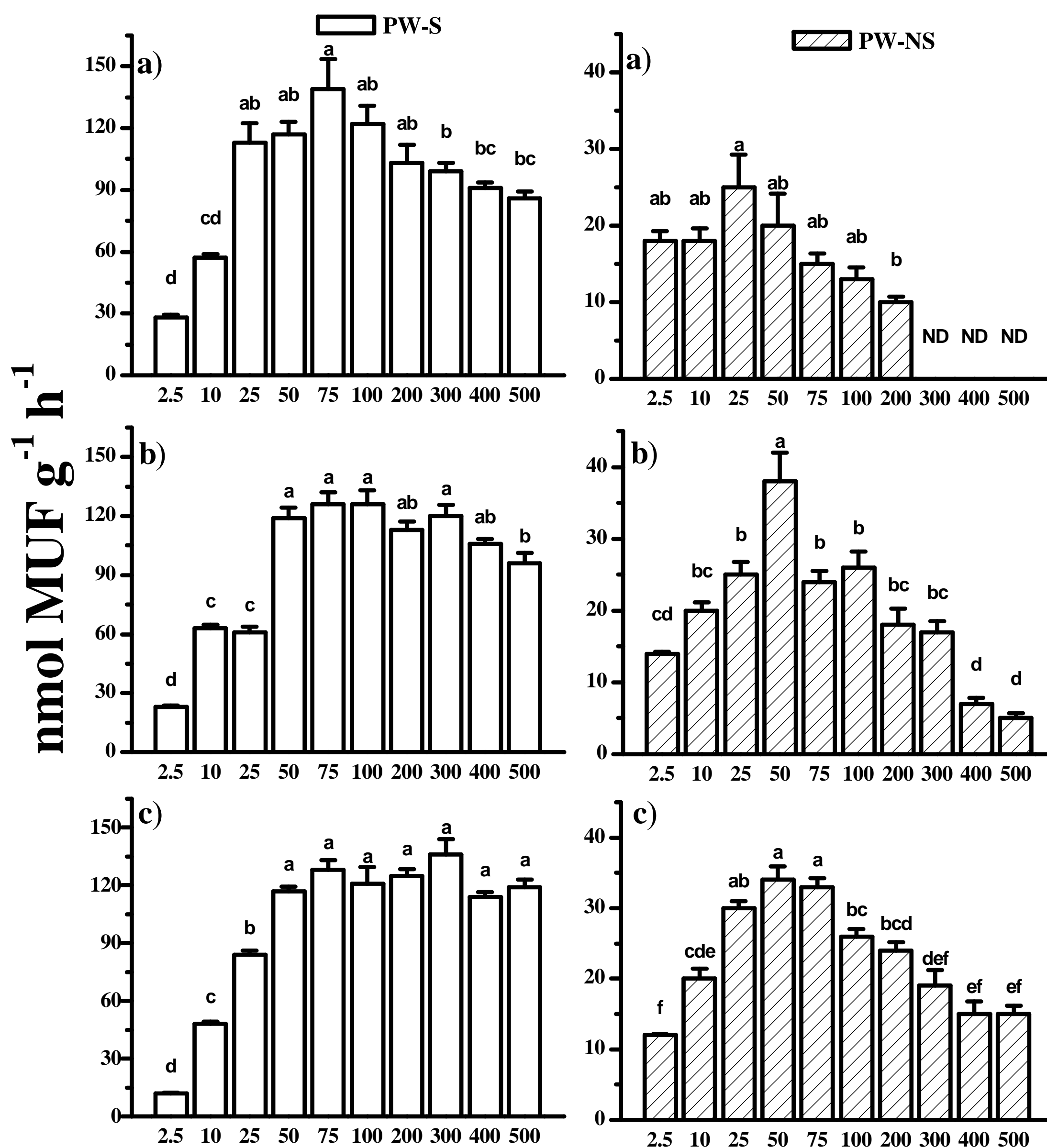

Fig. 2.3. β-cellobiohydrolase activity at increasing substrate concentrations (μM) measured in saline-sodic (PW-S) and not saline (PW-NS) pinewood composite soil samples at different levels of soil slurry dilution: a) 1:100; b) 2:100; c) 4:100. Means of eight analytical replicates. Error bars represent standard errors (n = 8). Different letters indicate significant differences (P < 0.05) as determined by LSD. ND = not detectable.

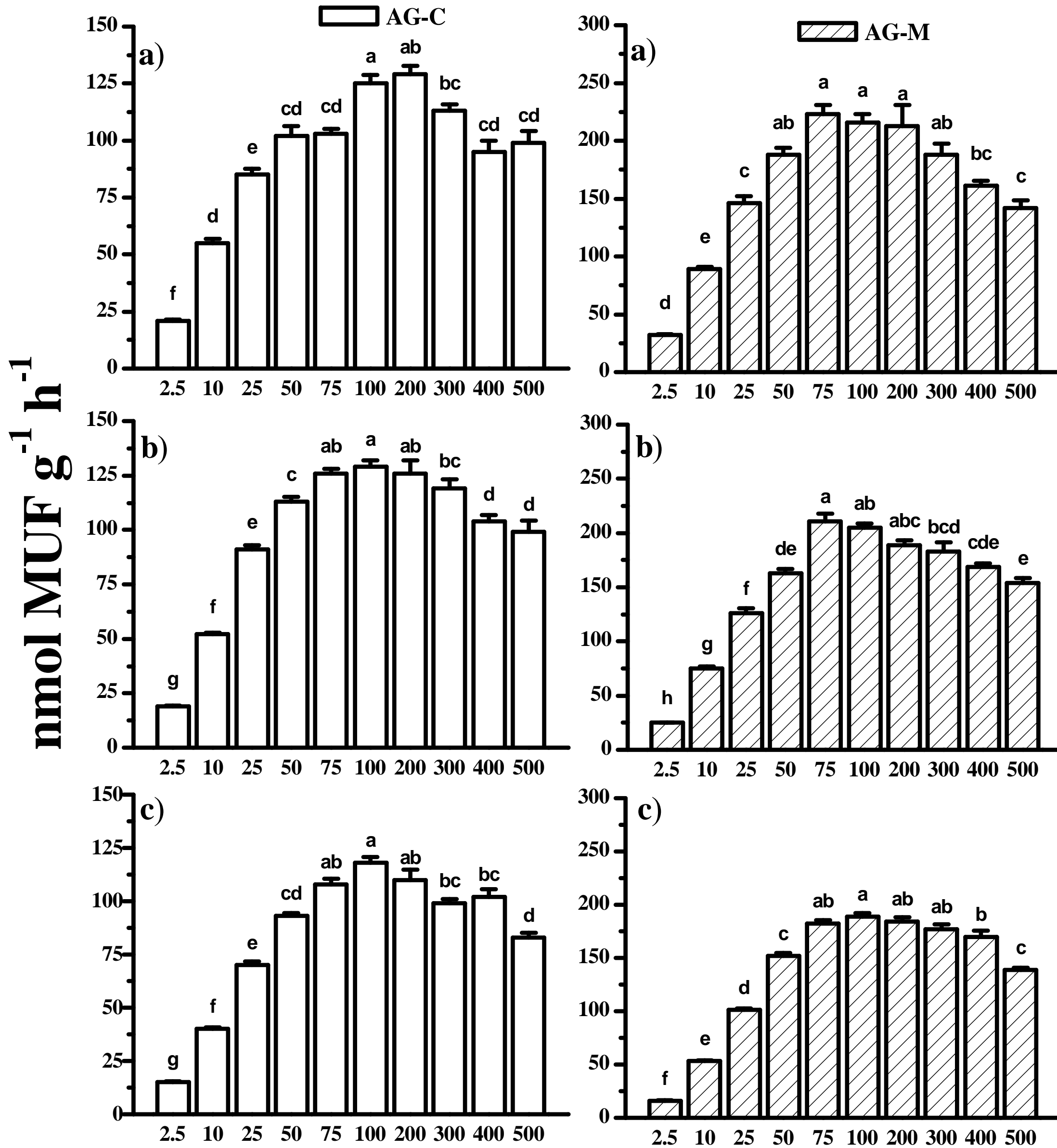

Fig. 2.4. β-glucosidase activity at increasing substrate concentrations (μM) measured in unfertilized control (AG-C) and manured (AG-M) agricultural composite soil samples at different levels of soil slurry dilution: a) 1:100; b) 2:100; c) 4:100. Means of eight analytical replicates. Error bars represent standard errors (n = 8). Different letters indicate significant differences ($P < 0.05$) as determined by LSD.

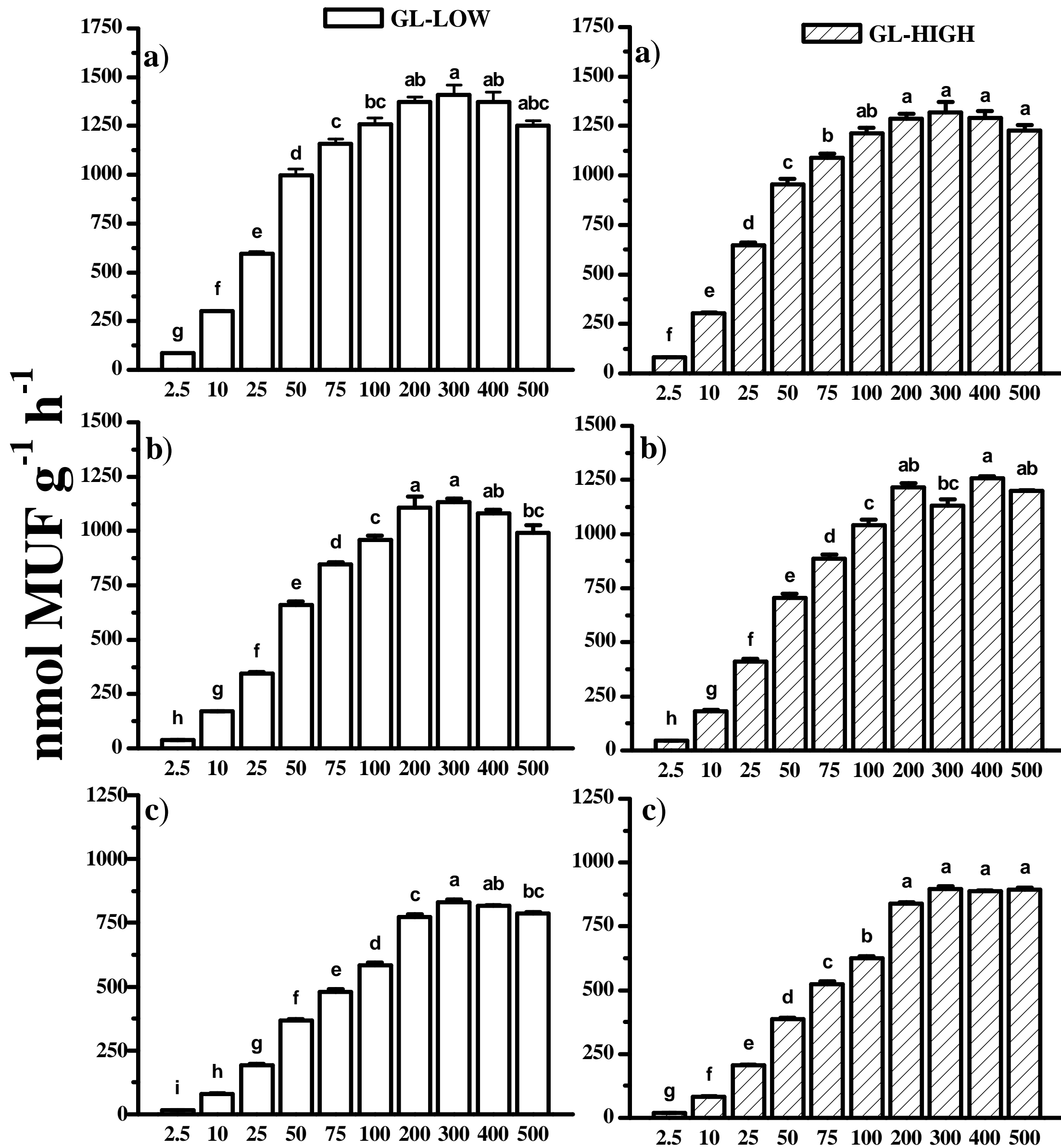

Fig. 2.5. β-glucosidase activity at increasing substrate concentrations (μM) measured in low (GL-LOW) and high (GL-HIGH) land-use intensity grassland composite soil samples at different levels of soil slurry dilution: a) 1:100; b) 2:100; c) 4:100. Means of eight analytical replicates. Error bars represent standard errors (n = 8). Different letters indicate significant differences ($P < 0.05$) as determined by LSD.

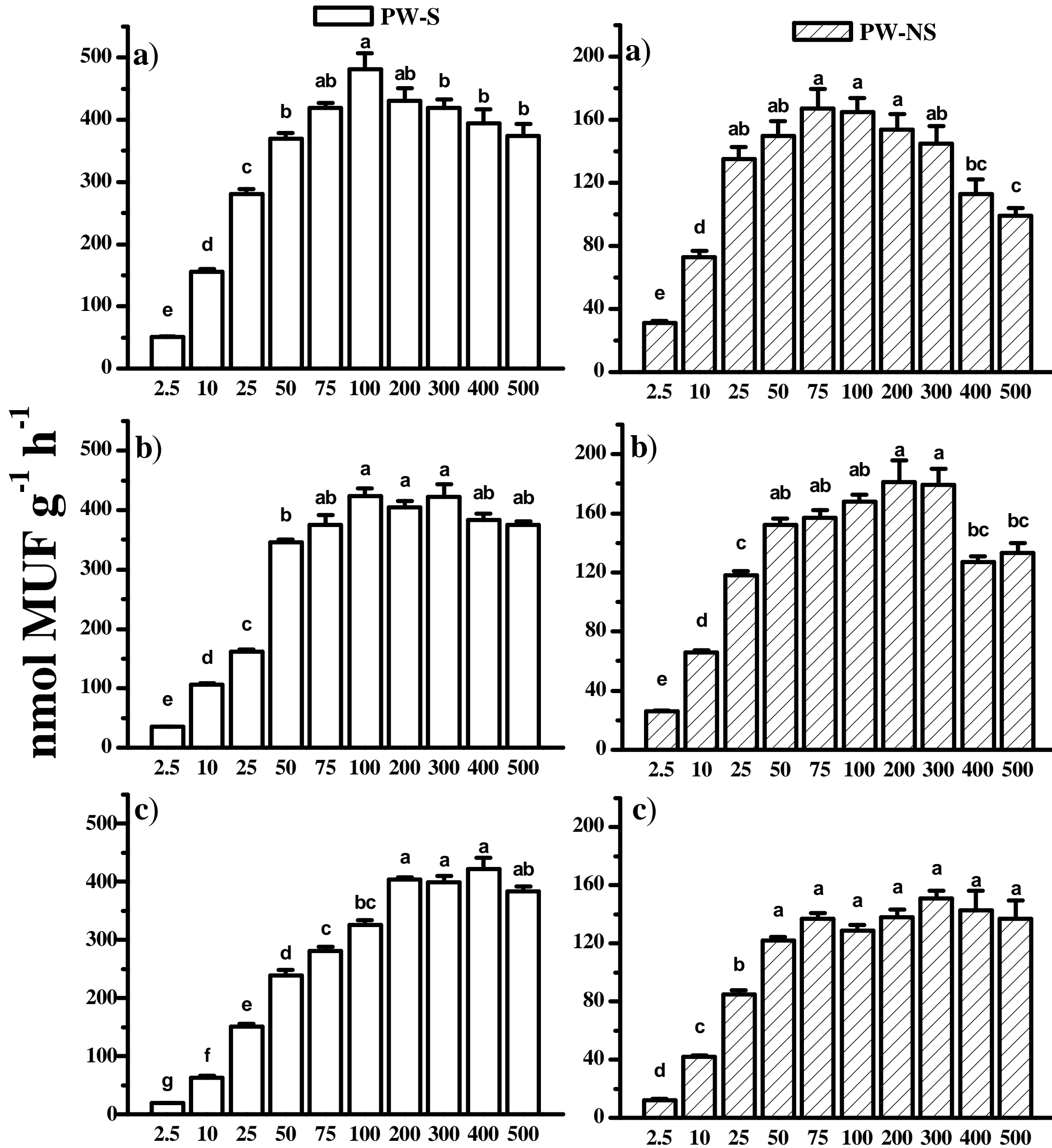

Fig. 2.6. β-glucosidase activity at increasing substrate concentrations (μM) measured in saline-sodic (PW-S) and not saline (PW-NS) pinewood composite soil samples at different levels of soil slurry dilution: a) 1:100; b) 2:100; c) 4:100. Means of eight analytical replicates. Error bars represent standard errors (n = 8). Different letters indicate significant differences (P < 0.05) as determined by LSD.

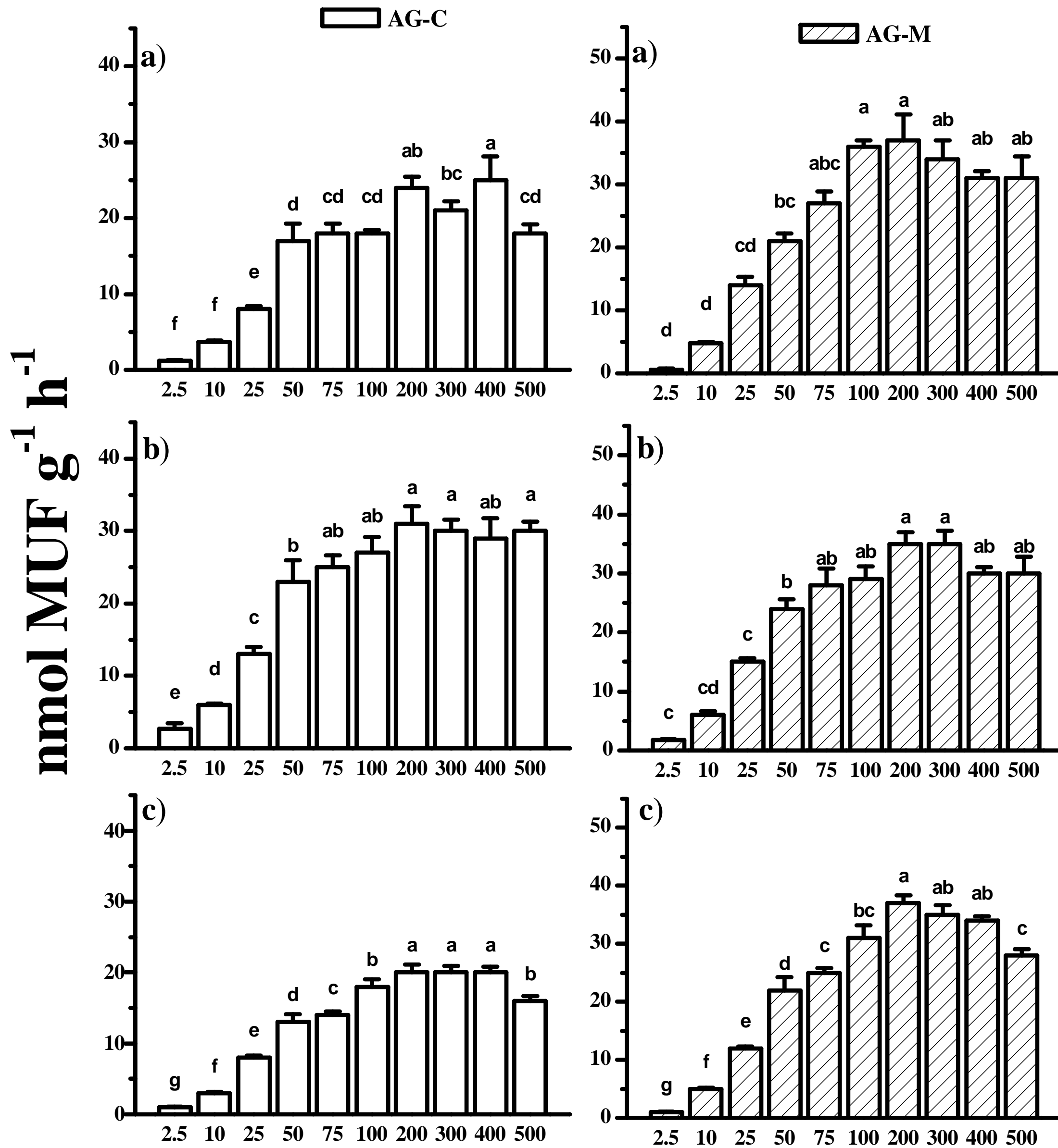

Fig. 2.7. *N*-acetil-β-glucosaminidase activity at increasing substrate concentrations (μM) measured in unfertilized control (AG-C) and manured (AG-M) agricultural composite soil samples at different levels of soil slurry dilution: a) 1:100; b) 2:100; c) 4:100. Means of eight analytical replicates. Error bars represent standard errors (n = 8). Different letters indicate significant differences ($P < 0.05$) as determined by LSD.

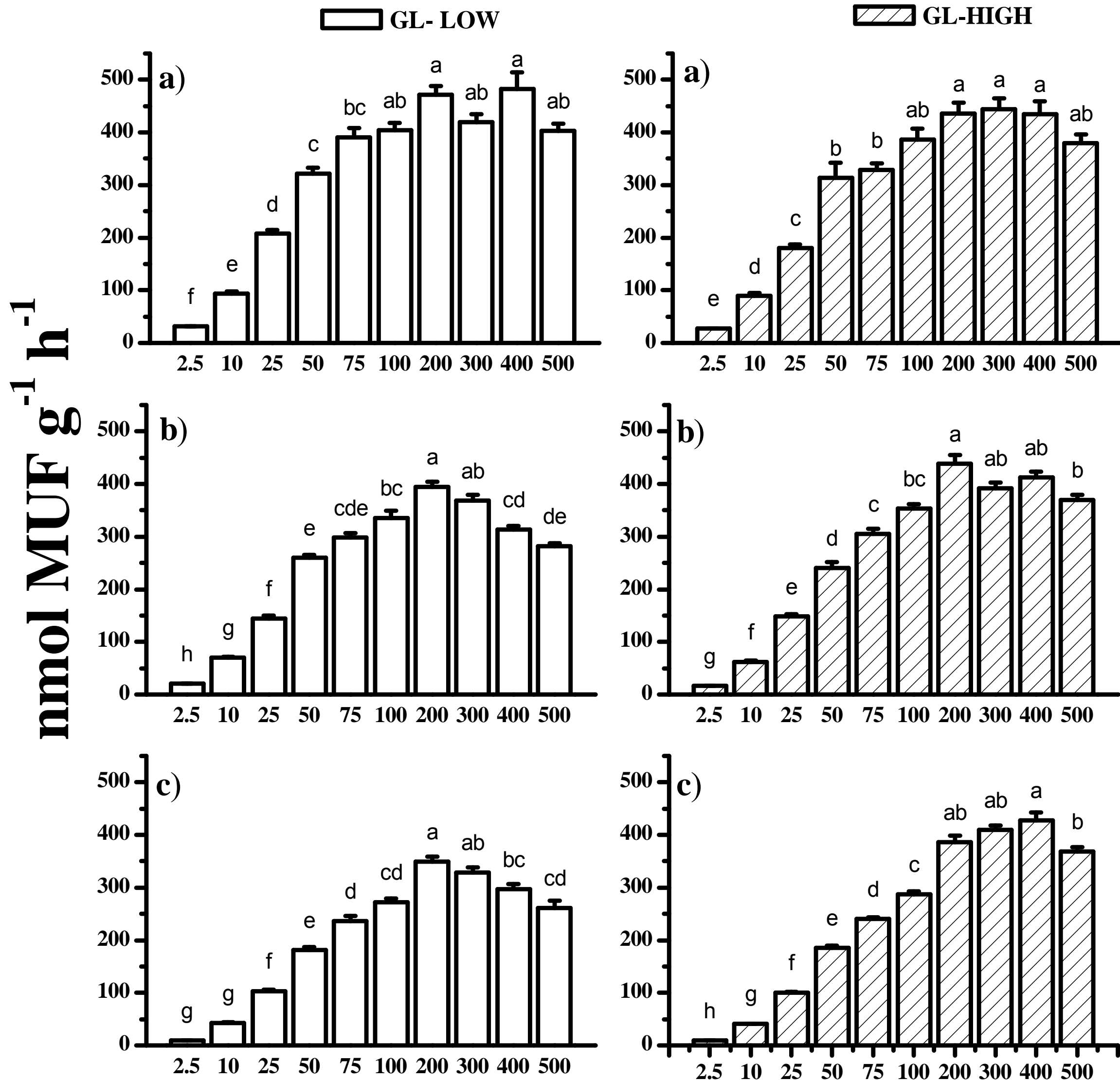

Fig. 2.8. *N*-acetil-β-glucosaminidase activity at increasing substrate concentrations (μM) measured in low (GL-LOW) and high (GL-HIGH) land-use intensity grassland composite soil samples at different levels of soil slurry dilution: a) 1:100; b) 2:100; c) 4:100. Means of eight analytical replicates. Error bars represent standard errors (n = 8). Different letters indicate significant differences ($P < 0.05$) as determined by LSD.

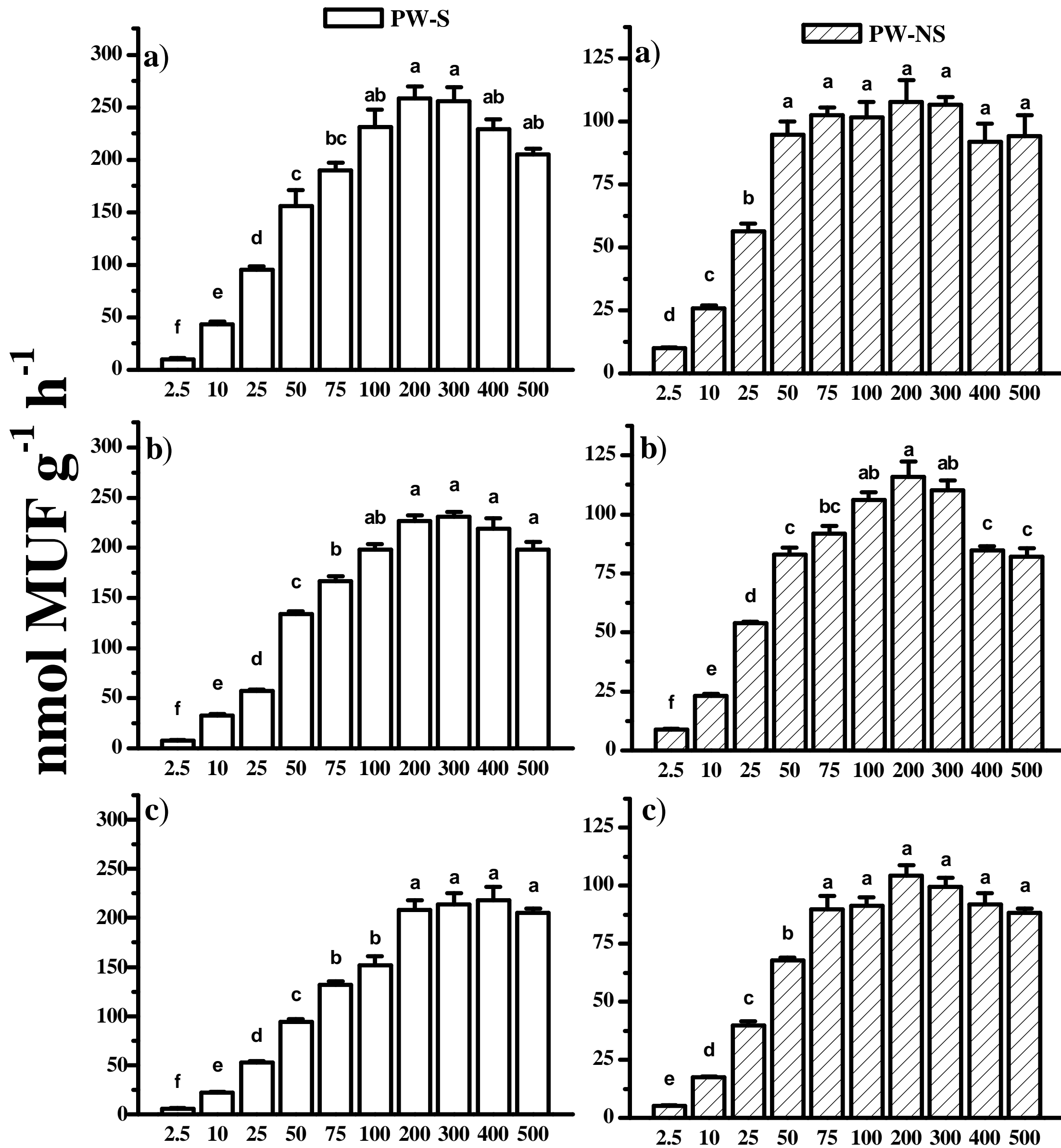

Fig. 2.9. *N*-acetil-β-glucosaminidase activity at increasing substrate concentrations (μM) measured in saline-sodic (PW-S) and not saline (PW-NS) pinewood composite soil samples at different levels of soil slurry dilution: a) 1:100; b) 2:100; c) 4:100. Means of eight analytical replicates. Error bars represent standard errors (n = 8). Different letters indicate significant differences ($P < 0.05$) as determined by LSD.

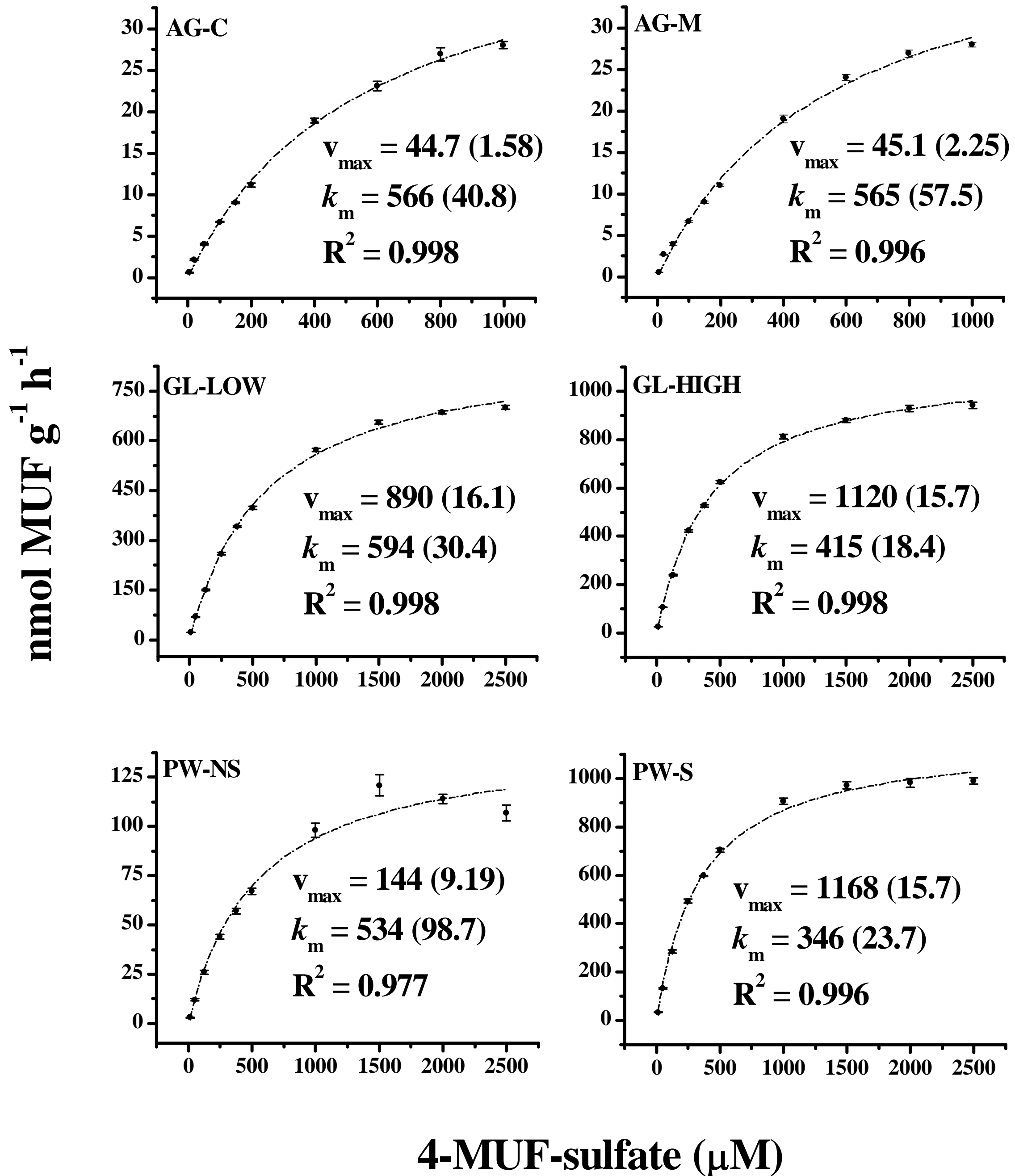

Fig. 2.10. Substrate saturation curves for arylsulfatase activity measured in composite soil samples using the soil slurry dilution of 4:100. Means of eight analytical replicates. Error bars represent standard errors (n = 8). Kinetic parameters V_{max} and K_m estimated using the Michaelis-Menten model. Values reported in parenthesis are errors associated with the estimated kinetic parameters.

2.3.3. Soils slurry dilution

The presence of soil suspension and the level of soil slurry dilution affected the slope of the MUF calibration curves (Fig. 2.11) and the quench coefficients (Table 2.6). Among the different environments, pinewood soil samples collected at the saline-sodic site (PW-S) were the ones causing the most pronounced quenching of fluorescence (i.e. lower slope of the calibration curve and lower quench coefficient), while agricultural soil samples (AG-C and AG-M) had limited effects on the MUF emitted fluorescence. The dilution level also affected the variability of the estimated enzyme activities. With regard to analytical replicates produced form the same soil slurry, lower levels of soil slurry dilution generally resulted in a reduction of the mean variability, while the effects on the number of analytical outliers were less clear (Table 2.7).

The results of the repeatability experiments are summarized in Table 2.8, 2.9 and 2.10. Mean enzyme activities varied strongly depending both on the soil and the enzyme considered. Generally lower values of activity were observed in agricultural soil samples (Table 2.8), and particularly in the unfertilized plots (AG-C), while maximum values were observed in grassland soil samples (Table 2.9). Pinewood soil samples showed values of enzyme activities related to C cycling within the same order of magnitude of agricultural soil samples (Table 2.10), while for other activities higher values were observed, particularly in PW-S.

In some cases a reduction of the soil slurry dilution was also accompanied by a reduction in the mean enzyme activity. This was particularly evident in grassland soil samples (Table 2.9), where, as compared to mean values observed for the dilution of 1:100, mean activities of the dilution of 2:100 and 4:100 were reduced by up to 40 % and 60 %, respectively.

The coefficient of variance shown by the mean activities of independent replicates of the agricultural and pinewood soil samples tended to decrease with decreasing levels of soil slurry dilution for most of the measured enzymes (Tables 2.8 and 2.10), while for grassland soil samples the trend was less clear (Table 2.9).

The comparison of the three soil slurry dilutions based on the variance shown by true independent replicates evidenced several significant differences. Within the eight enzyme activities under study, β-glucosidase resulted the most affected by the choice of the soil-to-buffer ratio, and statistically significant differences between the soil slurry dilutions were observed for most of the soil samples.

For the agricultural soil samples the variance shown by true independent replicates of the 1:100 dilution was significantly higher than the variance of the 2:100 dilution ($F = 24.5$, $P = 0.0391$*) in

AG-C, and of the 4:100 dilution in AG-C (F = 61.5, *P* = 0.016*) and AG-M (F = 38.9, *P* = 0.0251*). Similarly, for the grassland soil samples the variance shown by true independent replicates of the 1:100 dilution was significantly higher than the variance of the 2:100 dilution (F = 54.9, *P* = 0.0179*) and of the 4:100 dilution (F = 145, *P* = 0.00687**) in GL-LOW. Moreover, in GL-HIGH the variance shown by the 2:100 dilution was significantly higher than that of the 4:100 dilution (F = 19.4, *P* = 0.049*). In the case of pinewood soil samples the variance shown by true independent replicates of the 1:100 dilution was significantly higher than the variance of the 2:100 dilution (F = 317, *P* = 0.00315**) and of the 4:100 dilution (F = 41.1, *P* = 0.0238*) in PW-S. As observed for β-glucosidase activity, β-xylosidase, phosphomonoesterase and phosphodiesterase activities resulted affected by the choice of the soil-to-buffer ratio. With regard to β-xylosidase, the variance shown by the true independent replicates of the 1:100 dilution was significantly higher than the variance of the 4:100 dilution in AG-C (F = 22.1; *P* = 0.0433*), and of the 2:100 dilution in PW-NS (F = 2045, *P* = 0.000489***), respectively. In the case of phosphomonoesterase, the variance shown by the true independent replicates of the 1:100 dilution was significantly higher than the variance of the 4:100 dilution in GL-LOW (F =33.8; *P* = 0.0287*), and PW-NS (F = 749, *P* = 0.00133**). In PW-NS differences were observed also between the 2:100 dilution and the 4:100 dilution, with the later showing significantly lower variance (F = 81.5, *P* = 0.0121*). Similarly to phosphomonoesterase, for phosphodiesterase activity the variance shown by the true independent replicates of the 1:100 dilution was significantly higher than the variance of the 4:100 dilution in AG-C (F =231, *P* = 0.00431**), and PW-NS (F = 40.2, *P* = 0.0243*). In the case of α-glucosidase activity, the influence of soil slurry dilution on the variance shown by true independent replicates was far less evident. Significant differences were observed only in AG-M, where the variance shown by the 2:100 dilution was significantly higher than that of the 4:100 dilution (F = 134, *P* = 0.00738**) and in GL-HIGH where the variance shown by the 1:100 dilution was significantly lower than that of the 2:100 (F = 96.2, *P* = 0.0100*) and 4:100 dilutions (F = 78.2, *P* = 0.0130*). Significant differences between the three soil slurry dilutions were never observed for *N*-acetyl-β-glucosaminidase, β-cellobiohydrolase and arylsulfatase activities. The soil slurry dilution of 1:100 was the one resulting in the higher analytical replicates variability (Table 2.7), independent of the considered environment and sampling site. Moreover, the comparison of the variance of true independent replicates evidenced as the soil slurry dilution of 1:100 showed significantly higher

variance as compared to the soil slurry dilution of 4:100 in at least two cases for each of the considered environments. Likely, the soil slurry dilution of 1:100 showed significantly higher variance as compared to the soil slurry dilution of 2:100 in at least one case for each of the considered environments. As a consequence the soil slurry dilution of 1:100 was considered the one giving the poorer repeatability, and was excluded from the following reproducibility experiments.

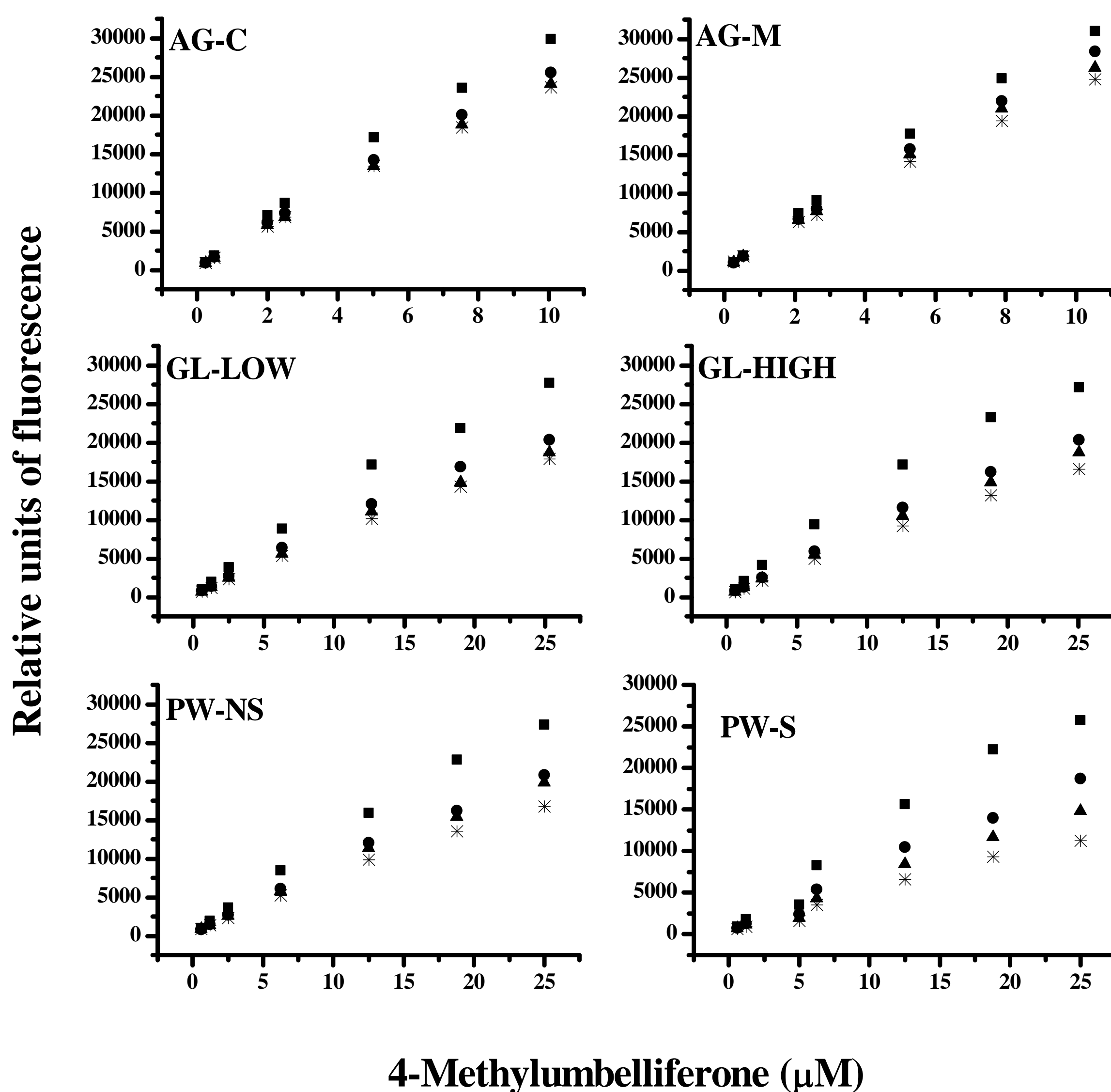

Fig. 2.11. Relative fluorescence signal of MUF for reference standards (■), and quench controls at different levels of soil slurry dilution: 1:100 (●), 2:100 (▲), 4:100 (∗). Reference standards data are means of eight analytical replicates. Quench controls data are means of three independent replicates produced by three different soil suspensions, which in turn are means of eight analytical replicates.

Table 2.6. Effects of soil slurry dilution on the quench coefficient*.

Site ID	Dilution 1:100	Dilution 2:100	Dilution 4:100
AG-C	0.90	0.85	0.82
AG-M	0.95	0.88	0.82
GL-LOW	0.77	0.69	0.63
GL-HIGH	0.74	0.66	0.56
PW-NS	0.73	0.69	0.57
PW-S	0.66	0.55	0.42

*Quench coefficients calculated as reported in section 2.2.8. Values are means of three independent replicates produced from different soil suspensions, which in turn are means of eight analytical replicates.

Table 2.7. Effects of soil slurry dilution on outliers number and variability of analytical replicates*.

Site ID	Dilution 1:100		Dilution 2:100		Dilution 4:100	
	Outliers	Mean CV	Outliers	Mean CV	Outliers	Mean CV
AG-C	5	20	6	13	6	9
AG-M	4	19	4	13	4	10
GL-LOW	6	14	3	8	2	9
GL-HIGH	3	11	5	11	4	7
PW-NS	8	26	2	20	2	16
PW-S	11	15	7	15	3	11

*Based on eight analytical replicates and eight enzyme activity; Outliers = total number of outliers as determined by Grubbs' test (n); Mean CV = mean coefficient of variance of the eight enzyme activities (%).

Table 2.8. Method repeatability at saturating substrates concentrations and different soil slurry dilutions for the agricultural soil samples*.

Activity	Site ID	Dilution 1:100		Dilution 2:100		Dilution 4:100	
		Mean	**CV**	**Mean**	**CV**	**Mean**	CV
β-GLU	AG-C	145.0	7.5	141.3	1.5	125.1	1.1
	AG-M	215.4	5.5	233.1	2.6	181.1	1.1
α-GLU	AG-C	12.1	7.1	8.7	4.4	6.5	5.1
	AG-M	12.6	3.3	10.6	14.2	8.1	1.6
N-AG	AG-C	31.7	13.6	31.7	10.5	28.5	4.4
	AG-M	47.2	23.2	47.4	19.3	45.9	7.6
β-XYL	AG-C	21.1	7.6	21.1	2.4	20.6	1.6
	AG-M	28.6	8.6	30.4	5.5	30.1	3.1
β-CEL	AG-C	22.2	6.6	26.0	10.9	29.0	5.9
	AG-M	40.3	9.4	57.7	8.2	55.7	9.7
SULF	AG-C	24.7	4.5	24.8	1.8	23.2	1.6
	AG-M	26.2	0.7	25.4	4.8	22.4	0.8
PME	AG-C	138.0	2.7	137.3	0.7	122.6	0.8
	AG-M	134.8	4.2	129.9	2.6	117.1	1.9
PDE	AG-C	45.5	3.7	45.5	1.8	42.1	0.3
	AG-M	49.5	1.8	50.9	1.4	47.3	3.8

*Based on three independent replicates produced from three different soil suspensions, which in turn are means of eight analytical replicates. Activities are expressed as nmol MUF g^{-1} soil h^{-1}. CV = coefficient of variance (%).

Table 2.9. Method repeatability at saturating substrates concentrations and different soil slurry dilutions for the grassland soil samples*.

Activity	Site ID	Dilution 1:100		Dilution 2:100		Dilution 4:100	
		Mean	**CV**	**Mean**	**CV**	**Mean**	CV
β-GLU	GL-LOW	2055.8	18.4	1434.6	3.6	834.5	3.8
	GL-HIGH	2142.0	2.8	1347.2	5.2	908.6	1.8
α-GLU	GL-LOW	57.4	2.8	50.9	5.7	43.7	8.8
	GL-HIGH	78.8	0.6	62.5	7.0	49.2	8.0
N-AG	GL-LOW	564.8	6.3	529.9	4.8	382.9	5.7
	GL-HIGH	581.1	4.0	517.1	5.7	392.6	9.1
β-XYL	GL-LOW	309.3	6.3	332.6	5.7	273.6	6.3
	GL-HIGH	364.5	5.0	329.4	4.6	282.0	5.4
β-CEL	GL-LOW	456.6	5.8	490.4	3.7	371.2	4.3
	GL-HIGH	498.2	6.6	455.5	3.0	370.6	5.1
SULF	GL-LOW	1096.5	1.8	966.3	3.0	687.5	2.8
	GL-HIGH	1628.1	2.5	1261.6	2.2	853.4	1.0
PME	GL-LOW	1294.5	8.4	1089.3	8.4	688.9	2.7
	GL-HIGH	1475.8	3.0	1171.6	2.9	797.1	1.9
PDE	GL-LOW	487.7	4.3	509.6	10.9	425.0	5.7
	GL-HIGH	594.6	5.4	507.1	4.6	448.0	10.2

*Based on three independent replicates produced from three different soil suspensions, which in turn are means of eight analytical replicates. Activities are expressed as nmol MUF g^{-1} soil h^{-1}. CV = coefficient of variance (%).

Table 2.10. Method repeatability at saturating substrates concentrations and different soil slurry dilutions for the pinewood soil samples*.

Activity	Site ID	Dilution 1:100		Dilution 2:100		Dilution 4:100	
		Mean	**CV**	**Mean**	**CV**	**Mean**	CV
β-GLU	PW-NS	363.4	20.1	262.5	13.5	262.1	8.7
	PW-S	605.0	29.1	577.7	1.7	439.6	6.2
α-GLU	PW-NS	10.9	10.6	9.7	5.7	8.39	7.7
	PW-S	24.5	17.4	26.5	5.4	26.7	3.8
N-AG	PW-NS	185.9	6.2	146.2	5.8	134.4	5.5
	PW-S	280.7	10.9	269.3	7.1	238.7	3.2
β-XYL	PW-NS	21.5	10.3	21.4	0.3	22.2	2.8
	PW-S	70.2	8.8	86.4	7.4	82.2	3.1
β-CEL	PW-NS	23.6	13.2	25.3	8.4	28.9	2.5
	PW-S	83.2	4.0	102.6	10.1	100.7	4.4
SULF	PW-NS	150.8	3.3	129.4	5.5	126.1	4.9
	PW-S	1439.7	1.5	1212.0	5.4	1015.5	3.2
PME	PW-NS	312.1	9.5	273.6	3.6	276.6	0.4
	PW-S	1129.3	8.5	1029.1	6.5	879.6	3.1
PDE	PW-NS	53.6	8.9	56.6	4.7	67.2	4.1
	PW-S	175.8	8.1	191.9	11.8	199.1	10.9

*Based on three independent replicates produced from three different soil suspensions, which in turn are means of eight analytical replicates. Activities are expressed as nmol MUF g^{-1} soil h^{-1}. CV = coefficient of variance (%).

The results of the reproducibility experiments are reported in Table 2.11, 2.12, 2.13. Methods using soil slurry dilutions of 2:100 and 4:100 were compared based on the mean repeatability shown by true independent replicates, on their reproducibility and on their overall precisions over four consecutive days of analysis. Independent of the soil slurry dilution and on the environment considered, the coefficients of variance between the activities estimated over the four replicated days of analysis were generally under 10 %. Larger coefficients of variance were found for *N*-acetyl-β−glucosaminidase in AG-C soil samples (Table 2.11), for phosphodiesterase in GL-LOW soil samples (Table 2.12), and for α-glucosidase in PW-NS and PW-S soil samples (Table 2.13). Few relevant differences between the variability shown by the two soil slurry dilutions were observed in the agricultural and pinewood environments. As compared to the results obtained with the dilution of 2:100, the use of the dilution of 4:100 resulted in an lower mean repeatability variance for arylsulfatase activity (F = 0.333, P = 0.0408*) and in a lower reproducibility variance for phosphomonoesterase activity (F = 0.0511, P = 0.0179*) in AG-M. Similarly, compared to the results obtained with the dilution of 2:100, the use of the dilution of 4:100 resulted in an lower mean repeatability variance for phosphodiesterase activity in PW-NS (F = 0.261, P = 0.0376*). Further, a significantly higher overall precision in the estimation of phosphomonoesterase and phosphodiesterase activities was observed with the dilution of 4:100 in PW-S (F = 0.336, P = 0.0420*) and PW-NS (F = 0.284, P = 0.0238*), respectively.

The influence of soil slurry dilution on the mean repeatability and reproducibility variances of the estimated activities resulted more evident in grassland soil samples. The reproducibility variance of α-glucosidase, *N*-acetyl-β−glucosaminidase, β-xylosidase and phosphodiesterase resulted significantly lower when the activities were assayed on the soil slurry dilution of 4:100 in GL-HIGH (F = 0.0244, P = 0.00618**; F = 0.0120, P = 0.00218**; F = 0.0843, P = 0.0359*; F = 0.171, P = 0.00336**, respectively). Further, GL-HIGH samples showed significantly lower mean repeatability variance for arylsulfatase, phosphomonoesterase and phosphodiesterase when the activities were assayed on the soil slurry dilution of 4:100 (F = 0.183, P = 0.0134*; F = 0132, P = 0.00482**; F = 0.205, P = 0.0191*, respectively).

Since either the mean repeatability variance or the reproducibility variance shown by the dilution of 4:100 were significantly lower as compared to that shown by the soil slurry dilution of 2:100 in at least one case for each of the considered environments, the dilution of 4:100 was considered as the optimal one and used for the following comparison with the benchmark method.

Table 2.11. Method reproducibility at saturating substrate concentrations and different soil slurry dilutions for agricultural soil samples*.

Activity	Site ID	Dilution 2:100		Dilution 4:100	
		Mean	CV	Mean	CV
β-GLU	AG-C	139.9	5.6	123.0	3.5
	AG-M	217.7	2.9	184.6	3.5
α-GLU	AG-C	7.7	4.2	6.1	8.9
	AG-M	9.4	6.6	7.3	1.4
N-AG	AG-C	27.6	9.0	27.8	12.7
	AG-M	45.8	9.2	46.9	5.1
β-XYL	AG-C	19.0	7.4	18.9	6.5
	AG-M	30.3	5.0	29.8	4.5
β-CEL	AG-C	23.9	2.7	25.9	1.6
	AG-M	54.0	3.1	59.1	4.0
SULF	AG-C	21.5	6.6	20.8	3.7
	AG-M	25.6	6.5	22.0	4.6
PME	AG-C	128.2	2.3	114.0	3.5
	AG-M	139.2	4.0	116.6	1.1
PDE	AG-C	44.4	4.2	41.2	5.0
	AG-M	50.6	4.4	46.9	2.6

*Based on four replicated days of analysis of three independent replicates. Activities are expressed as nmol MUF g^{-1} soil h^{-1}. CV = coefficient of variance between replicated days of analysis (%).

Table 2.12. Method reproducibility at saturating substrate concentrations and different soil slurry dilutions for grassland soil samples*.

Activity	Site ID	Dilution 2:100		Dilution 4:100	
		Mean	CV	Mean	CV
β-GLU	GL-LOW	1553.6	5.1	856.0	2.9
	GL-HIGH	1385.9	4.3	866.5	3.4
α-GLU	GL-LOW	61.6	4.2	45.5	8.9
	GL-HIGH	64.8	6.6	47.3	1.4
N-AG	GL-LOW	572.5	5.7	410.7	6.0
	GL-HIGH	519.2	7.6	389.6	1.1
β-XYL	GL-LOW	323.4	5.2	275.2	6.0
	GL-HIGH	336.1	5.0	271.2	1.8
β-CEL	GL-LOW	512.8	5.3	392.3	3.8
	GL-HIGH	456.6	2.5	360.1	2.7
SULF	GL-LOW	968.8	2.9	747.4	2.9
	GL-HIGH	1288.3	2.5	841.4	6.0
PME	GL-LOW	1134.3	4.3	770.5	2.2
	GL-HIGH	1208.2	2.3	791.6	6.5
PDE	GL-LOW	492.6	3.1	436.4	10.2
	GL-HIGH	495.6	6.5	427.9	2.1

*Based on four replicated days of analysis of three independent replicates. Activities are expressed as nmol MUF g^{-1} soil h^{-1}. CV = coefficient of variance between replicated days of analysis (%).

Table 2.13. Method reproducibility at saturating substrate concentrations and different soil slurry dilutions for pinewood soil samples*.

Activity	Site ID	Dilution 2:100		Dilution 4:100	
		Mean	CV	Mean	CV
β-GLU	PW-NS	305.9	7.6	246.6	10.3
	PW-S	515.6	4.5	442.0	3.1
α-GLU	PW-NS	10.9	10.9	9.3	21.0
	PW-S	27.7	8.1	25.7	10.9
N-AG	PW-NS	165.6	5.4	136.1	3.6
	PW-S	275.4	6.2	248.0	3.5
β-XYL	PW-NS	23.6	6.5	22.3	5.8
	PW-S	82.6	6.1	84.5	2.5
β-CEL	PW-NS	27.8	9.6	29.3	3.7
	PW-S	109.2	5.8	104.1	3.7
SULF	PW-NS	140.9	1.9	131.2	3.1
	PW-S	1314.6	5.4	1041.7	4.0
PME	PW-NS	318.3	4.5	300.7	4.8
	PW-S	1137.8	4.4	941.6	2.9
PDE	PW-NS	69.5	6.3	69.4	3.8
	PW-S	192.0	8.3	195.0	5.4

*Based on four replicated days of analysis of three independent replicates. Activities are expressed as nmol MUF g^{-1} soil h^{-1}. CV = coefficient of variance between replicated days of analysis (%).

2.3.4. Comparison between optimized methods and benchmark method

The optimized methods obtained for the agricultural, grassland and pinewood environments (see sections 2.3.2 and 2.3.3) were applied to field replicates in order to account for spatial variability occurring within each sampling site and compare the contrasting land uses, fertilization or constrains considered in each environment. A method using substrate final concentrations equal to 500 μM and a soil slurry dilution of 1:100 (ISO/TS 22939, 2010) was used as a benchmark for comparison with the optimized method.
Results regarding the agricultural soil samples are reported in Fig. 2.12 and Table 2.14. The optimized method lent evidence to differences in β-glucosidase and β-cellobiohydrolase activities, with the treatment fertilized with manure (AG-M) showing significantly higher values as compared to the unfertilized treatment (AG-C). On the contrary, only weakly significant differences in β-glucosidase and β-cellobiohydrolase activity were observed between AG-C and AG-M with the benchmark method. On the other hand, the optimized method reduced the significance of the difference observed between AG-C and AG-M for β-xylosidase activity compared to the benchmark method. Similarly, significant differences between arylsulfatase activity of AG-C and AG-M were observed only with the benchmark method. With regard to correlation of the enzyme activities with soil chemical and microbiological properties, β-glucosidase and β-cellobiohydrolase activity resulted significantly correlated with TOC and C_{mic} when data obtained with the optimized method were considered (Table 2.14 b) while correlations were either poorer or not significant when data obtained with the benchmark method were used for the linear regression (Table 2.14 a). Similar negative correlation coefficients and significance of the association between soil pH and P-cycling related activities were observed with the benchmark and the optimized methods. β-glucosidase, β-xylosidase and β-cellobiohydrolase resulted significantly intercorrelated, independently of the considered method, while contrasting results were observed for arylsulfatase activity association with other activities.
With regard to grassland soil samples, few differences between the low land use intensity (GL-LOW) and the high land use intensity (GL-HIGH) grassland sites were observed (Fig. 2.13). Arylsulfatase activity was significantly higher in GL-HIGH than in GL-LOW, regardless of the applied method. Similarly to what observed for the agricultural soil samples, in grassland soil samples β-xylosidase activity was significantly higher in GL-HIGH as compared to GL-LOW only

when the benchmark method was applied. β-glucosidase, β-xylosidase and β-cellobiohydrolase estimated with the optimized method resulted significantly intercorrelated (Table 2.15 b) while either poor or no correlations were observed when data obtained with the benchmark method were used for the linear regression (Table 2.15 a).

Large differences between not saline (PW-NS) and saline-sodic (PW-S) pinewood soil samples were observed for all the considered enzyme activities (Fig. 2.14), with the latter showing always higher values. The significance of the differences observed between the PW-NS and PW-S was limitedly affected by the method applied. The optimized method increased the significance of the difference observed between PW-NS and PW-S only for β-cellobiohydrolase activity compared to the benchmark method. Significant correlations of the enzyme activities with soil chemical and microbiological properties and enzyme activities intercorrelations were observed with both the benchmark and the optimized methods (Table 2.16).

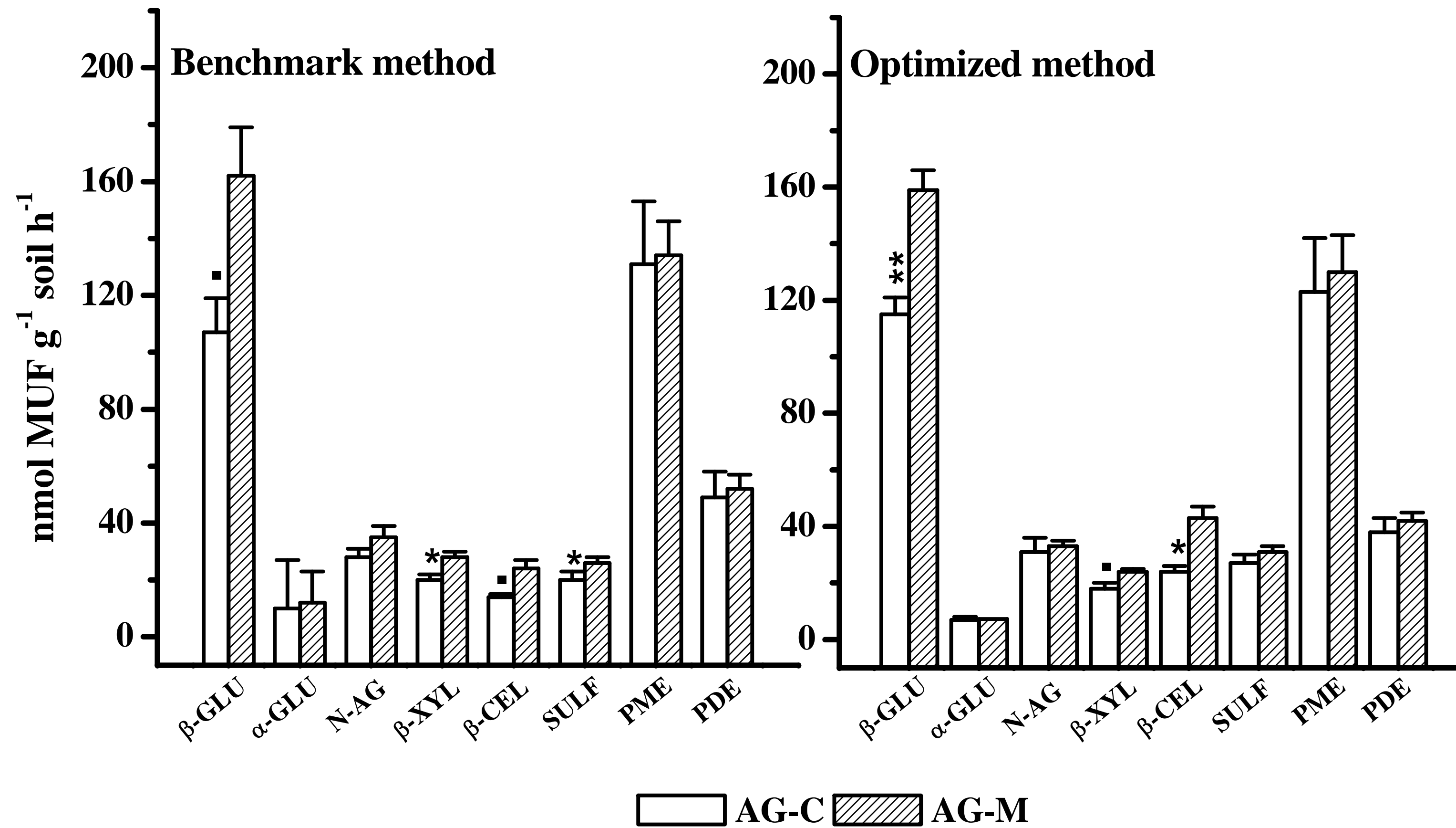

Fig. 2.12. Enzyme activities in unfertilized control (AG-C) and manured (AG-M) agricultural soil samples as determined by the benchmark and the optimized methods. Bars represent standard errors of four field replicates (n = 4). ▪ $0.10 < P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$.

Table 2.14. Pearson's correlation coefficients (r) between soil properties and enzyme activities as determined by (a) the benchmark method and (b) the optimized method for agricultural soil samples*.

	pH	TOC	C_{mic}	β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SULF	PME	PDE
a)											
β-GLU	0.24	0.83 *	0.58	1							
α-GLU	- 0.58	0.17	- 0.17	0.57	1						
N-AG	0.01	0.68	0.51	0.81 *	0.46	1					
B-XYL	- 0.11	0.66	0.36	0.86 **	0.75 *	0.72 *	1				
β-CEL	0.12	0.82 *	0.58	0.90 **	0.49	0.85 *	0.88 **	1			
SULF	- 0.12	0.58	0.22	0.88 **	0.74 *	0.74 *	0.91 **	0.79 *	1		
PME	- 0.90 **	- 0.23	- 0.47	0.15	0.80 *	0.36	0.43	0.27	0.40	1	
PDE	- 0.91 **	- 0.23	- 0.48	0.15	0.82 *	0.32	0.48	0.28	0.44	0.98 ***	1
b)											
β-GLU	0.40	0.91 **	0.74 *	1							
α-GLU	- 0.70	- 0.02	- 0.19	0.16	1						
N-AG	0.41	0.22	0.04	0.37	0.87 **	1					
B-XYL	- 0.14	0.68	0.38	0.83 *	0.58	0.66	1				
β-CEL	0.35	0.91 **	0.70 *	0.96 ***	0.20	0.41	0.85 **	1			
SULF	0.42	0.16	- 0.21	0.45	0.52	0.52	0.71	0.44	1		
PME	- 0.91 **	- 0.24	- 0.50	0.00	0.87 **	0.62	0.51	0.06	0.64	1	
PDE	- 0.82 *	- 0.08	- 0.37	0.17	0.88 **	0.67	0.64	0.22	0.72 *	0.98 ***	1

*Significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

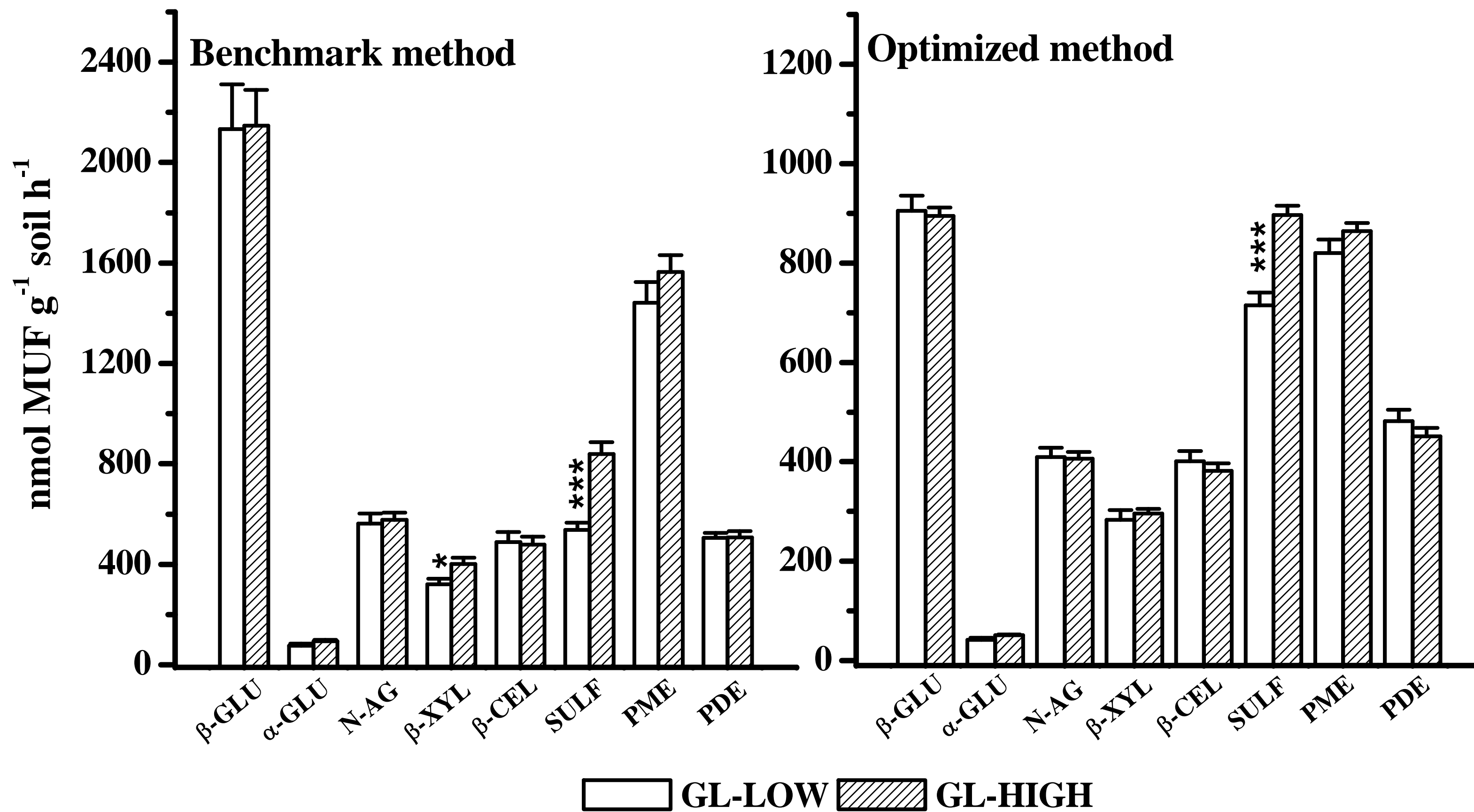

Fig. 2.13. Enzyme activities in low (GL-LOW) and high (GL-HIGH) land-use intensity grassland soil samples as determined by the benchmark and the optimized methods. Bars represent standard errors of six field replicates (n = 6). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Table 2.15. Pearson's correlation coefficients (r) between soil properties and enzyme activities as determined by (a) the benchmark method and (b) the optimized method grassland soil samples[*].

	pH	TOC	C_{mic}	β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SULF	PME	PDE
a)											
β-GLU	0.22	0.45	0.44	1							
α-GLU	- 0.44	- 0.28	- 0.12	0.13	1						
N-AG	0.07	0.23	0.35	0.38	0.74 **	1					
B-XYL	- 0.42	- 0.13	- 0.09	0.45	0.81 **	0.78 **	1				
β-CEL	0.24	0.29	0.39	0.35	0.51	0.86 ***	0.60 *	1			
SULF	- 0.80 **	- 0.54	- 0.40	0.08	0.71 **	0.42	0.81 **	0.23	1		
PME	- 0.13	0.11	0.20	0.35	0.74 **	0.92 ***	0.87 ***	0.76 **	0.64 *	1	
PDE	- 0.13	0.51	0.46	0.41	0.54	0.64 *	0.56	0.55	0.30	0.71 **	1
b)											
β-GLU	0.25	0.38	0.19	1							
α-GLU	- 0.59 *	- 0.38	- 0.38	0.48	1						
N-AG	0.06	0.15	0.06	0.73 **	0.60 *	1					
B-XYL	- 0.03	0.24	0.15	0.89 ***	0.70 *	0.69 *	1				
β-CEL	0.33	0.44	0.25	0.93 ***	0.40	0.81 *	0.79 **	1			
SULF	- 0.76 **	- 0.49	- 0.48	0.24	0.66 *	0.28	0.50	0.11	1		
PME	- 0.22	0.05	- 0.11	0.78 **	0.61 *	0.64 *	0.84 ***	0.66 *	0.73 **	1	
PDE	0.58 *	0.66 *	0.66 *	0.78 *	- 0.11	0.36	0.84 ***	0.52	0.02	0.49	1

[*]Significance levels: *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

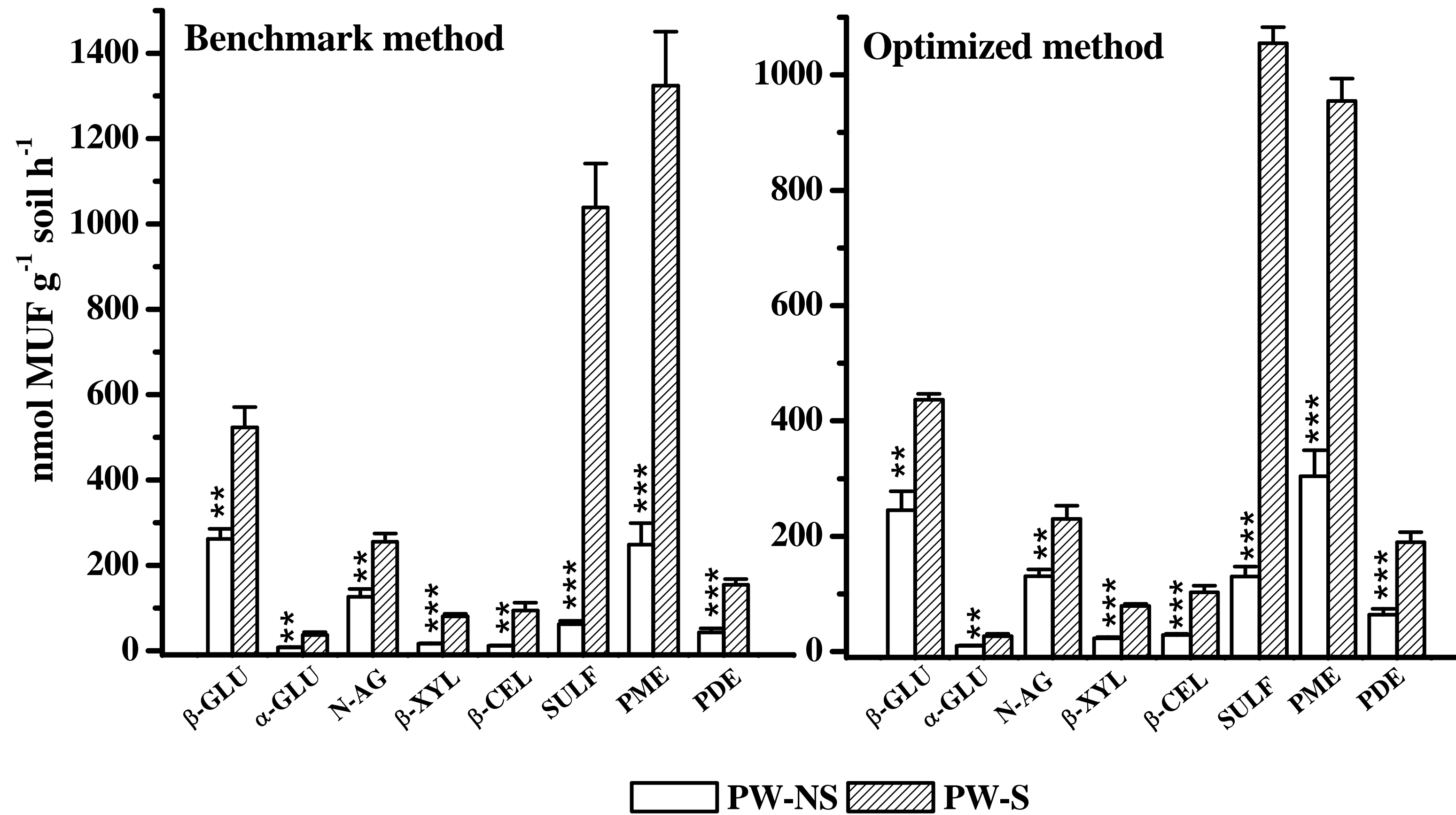

Fig. 2.14. Enzyme activities in not saline (PW-NS) and saline-sodic (PW-S) pinewood soil samples as determined by the benchmark and the optimized methods. Bars represent standard errors of four field replicates (n = 4). *$P \leq 0.05$; **$P \leq 0.01$; *** $P \leq 0.001$.

Table 2.16. Pearson's correlation coefficients (r) between soil properties and enzyme activities as determined by (a) the benchmark method and (b) the optimized method for pinewood soil samples.

	pH	TOC	C_{mic}	β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SULF	PME	PDE
a)											
β-GLU	0.55	0.73 *	0.93 ***	1							
α-GLU	0.38	0.93 **	0.81 *	0.79 *	1						
N-AG	0.30	0.78 *	0.86 **	0.73 *	0.90 **	1					
B-XYL	0.47	0.94 ***	0.90 **	0.82 *	0.96 ***	0.93 ***	1				
β-CEL	0.49	0.80 *	0.85 **	0.78 *	0.95 ***	0.94 ***	0.94 ***	1			
SULF	0.55	0.77 *	1.00 ***	0.92 **	0.80 *	0.86 **	0.90 **	0.84 **	1		
PME	0.46	0.76 *	0.99 ***	0.93 ***	0.82 *	0.88 **	0.89 **	0.85 **	0.99 ***	1	
PDE	0.40	0.76 *	0.95 ***	0.81 *	0.85 **	0.97 ***	0.92 **	0.91 **	0.95 ***	0.95 ***	1
b)											
β-GLU	0.56	0.85 **	0.91 **	1							
α-GLU	0.38	0.91 **	0.76 *	0.75 *	1						
N-AG	0.34	0.80 *	0.79 *	0.79 *	0.87 **	1					
B-XYL	0.52	0.92 **	0.93 ***	0.90 **	0.94 ***	0.89 **	1				
β-CEL	0.46	0.88 **	0.91 **	0.91 **	0.81 *	0.92 **	0.92 **	1			
SULF	0.51	0.88 **	0.98 ***	0.91 **	0.86 **	0.86 **	0.98 ***	0.95 ***	1		
PME	0.38	0.88 **	0.96 ***	0.89 **	0.82 *	0.85 **	0.94 ***	0.96 ***	0.98 ***	1	
PDE	0.42	0.75 *	0.96 ***	0.84 **	0.77 *	0.89 **	0.91 **	0.93 ***	0.96 ***	0.95 ***	1

[*]Significance levels: *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

2.4. Discussion

2.4.1. Main soil properties

The soils samples included in the study covered a wide range of main soil properties (Table 2.4). Soil pH ranged from the sub-acid to the moderately alkaline, while TOC, TN and microbial biomass changed of an order of magnitude from the lowest to the highest value. Within each environment two contrasting fertilization, land uses, or constrains were considered.

In the agricultural environment the treatments differed for the fertilization management. An unfertilized control treatment (AG-C) was compared with a treatment annually fertilized with manure (AG-M). As a result of this contrasting fertilization management, the treatments differs for SOM and microbial biomass content, with AG-M showing higher values. These results are in accordance with what observed in previous samplings. For a more detailed discussion of fertilization effects on soil properties see Chapter 1 and Giacometti et al. (2013).

In the grassland environment the treatments differed for both the land use and the fertilization management. An unfertilized grassland used as sheep pasture and classified as low land-use intensity (GL-LOW), was compared with a fertilized meadow, mown twice a year and classified as high land-use intensity (GL-HIGH) (Berner et al., 2011). The treatments mainly differed for soil reaction, with GL-HIGH showing lower values of pH as compared to GL-LOW. This difference was probably the result of mineral fertilization. In fact soil reaction may be affected by mineral N fertilization through acidification mechanisms (Clegg, 2006). Soil organic matter and microbial biomass content were consistently higher in GL-LOW compared to GL-HIGH, probably due to the higher organic inputs and the lower plant biomass removal associated with pasture (Murphy et al., 2006). However, differences between the treatments were quite small and exceed the 15 % only in the case of N_{mic} (Table 2.4).

In the pinewood environment the two sites differed for the location and sodicity of the water table as well as for the vegetation cover. A site covered by thermophilic submediterranean forest, occasionally affected by the water table but not by sodicity (PW-NS) was compared with a site covered by wood and swamp forest periodically saturated with sodic water (PW-S). The two sites strongly differ for TOC, TN and microbial biomass content, with PW-S showing higher values than PW-NS (Table 2.4). The differences in TOC observed between the two sites were within the same order of magnitude of the ones reported by Marinari et al. (2012) in a previous study. Salt affected soils usually have a low organic matter content due to poor plant growth which in turn results into

low inputs of organic materials into the soil (Wong et al., 2012). However, this was not observed in pinewood saline-sodic soil (Table 2.4). According to Marinari et al. (2012), plant C input may have not been reduced by stresses on plants with increasing soil sodicity. In contrast to what reported by Marinari et al. (2012) pronounced differences in microbial biomass between the two pinewood sites were observed in our study. In particular PW-NS showed lower values of C_{mic} as compared to the ones reported by Marinari et al. (2012). Microbial biomass is strongly affected by soil moisture and a reduction of soil water content is generally accompanied by a decline of microbial biomass (Geiseller and Horwath, 2009). At the PW-NS site the water table is within 1 m of the surface and its level fluctuate seasonally. At the time of sampling the top 10 cm of soil at the PW-NS site were extremely dry (soil water content of 2.5 % w:w) probably as a result of both a low level of the water table and a long period of drought, while the soil at the PW-S site was water saturated (soil water content of 46 % w:w). We therefore believe that the lower values of microbial biomass found in our study were due to the extreme dryness showed by PW-NS soil at the time of our sampling.

2.4.2. Substrate saturating concentrations

The kinetic experiments evidenced as substrate saturating concentrations may be both enzyme and site specific (Table 2.5), and that the use of a unique substrate concentration for all the enzyme activities included in the assay could be inappropriate for a correct estimation of maximum potential enzyme activities (German et al., 2011; Burns et al., 2013).

For all the soil samples and enzymes tested it was possible to establish a substrate concentration at which maximum enzyme activity (V_{max}) was observed. However, at higher substrate concentrations a significant reduction in the enzyme activity was observed in four out of eight of the tested enzymes. When the reaction rate reaches the maximum (V_{max}) the enzymes present in the reaction mixture are believed to be substrate saturated (Dick, 2011b). At higher concentrations substrate inhibition may occur (Freeman et al., 1995). Different mechanisms of substrate inhibition are reported in literature (Purich, 1979). High concentrations of substrate may cause alterations of the ionic force of the reaction mixture and a change in the ionic state of the amino acid residues of the enzyme, causing a non specific inhibition of the activity. The substrate can also bind to the enzyme allosteric site or form a so called dead-end complex with the enzyme, hampering its normal functioning. Finally, at high substrate concentrations alterations of the reaction order or metabolic pathway can occur. Working with soil enzymes and not with purified enzymes it is possible that not all the assumptions of Michaelis-Menten equation are met and it is also difficult to establish which

mechanism is responsible of the observed activity reduction. Therefore in this work the term apparent substrate inhibition will be used.

The results regarding *N*-acetil-β-glucosaminidase (Figs. 2.7, 2.8, and 2.9) confirmed what reported by the technical specification ISO/TC 22939 (2010), according to which chitinase activity should be assayed at a substrate concentration of 200 μM instead of 500 μM in order to avoid substrate inhibition. In our study also the enzymes involved in the breakdown of cellulose seemed to be subject to apparent substrate inhibition. In the case of β-cellobiohydrolase activity (Figs. 2.1, 2.2, and 2.3) the phenomenon was observed in all the samples even though the estimated substrate saturating concentrations slightly differed depending on the considered environment. At substrate concentrations equal or higher than 200 μM, inhibition was observed also for β-glucosidase activity, but only in the agricultural and pinewood samples (Figs. 2.4 and 2.6). Similarly, Freeman et al. (1995) reported that at substrate concentrations higher than 150 μM substrate inhibition was observed in low-active peatland samples. In grassland soil samples, β-glucosidase activity increased with increasing substrate concentrations and no apparent inhibition seemed to occur over the tested concentration range (Fig 2.5). The maximum activity reached in grassland soils were approximately an order of magnitude higher than the ones observed in agricultural and pinewood soils. Therefore it can be inferred that β-glucosidase enzyme concentration in grassland soils was much more higher as compared to agricultural and pinewood soils, and as a consequence no inhibition was observed in the former over the tested substrate concentration range. Also in the case of phosphomonoesterase activity substrate concentrations equal or higher than 200 μM caused apparent substrate inhibition in low-active agricultural samples, while no inhibition was observed in grassland and pinewood soil samples. As for β-glucosidase activity, the contrasting results found in the different environments could be due to the strong differences in the level of activity and therefore enzyme concentrations observed among agricultural, grassland and pinewood soil samples. Similarly to our findings, German et al. (2012) reported that in a recent study of enzymes Michaelis-Menten kinetic parameters in different soils, the range of substrate concentrations used varied by enzyme and in some cases even by location, due to substrate inhibition. The substrate concentration necessary to ensure substrate saturation for arylsulfatase activity in grassland and pinewood soil samples (Table 2.5) were five times higher than the ones reported in literature (Marx et al., 2001, 2005; Vepsäläinen et al., 2001; ISO/TS 22939, 2010) and could be the results of both a high level of activity and a low apparent substrate affinity (Fig. 2.10).

According to Burns et al. (2013) in the case of soils with very high enzyme concentrations, the substrate concentration commonly reported in literature might be insufficient to ensure substrate saturating conditions. In addition to this, the higher is the K_m value the lower is the affinity of the enzyme for the substrate, and the higher is the concentration of substrate required to achieve the 50 % of the maximum reaction rate (V_{max}) (Dick, 2011b). In the poorly active agricultural soil samples the low apparent affinity of the enzymes for the substrate of arysulfatase was probably the main factor determining the relatively high substrate concentration needed to attain saturation (Fig. 2.10, Table 2.5).

For α-glucosidase and β-xylosidase activities, substrate saturating concentrations were equal or close to 500 μM, which correspond to the substrate concentration used in most of the available analytical procedures (Marx et al., 2001, 2005; Vepsäläinen et al., 2001; ISO/TS 22939, 2010).

Similar results were observed for phosphodiesterase (Table 2.5) in agricultural soil samples, while for grassland soil samples, the substrate concentration of 500 μM was insufficient to ensure substrate saturating conditions, probably due to the higher enzyme concentrations of the latter (Burns et al., 2013). Likewise, in pinewood soil samples collected at the saline-sodic site (PW-S) the concentration of bis-4-MUF-phosphate had to be increased to 1000 μM to ensure saturation. In the PW-NS samples, saturation was attained at lower substrate concentrations but, since over-saturating concentrations did not cause apparent inhibition a unique substrate concentration of 1000 μM was used for all the pinewood soil samples.

2.4.3. Soils slurry dilution effect on quenching of fluorescence, repeatability and reproducibility

Fluorescence measurements are less affected by interferences due to soil turbidity compared to absorption-based detection methods (Deng et al., 2011). The emission of fluorescence is highly specific since is uncommon that non-target compounds present in the reaction mixture adsorb and reemit light at the same frequency of the target reaction product MUF (Deng et al., 2013). However, quenching of MUF due to the presence of soil particles is a well documented phenomenon, which could lead to a substantial reduction of the relative fluorescence emitted by MUF (Freeman et al., 1995; Marx et al., 2001; Deng et al., 2011, 2013; German et al., 2011).

In our study, the relative fluorescence emitted by MUF was affected by the presence of soil suspension (Fig. 2.11 and Table 2.6). The use of less diluted soil slurries increased the turbidity of the reaction mixture, which in turn increased the quenching of fluorescence (German et al., 2011).

Moreover, the quenching of MUF varied depending on the considered soil sample (Table 2.6), confirming the importance of developing a specific calibration curve for each soil tested (Deng et al., 2013; Marx et al., 2001). In agricultural soil samples the quench coefficient never exceed 0.80 (Table 2.6). Similar values were reported by German et al. (2011), using a 1:125 soil-to-buffer ratio. In grassland and pinewood soil samples the degree of quenching was higher, and the dilution level of 4:100 lead to a reduction of the fluorescence signal of up to 68 % in pinewood saline-sodic soil. This values are in accordance with the findings of Freeman et al. (1995), and were probably due to the presence of larger amount of phenolic compounds (Freeman et al., 1995) and particulate or dissolved organic matter (German et al., 2011) in pinewood and grassland soils as compared to agricultural soils.

The soil slurry dilution affected the variability shown by analytical replicates, with the highest level of dilution (1:100 w:v) resulting in the largest variability across all the enzyme activities and soil samples (Table 2.7). According to German et al. (2011) soil slurries that are too diluted may lead to an increase in the analytical variability due to fine scale heterogeneity of the soil slurry.

Replicated wells of the same soil suspension (i.e. analytical replicates) provide an estimation of the degree of soil slurry homogenization (German et al., 2011) and of the errors generated in pipetting (Deng et al., 2013). However due to the heterogeneous nature of soil, in addition to analytical replicates, it is crucial to conduct assay replications using also different soil suspensions (i.e. true independent replicates) (Deng et al., 2013).

In our experiments we considered repeatability and reproducibility, two different conditions of precision, both useful to compare and describe the variability of measurement methods. The variability observed under repeatability conditions may be attributed to unavoidable random errors, to inherent variation in the measurement procedure and to the soil sample heterogeneity. Under reproducibility conditions time-different variations also contribute to the observed variability. Assuming that deep freezing (-20 ± 2 °C) is a suitable soil storage technique that do not cause relevant variations in enzyme activity levels over at least 4 months (ISO/TS 22939, 2010), the most relevant time-different variation occurring in microplate-based fluorimetric assays within a laboratory is the use of daily prepared reagents. In fact, since MUF and MUF conjugates appear to be unstable once dissolved (ISO/TS 22939, 2010) new substrates and standard solutions should be produced for each day of analysis.

In our study the comparison of methods using different soil slurry dilutions based on the variability shown by true independent replicates evidenced as the soil slurry dilution of 1:100 led to the poorer repeatability of the results, independent of the considered soil samples, while no clear differences in the repeatability of the methods using a soil slurry dilution of 2:100 and 4:100 were observed. However, when the soil slurry dilutions of 2:100 and 4:100 were compared based on data obtained over 4 consecutive days of measurements, results indicated as the 4:100 dilution improved the mean repeatability and reproducibility of some of the estimated activities as compared to the 2:100 dilution, and was therefore defined as the optimal one. These findings confirm that, independently of the soil-to-buffer ratio the use of a relatively large mass of soil for the soil slurry preparation may increase the sample representativeness (DeForest, 2009) and therefore reduce the variability observed between true independent replicates of the same soil sample and between different days of analysis.

The methods using saturating substrate concentrations defined in the kinetic experiment (Table 2.5) and the soil slurry dilution of 4:100 were considered as the optimized ones. Noteworthy, the coefficient of variance shown by true independent replicates and by repeated days of analysis of the optimized methods rarely exceeded the 10 % (Tables 2.8-2.13). These results indicate that the repeatability and reproducibility of the optimized methods were acceptable. Under the conditions of this study, the optimized methods could therefore be considered suitable for a precise estimation of soils enzyme potential activities.

Since enzyme activities were expressed on a soil dry mass base (see section 2.2.8), different dilution levels were expected to provide similar results. However, the soil-to-buffer ratio appeared to affect the values estimated, and in some cases a reduction of the soil slurry dilution was accompanied by a reduction of the mean enzyme activities (Tables 2.8, 2.9 and 2.10). However, the phenomenon seemed to vary by site and enzyme. The reduction become more evident for soil samples characterized by a high level of enzyme activity, such as grassland soil samples, where mean activities decreased by up to 60 %. It has been reported that different dilutions do not give exactly comparable results (ISO/TS 22939, 2010). Lower levels of soil slurry dilution may reduce the exposure of enzymes to substrates (ISO/TS 22939, 2010) and possibly limit substrate diffusion in the reaction mixture. As a result the rate of the enzyme-catalyzed reaction may decrease as compared to that measured in a more diluted soil slurry and this probably become more evident in soil samples with high enzyme concentrations.

2.4.4. Comparison between optimized methods and benchmark method

Most differences in enzyme activities among sites or treatments are likely the result of differences in soil organic matter or microbial biomass content (Sinsabaugh et al., 2008).

In the agricultural environment, the treatment fertilized with cattle manure (AG-M) had roughly 40 % more TOC and 70 % more C_{mic} as compared to the unfertilized control (AG-C). Differences observed in the pinewood environment were even larger, with saline-sodic pinewood soil (PW-S) showing four times as TOC and C_{mic} than the not saline pinewood soil (PW-NS). As a consequence, the differences in enzyme activities observed within the agricultural and pinewood environments can be mainly ascribed to differences in SOM and microbial biomass content (Table 2.4).

In the agricultural environment the optimized method lent evidence to differences in β-glucosidase and β-cellobiohydrolase activities, with the treatment fertilized with manure (AG-M) showing significantly higher values as compared to the unfertilized treatment (AG-C), while only weakly ($0.10 < P > 0.05$) significant differences in β-glucosidase and β-cellobiohydrolase activity were observed with the benchmark method (Fig. 2.12). Noteworthy, in agricultural soil samples the optimized method of β-glucosidase and β-cellobiohydrolase activities strongly differ from the benchmark method for both the substrate final concentration and the soil slurry dilution used. In the optimized method, substrate final concentrations were equal to 100 and 75 µM, respectively, since at higher concentrations apparent substrate inhibition was observed (Figs. 2.1 and 2.4). On the contrary, in the benchmark method substrate final concentration was kept constant to 500 µM across all the enzyme activities. Moreover, in the case of β-glucosidase activity results of the repeatability experiment evidenced as the soil slurry dilution of 1:100, employed in the benchmark method, led to a significantly lower repeatability as compared to the soil slurry dilution of 4:100 of the optimized method. As reported by Steen and Ziervogel (2012), when substrate inhibition is an issue, the use of too large substrate concentrations may lead to underestimates of maximum reaction velocity and increased likelihood of Type II errors, just as the use of sub-saturating substrate concentrations will. Besides, differences among treatments are more likely to be detected if the uncertainty associated with the measurement is decreased by properly selecting the soil-to-buffer ratio. Further, as compared to the benchmark method, the use of the optimized method revealed closer associations between β-glucosidase and β-cellobiohydrolase activities and soil TOC and microbial biomass content (Table 2.14). These soil properties are known to play a key role in the

regulation of microbial activity (Sinsabaugh et al., 2008) and a correlation with soil enzyme activities is therefore expected. On the other hand, the optimized method slightly reduced the significance of the difference observed between AG-C and AG-M for β-xylosidase activity compared to the benchmark method (P = 0.089 and P = 0.012*, respectively). However, in agricultural soil samples, the optimized method of β-xylosidase activity strongly differ from the benchmark method only for the soil slurry dilution levels. Substrate final concentrations of the optimized and the benchmark methods were quite similar, and equal to 400 and 500 μM, respectively. Therefore small differences between the results obtained with the two methods may be expected.

Significant differences between arylsulfatase activity of AG-C and AG-M were observed only with the benchmark method. Moreover, when data obtained with the benchmark method were considered, arylsulfatase and C-cycle enzyme activities resulted intercorrelated (Table 2.14 a). On the contrary arylsulfatase activity resulted weakly associated only with P-related enzyme activities and no differences between AG-C and AG-M were found if data obtained with the optimized method were considered (Table 2.14 b). In the optimized method the substrate final concentration for arylsulfatase was set to 1000 μM, since lower concentrations proved to be insufficient to attain substrate saturation (Fig. 2.10), while in the benchmark method substrate concentration was kept constant to 500 μM across all the enzyme activities. The use of sub-saturating substrate concentrations causes underestimation of the enzyme potential activity which in turn may reduce the likelihood to detect significant differences between treatments (German et al., 2011). However the opposite was observed for arylsulfatase activity in agricultural soil samples. Even though arylsufatase activity values estimated with the benchmark method were lower than the ones estimated with the optimized method, confirming that underestimation of the activity occurred due to the sub-saturating substrate concentrations, significant differences between the treatments were found only with the benchmark method.

With regard to pinewood environment, all the activities involved in the study resulted markedly higher in the saline-sodic pinewood site (Fig. 2.14). In this environment method optimization improved the statistical significance of the differences observed between the two sites for β-cellobiohydrolase activity. Similarly to what observed for the agricultural environment, in the optimized method substrate final concentration was equal to 50 μM, since at higher concentrations apparent substrate inhibition was observed (Table 2.5), while in the benchmark method substrate

final concentration was kept constant to 500 μM across all the enzyme activities. Thus, this results further confirms as over-saturating substrate concentrations can negatively affect the statistical significance of differences observed between treatments (Steen and Ziervogel, 2012). For the other enzyme activities no relevant differences between the significance levels obtained with the benchmark and the optimized methods were observed. This was probably due to the fact that the magnitude of the differences was to big to appreciate any improvement.

In the case of grassland soils, the treatments had quite similar TOC and C_{mic} content (Table 2.4) and soil pH was probably the main factor influencing soil catalytic behavior and determining the differences observed between the two treatments. Mineral fertilization lowered soil pH in GL-HIGH, which in turn influenced arylsulfatase activity, as confirmed by the highly significant difference in the activity of GL-LOW and GL-HIGH (Fig. 2.13) and by the strong negative correlation observed between arylsulfatase activity and soil pH (Table 2.12). Arylsulfatase pH optimum determined with *p*-nitrophenyl sulfate as model substrate has been reported to vary from 5.5 to 6.2 (Klose et al., 2011). Values ranging from pH 4.0 to 4.5 were observed by Niemi et al. (2005) using the MUF conjugate. Therefore GL-HIGH acidic pH might be more favorable as compared to the sub-neutral pH of GL-LOW (Speir et al., 1999). Differences in the quality of the organic materials entering the soil due to a diverse plant community composition characterizing the two treatments (Berner et al., 2011) could as well have contributed to the observed differences. Arylsulfatase activity is known to be related to soil organic carbon content (Deng and Tabatabai, 1997). However in grassland environment no correlation was observed, probably because the TOC and TN values of the grassland soils were in a too narrow range. The statistical significances of the differences observed between GL-LOW and GL-HIGH arylsulfatse activities obtained with the benchmark and the optimized methods were comparable (Fig. 2.13). As observed for pinewood soils, this was probably due to the fact that the magnitude of the differences between the treatment was to big to appreciate any significance improvement. A trend similar to that shown by arylsulfatase was observed also for phosphomonoesterase activity but the differences between the treatments were not significant, independent of the considered method.

Significantly higher β-xylosidase activity was observed in GL-LOW compared to GL-HIGH only with the benchmark method. It should be noticed that in this instance the benchmark and the optimized method differed only for soil slurry dilution level. The use of different soil slurry dilution levels did not affect the repeatability of β-xylosidase activity measurements in grassland soil samples. As a consequence the exclusion of the dilution of 1:100, used in the benchmark method,

was based on the results of other enzyme activities, for which the choice of the soil slurry dilution significantly affected the repeatability of the measurements. However, in grassland soil samples a reduction of the soil slurry dilution was accompanied by a decrease of the mean enzyme activity (Table 2.9), which in turn could have reduced the power to detect differences in β-xylosidase activity between the grassland treatments.

2.5. Conclusions

Apparent substrate inhibition affected four out of eight of the enzyme activities involved in the study. For *N*-acetil-β-glucosaminidase and β-cellobiohydrolase apparent substrate inhibition occurred across all the considered environments, while for β-glucosidase and phosphomonoesterase the phenomenon was site-specific. Phosphodiesterase and arylsulfatase activities reached saturation at up to 5 times the concentration of substrate commonly reported in literature. Therefore, for a correct estimation of maximum potential enzyme activities, substrate concentrations require optimization since saturating concentrations vary by site and enzyme.

The choice of the soil-to-buffer ratio for the soil slurry preparation affected the quenching of fluorescence, the estimated enzyme activities and the variability associated with the measurements. The use of less diluted soil slurries increased the turbidity of the reaction mixture which in turn increased the quenching of fluorescence emitted by MUF. However, chemical soil properties also affected the degree of quenching that hence varied among sites and treatments, confirming the importance of developing specific calibration curves for each soil tested.

The soil slurry dilution level affected the enzyme-catalyzed reaction rates, thought modifications in the exposure of enzymes to substrate and in substrate diffusion, particularly in grassland samples. For meaningful data comparison and interpretation we thus suggest that the same soil-to-buffer ratio is used. Of the three considered soil slurry dilution levels, the dilution of 4:100 generally resulted in lower analytical replicates variability as well as in lower repeatability and reproducibility variance across all the considered environments and was therefore defined as the optimal in terms of precision of the measurements. As compared to results obtained with the benchmark method, the optimized methods improved the statistical significance of differences observed between contrasting treatments and revealed closer associations between enzyme activities and soil properties. However, this improvement was evident only when apparent substrate inhibition was an issue.

Based on the evaluation of eight enzyme activities in three different environments and six diverse sampling sites, the repeatability and reproducibility of the optimized methods were acceptable, indicating the potential of microplate-scale fluorometic assays to be used as precise and throughput methods for the simultaneous determination of multiple soil enzyme activities.

CHAPTER 3

Measuring soil enzyme activities as soil quality indicators using microplate-based fluorimetry

CHAPTER 3

3.1. Introduction

Soil enzymes catalyze a complex web of chemical reactions necessary for life processes of microorganisms, depolymerization of macromolecular organics, cycling of nutrients and formation of organic matter and soil structure (Dick, 1994). Among the different activities catalyzed by soil enzymes, extracellular enzyme activities are required for the degradation of complex polymers such as cellulose, hemicellulose, lignin, starch, proteins, and chitin of which the organic compounds entering the soil are mostly composed of. Therefore, soil extracellular enzymes catalyze the rate-limiting steps of organic matter degradation (Sinsabaugh et al., 2008). The measurement of their activity may provide insights into the biochemistry of organic matter decomposition and nutrient cycling in soil (Sinsabaugh et al., 2008).

Enzymes synthesis and secretion is an energy and nutrient intense process, thus cells will spend this precious resources only if the benefit of increased availability of assimilable mineral nutrients, energy and low molecular mass organics counterbalance the costs (Burns at al., 2013). Most of the extracellular soil enzymes are induced by the presence of their target substrate and their activity is considered an indicator of substrate availability (Geisseler and Horwath, 2009). However, factors other than substrate induction may also influence the synthesis and activity of soil extracellular enzymes, complicating the relationship between enzyme activity and substrate availability. Within this factors, inhibition of enzyme synthesis and activity in the presence of high levels of end products is a well documented phenomenon, probably based on cellular economics (Allison and Vitousek, 2005; Burns et al., 2013). Other mechanisms influencing the final level of enzyme activity independent of substrate availability are the presence of soil colloid-immobilized enzymes still retaining part of their catalytic activity (Burns, 1982), and the production of low constitutive levels of enzymes by soil microorganisms for substrate detection (Burns et al., 2013).

Changes in enzyme activities may provide early indication of changes in fundamental soil biochemical processes and thus could represent a useful tool to asses and monitor soil quality changes (Ekenler and Tabatabai, 2003a; Schloter et al., 2003).

Since the organic materials entering the soil are both structurally complex and highly diverse, their breakdown and subsequent mineralization requires the combined activity of many different microorganisms and enzymes (Burns et al., 2013). Involving in the study a large number of enzyme activities should yield more versatile information than the activity of only one enzyme, and provide

a better picture of the multiple biochemical processes occurring in soil (Vepsäläinen et al., 2001).
Traditional bench-scale colorimetric enzyme assays are time consuming and labor intensive analytical procedures that constrain the analysis to one enzyme for a limited number of samples at a time (Popova and Deng, 2010). As a consequence their application implies that either the samples storage time is increased or the number of activities tested is reduced.
Microplate-scale fluorimetric assays based on the use of 4-methylumbelliferone (MUF), overcome the main practical limitations posed by traditional bench-scale colorimetric assays, and are becoming prevalent in studies regarding soil enzymes (German et al., 2011). A number of conjugates of the fluorogenic compound MUF are nowadays available, allowing the determination of a variety of enzyme activities (German et al., 2011). Therefore, microplate-based fluorimetric assays appear to offer a fast and throughput approach for studying the response of multiple enzyme activities involved in main soil biochemical processes of organic substrate degradation and nutrient elements cycling.
In the present study the usefulness of microplate-based fluorimetric enzyme assays as a tool to asses soil functional diversity and quality was tested. A long-term agricultural field experiment established in 1966 in which cattle manure and crop residue are factorially combined with increasing levels of urea was selected with the aim of studying the changes in soil enzyme activity profiles in response to organic input and mineral N fertilization.
Eight extracellular hydrolytic enzymes were chosen based on their importance in nutrient cycling and organic matter decomposition, and on their potential sensitivity to differences in organic and mineral N fertilization management. Among the glycosidases involved in the study, β-cellobiosidase and β-glucosidase take part in the degradation of cellulose, the most abundant carbon rich substrate of plant origin entering the soil, catalyzing the hydrolysis of cellulose in cellobiose and of cellobiose in glucose, respectively. β-xylosidase is involved in the degradation of xylose, a polysaccharide derived from hemicellulose, the second most abundant plant derived carbohydrate found in soil, while α-glucosidase catalyze the hydrolysis of maltose, a disaccharide deriving from starch, a plant storage carbohydrate. These enzymes have a critical role in the degradation of organic compounds as well as in the formation and development of soil organic matter and structural components (Deng and Popova, 2011). Moreover, some of these glycosidases release low molecular weight sugars that can be readily used by soil microorganisms as energy sources (Bandick and Dick, 1999).

The measurement of glycosidases activity has been widely employed in soil enzymatic studies and is considered an indicator of management-induced effects in biodegradation of C compounds in arable soils (Bandick and Dick, 1999).

N-acetyl-β-glucosaminidase takes part in chitin degradation releasing aminosugars, labile sources of organic C and N in soil (Ekenler and Tabatabai, 2002, 2003b). Since *N*-acetyl-β-glucosaminidase is produced mainly by soil fungi and play an important role in fungal cell wall development and active growth (Geisselr and Horwath, 2009), the measurement of its activity may be used as an indirect indicator of fungal biomass (Ekenler and Tabatabai, 2003b).

Arylsulfatase is the enzyme that catalyze the hydrolysis of the O-S ester bond of arylsulfate. This enzyme is believed to be involved in the mineralization of ester sulfate in soils and therefore plays an important role in S cycling because it releases plant available SO_4^{2-} (Deng and Tabatabai, 1997). Moreover it can be considered an indicator of fungal biomass since only fungi and not bacteria contain ester sulfate (Bandick and Dick, 1999).

Phosphatases are a group of enzymes that catalyze the hydrolysis of phosphate esters: phosphodiesterase catalyzed the hydrolysis of phosphoric diesters as phospholipids and nucleic acids into phosphoric monoesters, while phosphomonoesterase hydrolyze phosphoric monoesters into inorganic phosphorus, which can be taken up by plants (Acosta-Martínez and Tabatabai, 2011).

According to Dick et al. (1994) the use of soil enzyme activities as soil quality indicators is ground on the fact that soil enzymes are closely related to other important physical, chemical and microbiological soil parameters linked to soil quality. Therefore, our second objective was to evaluate through both simple linear correlation and multivariate analysis the relationship existing between soil enzyme activities and soil properties such as soil reaction, soil organic matter and microbial biomass content.

3.2. Materials and methods

3.2.1. Study site and experimental design

The study site was a long-term field experiment started in 1966, located at the Experimental Farm of the University of Bologna, in the South-east of the Po valley (Italy, 44°33′ N, 11° 24′ E; 23 m.a.s.l.). The climate of the region was classified as sub-humid. The soil was classified as a fine silty, mixed, mesic Udic Ustochrepts (USDA Soil Taxonomy). The experimental design was a split-plot with four replicate blocks. Maize (*Zea mays* L.) and winter wheat (*Triticum aestivum* L.) were cropped in a 2-year rain fed rotation. Wheat straw or maize stalk (*crop residue*) and cattle manure (*manure*) were applied every year to the same plots and compared to unamended plots (*control*). Each plot was then split to receive 0 (N_0) or 200 (N_{200}) kg ha^{-1} of mineral N, supplied as urea. Manure and crop residue were added every year in October at the same dry matter rate, corresponding to 6.0 t dry matter ha^{-1} after wheat and 7.5 t dry matter ha^{-1} after maize crop, and incorporated into the soil by mouldboard ploughing to 0.40 cm depth. Expressed as a percentage of their dry weight the organic materials had the following composition (Triberti et al., 2008): (i) wheat straw: organic C 32.8 %, total N 0.47 %, C:N ratio 69.8; (ii) maize stalks: organic C 32.6 %, total N 0.70 %, C:N ratio 46.6; (iii) cattle manure: organic C 33.1 %, total N 2.64 %, C:N ratio 12.3. Urea was supplied in two applications: 30 % at the end of tillering and 70 % at the beginning of stem elongation in wheat; 50 % at seeding and 50 % at the 4th leaf stage in maize.

3.2.2. Soil sampling and characterization

Soil samples from the top 20 cm were collected in March 2012 before maize sowing. For each subplot of 33 m^2 nine soil samples were collected using an auger and combined to form composite samples. Freshly sampled soil was kept in a cooler for transportation back to the laboratory, sieved through a 2 mm sieve and thoroughly mixed. Roots and visible plant residue were removed by tweezers. Each field replicate was then divided into three aliquots. One aliquot was air dried and used for the soil chemical characterization. A second aliquot was stored at + 4 °C and used for the soil microbiological characterization. A third aliquot was stored at – 20 °C and used for the measurement of enzymatic activities. Soil water content was determined gravimetrically by drying soil samples to constant mass at 105 °C. Soil pH was measured using a glass electrode in 1:5 (v:v) suspensions of air dried soil in 10 mM $CaCl_2$ (ISO 10390, 2005). Traces of carbonates (< 4.00 g kg^{-1} $CaCO_3$) were detected in some of the collected samples (Dietrich-Frühling method).

Total organic carbon (TOC) and total nitrogen (TN) content were determined on air dried, finely ground soil aliquots. Soil subsamples of 10 mg were analyzed in triplicate by an elemental analyzer (CHNS-O Elemental Analyzer 1110, Thermo Scientific GmbH, Dreieich, DE) after pre-treatment with a 1:1 HCl solution to eliminate the traces of carbonates.

Microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) were determined within a week from soil collection on field moist soil samples stored at + 4 °C using the chloroform-fumigation extraction (CFE) method (Brookes et al., 1985). The equivalent of 4 g of oven dried soil was fumigated with ethanol-free chloroform for 24 h at room temperature in a desiccator. Fumigated and unfumigated samples were dispersed in 16 mL of 0.5 M K_2SO_4 and extracted on an horizontal shaker at 250 rev min^{-1} for 1 h. Extracts were filtered through Whatman no. 42 filter paper and analyzed for the organic carbon and total nitrogen content with an elemental analyzer (TOC-VCPH/CPN, Shimadzu, Kyoto, JP). C_{mic} was calculated as organic carbon in the fumigated minus organic carbon in the unfumigated soil extracts. Similarly, N_{mic} was calculated as total nitrogen in the fumigated minus total nitrogen in the unfumigated soil extracts. The organic carbon content of the unfumigated extracts was also used as an estimation of soil extractable organic carbon (C_{extr}).

3.2.3. Enzymatic assays

Activities of β-glucosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, β-xylosidase, β-cellobiosidase, arylsulfatase, phosphomonoesterase and phosphodiesterase were assayed using MUF (7-hydroxyl-4-methylcoumarin) conjugates. Final substrates concentrations were selected to ensure substrate saturating conditions, based on the results reported in Chapter 2 (Table 3.1). All the assays were conducted on deep frozen soil samples (-20 °C), within 4 months from the soil samples collection (ISO/TS 22939, 2010). A 0.5 M sodium acetate buffer solution was made by mixing sodium acetate trihydrate (analytical grade, crystalline, Carlo Erba) with deionized water. The pH was adjusted to 5.5 using glacial acetic acid (99.9 % v:v, Carlo Erba) (ISO/TS 22939, 2010). This buffer solution was then used to dilute standard, substrates and soil samples. To minimize variability due to reagents storage, substrates and standard solutions were prepared on the day of the assay. Freshly made solutions were kept away from light until use. To avoid microbial contamination, glassware, buffers and deionised water were sterilized in autoclave (121 ± 3 °C for 20 min.) before usage (ISO/TS 22929, 2010). Each substrate was pre-dissolved in DMSO (dimethyl sulfoxide; SIGMA). Sodium acetate buffer was then added to give the desired final concentration (Table 3.1).

Five mM 7-hydroxyl-4-methylcoumarin (MUF) standard solution was prepared in methanol and water (1:1, v:v). This stock solution was diluted to 1.00, 2.00, 4.00, 10.0, 20.0, 30.0, 40.0 μM in sodium acetate buffer.

Moist soil samples corresponding to 4 g of oven dried soil were weighted into sterilized Pyrex tall-form 150 mL becker. One hundred mL of 0.5 M acetate buffer was added and mixed using an Ultra Turrax IKA for 2 min at 9000 rpm (IKA-Werke, Staufen, DE). A magnetic stir bar was then added and soil was kept under continuous stirring. There was no delay between soil slurry preparation and dispensation in the micro-plate. The entire procedure of soil samples processing was staggered so that the time between soil slurry preparation and subsequent substrate addition never exceeded 40 min. Flat-well black polystyrene 96-well microplates with a well capacity of 350 μL were used throughout the experiment (Greiner Bio-One, Frickenhausen, DE). Buffer, soil slurry, standard solutions and substrate solutions were dispensed in the micro-plates in the following order: first 100 μL of sodium acetate buffer were dispensed in the wells that served as soil controls and substrate controls. Next 50 μL of sodium acetate were dispensed in the wells that served as quench controls. Then 150 μL of sodium acetate buffer was added in the wells that served as reference standards. Soil slurry aliquots of 100 μL were than withdrawn from the soil suspension under continuous stirring and dispensed in the wells that served as quench controls, soil controls and soil assays. After all the soil slurries included in the assay were processed and dispensed, 50 μL of MUF standard solutions were dispensed into wells that served as quench controls and reference standards. Lastly 100 μL of substrate solutions were dispersed into wells that served as substrate controls and soil assays. The total volume of the reaction mixture was 200 μL. Eight analytical replicates were used for soil assay and substrate controls wells. Four analytical replicates were used for reference standards, quench controls and soil controls wells. A list of the different component included in the assay and their function is given in Table 2.2 (Chapter 2). The addition of the substrates was considered the start of the incubation period.

The microplates were covered and incubated in the dark at 30 °C. The fluorescence intensity was measured using a microplate fluorometer (infinite200, TECAN, Männedorf, CH) with 365-nm excitation and 450-nm emission filters. Measurements were taken immediately after the plate set-up and from then on every 30 min over a 3 h incubation period. Before each reading the microplates were shaken for 5 s in order to homogenize the reaction mixture.

Enzyme activities were expressed in nmol product h^{-1} g^{-1}. Rates of fluorescence increase rather than absolute amount of fluorescence at the end of the incubation period were used for the calculation. Rates of fluorescence increase were converted into enzyme activity according to the following equations (adapted from Marx et al. 2001 and German et al., 2011):

$$\text{Activity (nmol MUF g}^{-1}\text{ h}^{-1}\text{)} = \frac{\text{Net fluorescence (RUF min}^{-1}\text{) x 100 (mL) x 200 (}\mu\text{L) x 60 (min h}^{-1}\text{)}}{\text{Emission coefficient (RUF/ }\mu\text{mol L}^{-1}\text{) x 100 (}\mu\text{L) x Soil dry mass (g)}}$$

Where:

$$\text{Net fluorescence} = \left(\frac{\text{Assay slope} - \text{Soil control slope}}{\text{Quench coefficient}} \right) - \text{Substrate control slope}$$

$$\text{Emission coefficient (RUF/ }\mu\text{mol L}^{-1}\text{)} = \text{Reference standard curve slope}$$

$$\text{Quench coefficient} = \frac{\text{Quench controls curve slope (RUF /}\mu\text{mol L}^{-1}\text{)}}{\text{Reference standards curve slope (RUF /}\mu\text{mol L}^{-1}\text{)}}$$

RUF = relative units of fluorescence

3.2.4. Data handling and statistics

All results are expressed on an oven-dried basis. Results represent the arithmetic means of the field replications (e.g. subplots), which in turn are the mean of analytical replications.

The experimental data was analyzed as a split-plot arrangement of treatments with organic fertilization as the main factor and mineral N fertilization as a subfactor using the aov procedure of R (R software version 2.13.1). The assumptions of the statistical models were tested for every data set. Normality of the residuals was evaluated graphically and with the Shapiro-Wilk test. Homogeneity of variance was tested with the Levene's test. Means were separated calculating least significant difference (LSD) at the significant level $P = 0.05$. Association between soil properties was estimated using linear regression. Normality of the residuals was evaluated graphically and

with the Shapiro-Wilk test. Pearson's product moment correlation coefficients (r) and significance of the associations (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$) are reported.

Principal component analysis (PCA) was carried out using all the measured soil properties to reveal differences among the treatments. Correlation between the first two principal components variates and the original variates were also calculated. Pearson's product moment correlation coefficients (r) and significance of the associations (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$) are reported.

Table 3.1. Enzymes included in the study, abbreviations, corresponding MUF model substrates and final substrate concentrations[*].

Enzyme	**EC**	**Substrate**	**Concentration (μM)**
β-1,4-glucosidase (β-GLU)	3.2.1.21	4-MUF-β-D-glucoside	100
α-1,4-glucosidase (α-GLU)	3.2.1.20	4-MUF-α-D-glucoside	400
N-acetyl-β-glucosaminidase (N-AG)	3.2.1.30	4-MUF-*N*-acetyl-β-D-glucosamide	200
β-1,4-xylosidase (β-XYL)	3.2.1.37	4-MUF-β-D-xyloside	400
β-D-1,4-cellobiosidase (β-CEL)	3.2.1.91	4-MUF-β-D-cellobioside	75
Arylsulfatase (SULF)	3.1.6.1	4-MUF-sulfate	1000
Phosphomonoesterase (PME)	3.1.3.2	4-MUF-phosphate	200
Phosphodiesterase (PDE)	3.1.4.1	bis-4-MUF-phosphate	500

[*]MUF = 4-methylumbelliferone. EC = enzyme code number defined by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB).

3.3. Results

3.3.1. Soil chemical and microbiological properties

Soil reaction was not significantly affected by organic or mineral N fertilization (Fig 3.1 a). However a clear trend was observed between blocks: soil samples from block 1 and 4 showed pH values around the neutrality, while soil samples from block 2 and 3 had a more acidic reaction (Fig. 3.1 b).

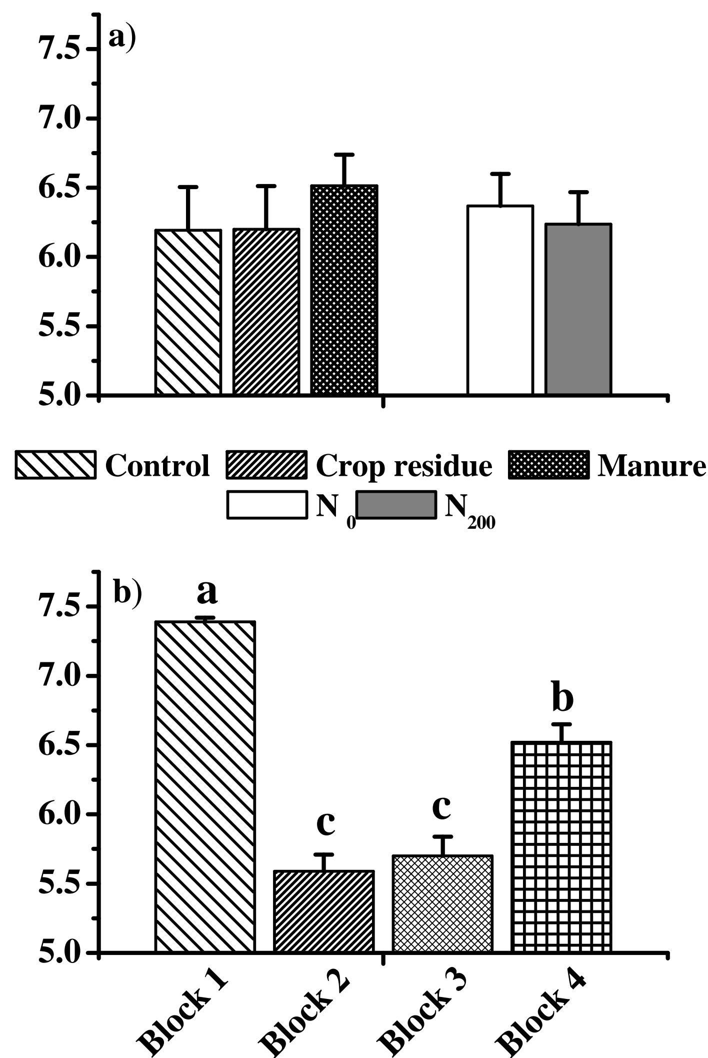

Fig. 3.1. Marginal means of soil pH for the effects of (a) fertilization and (b) block. Error bars represent standard errors. Different letters indicate significant differences ($P < 0.05$) as determined by LSD.

Long-term fertilization with manure significantly increased TOC and TN, while no significant differences were observed between the control and the crop residue treatments (Fig. 3.2). With regard to the organic carbon extractable fraction (C_{extr}) values were minimum in the plots amended with crop residue and maximum in the plots amended with manure. Conversely, C:N ratio was highest under crop residue amendment. The subplot factor mineral N fertilization did not affect TOC, C_{extr}, TN or the C:N ratio. Interactions between the main factor organic fertilization and the subfactor mineral N fertilization were never significant.

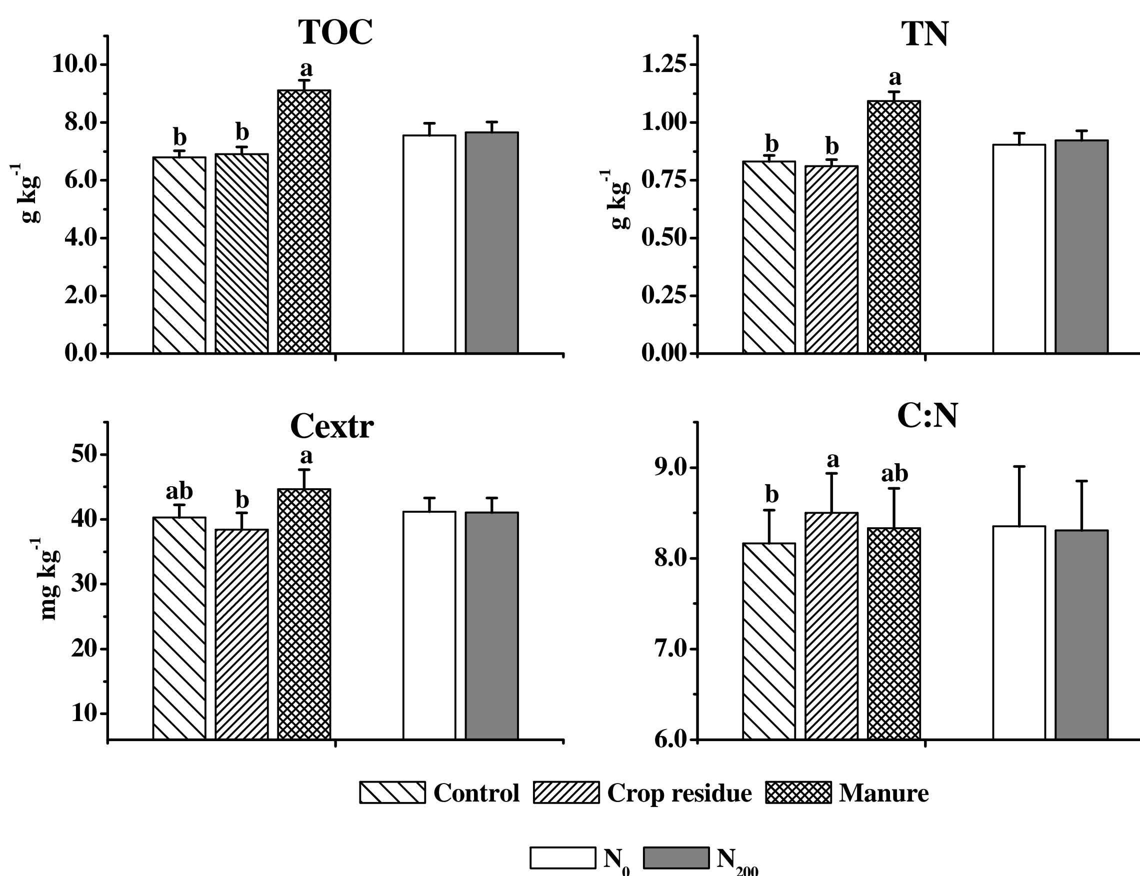

Fig. 3.2. Marginal means of total organic carbon (TOC), total nitrogen (TN), extractable organic carbon (C_{extr}) and organic carbon to total nitrogen ratio (C:N) for the effects of organic fertilization and mineral N fertilization. Error bars represent standard errors. Different letters indicate significant differences ($P < 0.05$) as determined by LSD.

As observed for soil organic matter content, treatments amended with manure showed higher values of C_{mic} as compared to the crop residue and control treatments (Fig 3.3). Similar results were observed for N_{mic}, even though in this instance crop residue showed intermediate values between the control and the manure treatments. The C_{mic}:N_{mic} ratio decreased as a consequence of organic fertilization, while an opposite trend was observed for N fertilization. However none of these differences were significant. Likely, the C_{mic}:TOC ratio increased under crop residue and manure treatments as compared to control treatment, but differences were too small to detect a significant effect of organic fertilization on this parameter. The subplot factor mineral N fertilization did not affect microbial biomass content, the C_{mic}:N_{mic} ratio and the C_{mic}:TOC ratio. Significant interactions between the main factor organic fertilization and the subfactor N fertilization were never observed.

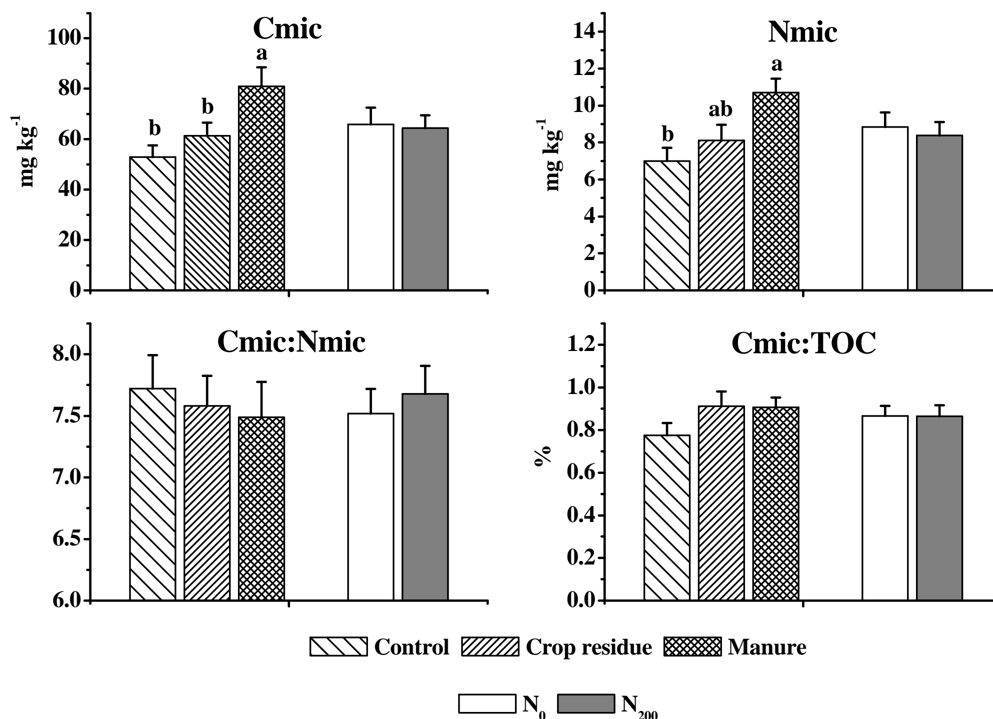

Fig. 3.3. Marginal means of microbial biomass carbon (C_{mic}), microbial biomass nitrogen (N_{mic}), microbial biomass C:N ratio (C_{mic}:N_{mic}) and percentage of microbial biomass carbon over total organic carbon (C_{mic}:TOC) for the effects of organic fertilization and mineral N fertilization. Error bars represent standard errors. Different letters indicate significant differences ($P < 0.05$) as determined by LSD.

3.3.2. Enzyme activities

Results regarding soil enzyme activities are reported in Fig. 3.4. β-glucosidase and β-cellobiosidase activities appeared affected by organic fertilization, with manure treatment showing higher activities as compared to crop residue and control treatments. A similar trend was observed for β-xylosidase and arylsulfatase activities but differences were not statistically significant.

With regard to the sub-plot factor mineral N fertilization, higher α-glucosidase, phosphomonoesterase and phosphodiesterase activities were found in N_{200} as compared to N_0 plots. *N*-acetyl-β-glucosaminidase activity was maximum in the crop residue treatment as compared to manure and control treatments. However the variability observed between blocks was high and no significant differences were found. Significant interactions between the main factor organic fertilization and the subfactor mineral N fertilization were not observed.

3.3.3. Correlation between variables

A correlation matrix including all the considered soil properties is reported in Table 3.2. Soil reaction was positively correlated with TOC and C_{extr}, as well as with TN, C_{mic} and N_{mic}. A positive correlation was also observed between soil pH and β-glucosidase activity. Conversely, correlations between soil pH and α-glucosidase, phosphomonoesterase and phosphodiesterase were negative. TOC and TN were strongly intercorrelated and showed positive correlations with C_{extr}, C_{mic} and N_{mic} as well as with β-glucosidase, β-xylosidase and β-cellobiosidase. Similar though weaker correlations were observed between C_{extr} and C_{mic} and N_{mic}. C_{extr} resulted also positively correlated with β-glucosidase and β-cellobiosidase, and negatively correlated with phosphomonoesterase. C_{mic} and N_{mic} resulted strongly intercorrelated and showed positive correlation with β-glucosidase and β-cellobiosidase and negative correlation with phosphomonoesterase. Enzyme activities that were positively correlated with TOC and TN such as β-glucosidase, β-xylosidase and β-cellobiosidase resulted also intercorrelated. Similarly, activities that were negatively correlated with soil pH, such as α-glucosidase, phosphomonoesterase and phosphodiesterase, were intercorrelated. Arylsulfatase and *N*-acetyl-β-glucosaminidase activities resulted positively correlated with enzyme activities involved in the C-cycle as β-glucosidase, β-xylosidase and β-cellobiosidase and with phosphodiesterase. *N*-acetyl-β-glucosaminidase activity resulted also positively correlated with α-glucosidase, while arylsulfatase was positively correlated with phosphomonoesterase activity.

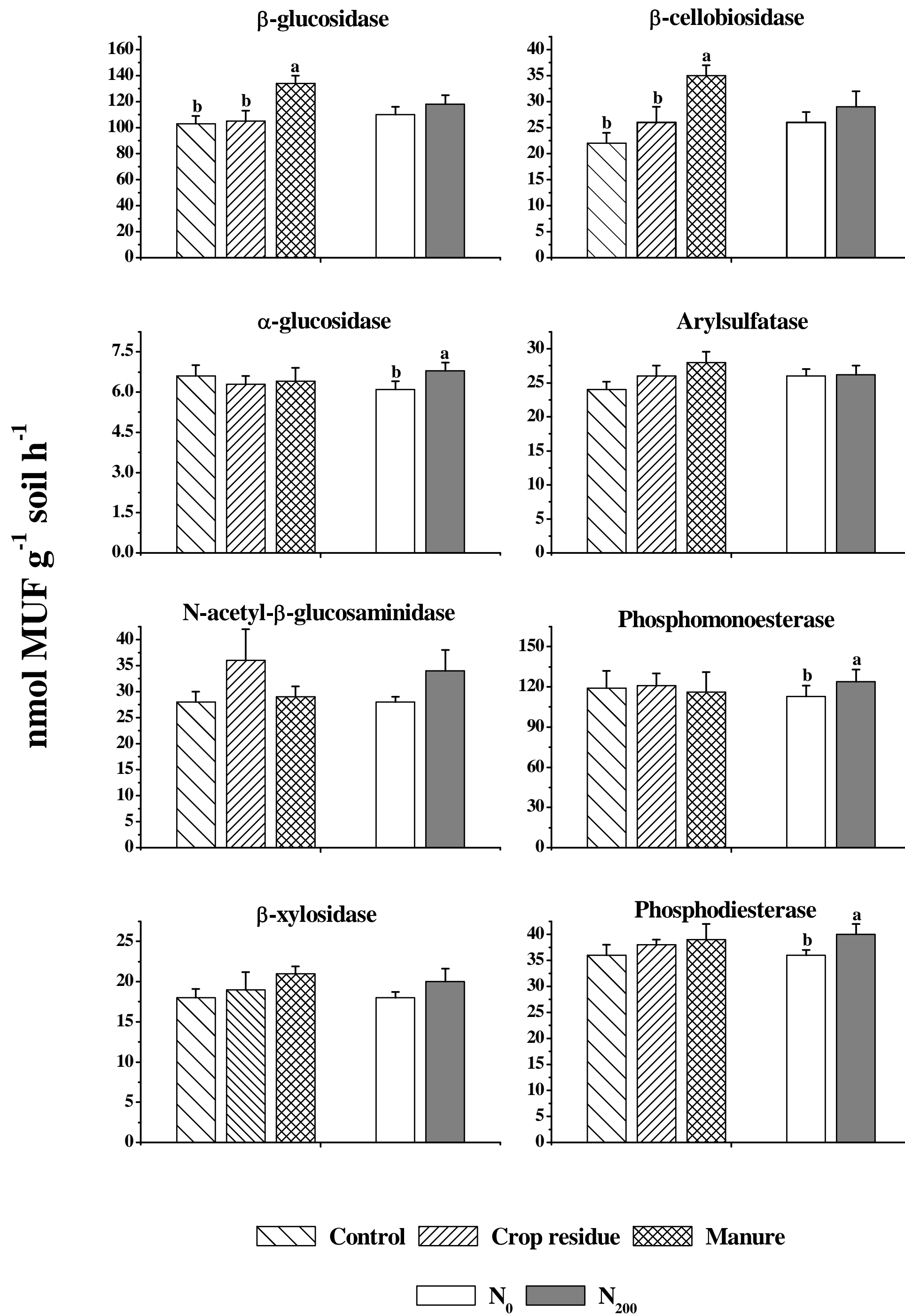

Fig. 3.4. Marginal means of enzyme activities for the effects of organic fertilization and mineral N fertilization. Error bars represent standard errors. Different letters indicate significant differences ($P < 0.05$) as determined by LSD.

Table 3.2. Correlations between variables*.

	pH	TOC	TN	C_{extr}	C_{mic}	N_{mic}	β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SULF	PME	PDE
pH	1													
TOC	0.48 *	1												
TN	0.48 *	0.98 ***	1											
C_{extr}	0.80 ***	0.64 ***	0.66 ***	1										
C_{mic}	0.77 ***	0.82 ***	0.81 ***	0.73 ***	1									
N_{mic}	0.78 ***	0.82 ***	0.79 ***	0.70 ***	0.95 ***	1								
β-GLU	0.42 *	0.84 ***	0.83 ***	0.58 **	0.72 ***	0.74 ***	1							
α-GLU	- 0.44 *	- 0.11	- 0.12	- 0.32	- 0.18	- 0.23	0.25	1						
N-AG	- 0.07	0.02	0.01	0.04	0.15	0.11	0.44 *	0.58 **	1					
β-XYL	0.01	0.41 *	0.40 *	0.21	0.33	0.35	0.76 ***	0.63 **	0.79 ***	1				
β-CEL	0.39	0.82 ***	0.81 ***	0.58 ***	0.69 ***	0.73 ***	0.80 ***	0.18	0.48 *	0.75 ***	1			
SULF	- 0.14	0.39	0.37	0.01	0.08	0.20	0.57 **	0.37	0.37	0.65 ***	0.64 **	1		
PME	- 0.91 ***	- 0.30	- 0.29	- 0.71 ***	- 0.61 **	- 0.58 **	- 0.12	0.81 ***	0.26	0.30	- 0.08	0.46 *	1	
PDE	- 0.57 **	0.01	0.01	- 0.38	- 0.24	- 0.23	0.25	0.69 ***	0.47 *	0.63 ***	0.29	0.64 ***	0.81 ***	1

*Significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

3.3.4. Principal component analysis

A principal component analysis was carried out including all the measured soil properties (Fig. 3.5). Pearson's product moment correlation coefficients (r) between the first two principal components variates and the original variates were used to obtain detailed information about the variables that contributed most to the separation of the treatments (Table 3.3). The first two principal components accounted for the 46 % and the 30 % of the total variance, respectively (Fig. 3.5). Significant correlations between PC1 and the original variates were observed for soil pH, TOC, C_{extr}, TN, C_{mic}, N_{mic} and enzyme activities related to the C-cycle as β-glucosidase, β-xylosidase and β-cellobiosidase, indicating that these variables contributed more to the separation of the samples along this component (Table 3.3). Soil samples of plots amended with manure were clearly separated from the other ones along PC1, while for other treatments no obvious differences were observed. PC2 was dominated by soil pH and by the enzyme activities α-glucosidase, *N*-acetyl-β-glucosaminidase, β-xylosidase, arylsulfatase, phosphomonoesterase and phosphodiesterase (Table 3.3). N_0 and N_{200} treatments means were respectively in the positive and in the negative part of the component. However, overlapping of the error bars was observed, thus no clear separation was obtained along this component.

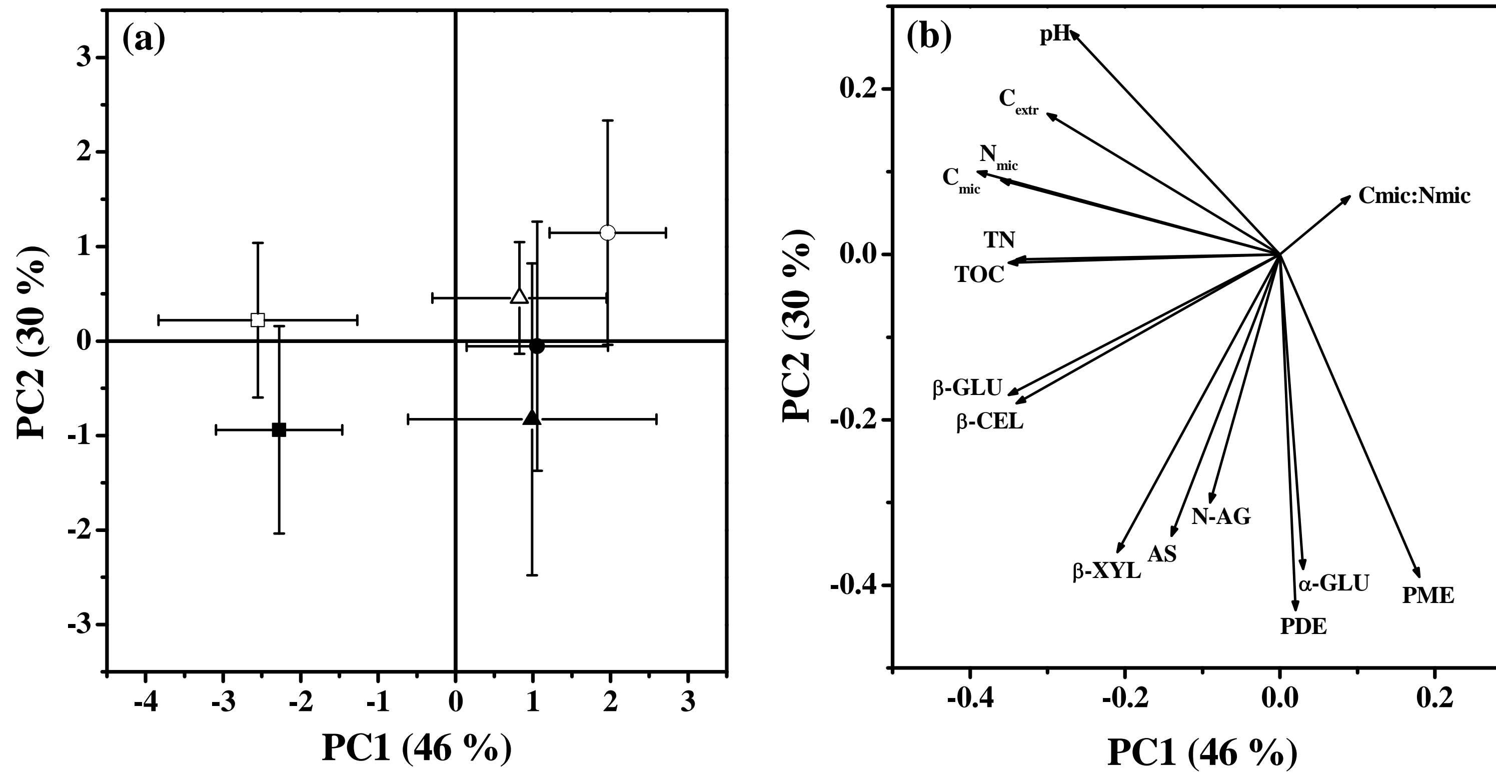

Fig. 3.5. Plot of PC1 and PC2 means (± standard error) produced from the principal component analysis of soil properties obtained from (○) control, (●) control + N, (Δ) crop residue, (▲) crop residue + N, ( ) manure and (■) manure + N soils (a) and relative loadings (b).

Table 3.3. Results of principal component analysis*.

	PC			
	1		**2**	
% of variance	46		30	
pH	- 0.70	***	0.58	**
TOC	- 0.91	***	- 0.02	
TN	- 0.90	***	- 0.01	
C_{extr}	- 0.79	***	- 0.36	
C_{mic}	- 0.91	***	0.22	
N_{mic}	- 0.93	***	0.19	
C_{mic}:N_{mic}	0.24		0.15	
β-GLU	- 0.90	***	0.36	
α-GLU	- 0.09		- 0.81	***
N-AG	- 0.25		- 0.65	***
β-XYL	- 0.54	**	- 0.76	***
β-CEL	- 0.89	***	- 0.38	
SULF	- 0.38		- 0.72	***
PME	0.46		- 0.83	***
PDE	0.06		- 0.91	***

*Significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

3.4. Discussion

Soil reaction was not significantly affected by the different treatments (Fig 3.1a). In a previous study regarding the same long-term experiment, mineral N fertilization affected soil pH trough acidification mechanisms (Chapter 1; Giacometti et al., 2013). However, the effect become evident only short after N fertilization. Since in the present study soil samples were collected in March, before maize sowing and urea application, no clear effect of N fertilization on soil pH was observed. The differences in soil reaction found between blocks (Fig. 3.1 b) were probably due to small differences in soil calcium carbonate content. The variability of soil pH between blocks could have act as a confounding factor hampering the identification of clear differences between the pH of the treatments.

As reported in other long-term experiments, manure's positive effects on SOM exceeded that of crop residue (Rasmussen and Collins, 1991; Reeves, 1997; Triberti et al., 2008), suggesting that not only the quantity but also the quality of the organic inputs added influenced their fait in soil. As observed in a previous study (Chapter 1; Giacometti et al., 2013), manure increased SOM and its more labile fraction and stimulated microbial growth (Figs. 3.2 and 3.3). On the contrary crop residue amendment and mineral N fertilization had little impact on soil organic matter as well as on soil microbial biomass.

The small changes of the C_{mic}:N_{mic} ratio observed under the different treatments (Fig. 3.3) may indicate a shift of the microbial community composition due to organic and mineral N fertilization. Changes in the composition of the soil microbial community are reported to occur as a response to differences in the quantity and chemical composition of the organic residue and nutrients entering the soil (Zelles, 1999). The changes of the microbial community PLFAs profiles observed under the different treatments in a previous study further support this hypothesis (Chapter 1; Giacometti et al., 2013).

Organic fertilization, and particularly soil amendment with manure, slightly increased the percentage of total organic carbon representing microbial biomass (Fig. 3.3). This imply that the soil microbial community made use of higher substrate and nutrient availability under this treatment (Berner et al., 2011).

According to Sinsabaugh et al. (2008) most differences in enzyme activities among sites or treatments are likely the result of differences in SOM or microbial biomass. SOM content could be related to substrate availability. Since most hydrolases are adaptive enzymes, they appear to be

primarily a function of the amount of substrate available and are therefore positively related to SOM content (Kandeler et al., 1999). Further, soil enzymes are mainly produced by the soil microbiota to degrade available substrates (Winding et al., 2005). Therefore the larger is the microbial biomass the higher is the quantity of enzymes that could be potentially synthesized (Geiseller and Horwath, 2009).

In our experiment microbial biomass and organic substrates availability appeared to be the key factors determining the response of β-cellobiosidase and β-glucosidase activities to the different treatments. In accordance with Dodor and Tabatabai (2005) these glycosidases were positively correlated with total and extractable organic C as well as with microbial biomass C and N (Fig. 3.4 and Table 3.2). Maximum values of β-cellobiosidase and β-glucosidase were observed under manure amendment. A similar trend was observed for β-xylosidase activity, even thought differences were not significant. Other investigations (Kandeler et al., 1999; Marinari et al., 2000, 2006; Böhme et al., 2005; Moscatelli et al. 2011) have also reported positive effects of manure on glycosidases activity.

The activity of α-glucosidase reacted differently from the other glycosidases, and seamed to be more influenced by mineral N fertilization than by soil amendment with organic materials (Fig. 3.4). In general, as compared to the enzymes targeting cellulose and hemicelluloses, the activity of α-glucosidase is more strongly related to the content of soluble saccharides and peaks earlier when fresh organic residue are added but also decline faster (Sinsabaugh et al., 2002). Moreover, it could be speculated than since starch is a storage polysaccharide, plants increase its synthesis when they are not N limited and therefore N_{200} plots contained plant material enriched in starch as compared to that of N_0 plots (Entry et al., 1998). The positive effect of N fertilization and the negative correlation with soil pH observed in our study are in accordance with Kiss et al. (1978) who reported that earlier woks on α-glucosidase found that soil liming and the subsequent increase in soil pH lowered the enzyme activity while NPK fertilization increased it.

N-acetyl-β-glucosaminidase is an enzyme produced mainly by fungi (Edwards and Zak, 2011), therefore the tendency of the activity to increase under crop residue amendment may be related to a larger or more active fungal biomass, probably stimulated by the quality of the organic mater added with straw. It is known that saprotrophic fungi are involved in the decomposition of organic matter, and particularly of complex substrates such as lignin and cellulose of which straw is rich (Poll et al., 2010). Similarly to what observed in our study, Ekenler and Tabatabai (2003b) reported that soil mulching with corn stalks increased the activity of *N*-acetyl-β-glucosaminidase.

Martens et al. (1992) observed an increase of *N*-acetyl-β-glucosaminidase activity when soil was amended with crop residue while no effect was observed after treating the soil with sewage sludge, and suggested that this different responses to organic inputs could be due to enzymes added to soil by plant residue.

Arylsulfatase activity is known to be related to soil organic carbon content (Deng and Tabatabai, 1997) and to substrate quality (Berner et al., 2011). In our study, a tendency of arylsulfatase activity to increase under organic fertilization was observed, although differences were not statistically significant. In accordance to what observed by Speir (1977), arylsulfatase activity was significantly correlated with phosphatases activity (Table 3.2). The positive correlations observed between arylsulfatase activity and C-cycling enzymes were probably indirect, and due to the fact that the activities of all these enzymes are influenced by substrate availability (Table 3.2).

For the enzymes involved in P-cycling factors other than microbial biomass and SOM content and quality also contributed to influence their activity levels. In the case of phosphomonoesterase, the positive effect of N fertilization was probably indirect and due to the slight acidification that resulted from urea distribution. Even though the effect of N fertilization on soil reaction was not significant in this sampling (Fig. 3.1), the strong negative correlation observed between soil pH and phosphomonoesterase activity (Table 3.2) suggested that the observed differences could be due to a response to soil pH. According to Tabatabai (1994), the inverse relationship between acid phosphatase activity and soil pH suggests that either its rate of synthesis and release by soil microorganisms or its stability is related to soil pH. Moreover, phosphatases are inducible enzymes and their synthesis is influenced by the quantity of orthophosphate available to plants and microorganisms, which in turn is strongly affected by soil pH (Acosta-Martínez and Tabatabai, 2000; Ekenler and Tabatabai, 2003a). Significant amount of P are reported to enter soil P cycle with application of organic amendments such as animal manure (Acosta-Martínez and Tabatabai, 2011). However in our study no clear effect of soil amendment with manure was observed on phosphatases. This results are in agreement with previous studies according to which, as compared to other enzyme activities the phosphatases are more strongly affected by changes in soil pH due to management, independent of organic matter content (Acosta-Martínez and Tabatabai, 2000; Ekenler and Tabatabai, 2003a). Phosphatases are also known to be related to phosphate availability, and activity inhibition has been observed following the addition of phosphate fertilizers (Dick, 1994). In our experiment all the plots receive the same amount of phosphate fertilizer, thus phosphate availability should have been equal across all the treatments. The negative correlation observed

between soil microbial biomass and phosphomonoesterase activity was probably an indirect effect of soil reaction (Table 3.2). In fact, while microbial biomass proliferation was favoured by neutral soil pH, phosphomonoesterase reached maximum levels of activity under more acidic conditions. Similar negative correlations between acid phosphatase and microbial biomass C and N were reported by Ekenler and Tabatabai (2003a), in a study investigating the effects of soil liming.

Phospodiesterase and phosphomonoestarase are tightly linked in the soil P cycling. The reactions they catalyze occur in a series in which the product released by phosphodiesterase may become the substrate for phosphomonoesterase, ultimately leading to free phosphates (Acosta-Martínez and Tabatabai, 2011). This close ecological link was observed also in our study, where phosphodiesterase and phosphomonoesterase resulted strongly correlated (Table 3.2).

In the principal component analysis, soil samples of plots amended with manure were clearly separated from the other ones along PC1. This separation was determined by total and extractable organic C and microbial biomass content and by enzyme activities related to the degradation of cellulose and hemicelluloses, all parameters that were clearly influenced by organic fertilization. Along PC2, N_0 plots were grouped in the positive part of the component, while N_{200} plots were grouped in the negative one. This separation was to a large extent due to soil pH and to enzyme activities that were directly or indirectly influenced by urea application. In other words, soil samples were separated along PC1 and PC2 according to their response to organic fertilization and mineral N fertilization, respectively.

3.5 Conclusions

Organic fertilization with cattle manure increased the activities of extracellular enzymes involved in the breakdown of cellulose and hemicellulose, while crop residue amendment had limited effects. These activities were strongly related to total and extractable organic C and to microbial C and N, confirming the key role that SOM and microbial biomass content have in determining soil catalytic potential.

The enzymes involved in the degradation of starch were more influenced by the quality than by the quantity of the available substrates and showed maximum activity under mineral N fertilization.

Mineral N fertilization also increased the activity of the enzymes involved in the P cycle, probably through acidification mechanisms following urea application, confirming that these activities are more affected by changes in soil reaction than by microbial biomass and SOM content, as also confirmed by the strong negative correlation observed between phosphatases activity and soil pH.

In conclusion the results of this study confirm that soil enzymes respond to differences in fertilization management and are closely related to other important physical, chemical and microbiological soil quality indicators. Their assay by means of microplate-based fluorimetry is a fast and throughput tool for the assessment of soil functional diversity and for soil quality monitoring.

GENERAL CONCLUSIONS

The results of this study showed as soil microorganisms respond to organic and mineral N fertilization by adjusting their biomass, activity and community composition, confirming that microbial and biochemical parameters are early and sensitive indicators of fertilization-induced changes of soil quality.

The soil microbial community responded to manure amendment by increasing its biomass and shifting its composition toward Gram-positive bacteria. Moreover, enzyme activities involved in the oxidation of organic substrates and in the hydrolysis of cellulose, hemicellulose, proteins and urea were maximum under manure treatment, as a result of an increased production of enzymes promoted by an enlarged microbial biomass and by a higher substrate availability. In contrast, soil amendment with crop residue had limited effects on soil microbiota. Mineral N fertilization stimulated crop residue mineralization and reduced the amount of straw-derived C retained into the soil. Further, mineral N fertilization increased the activity of P-cycling enzymes through acidification mechanisms and inhibited the activity of N-cycling enzymes soon after N fertilizer application.

Repeated samplings over time evidenced as microbial and biochemical parameters were generally affected by environmental conditions. However, even though their average levels fluctuated over time, the identification of fertilization treatments effects was not hampered. Therefore, under the conditions of our study microbial and biochemical parameters seasonal fluctuations did not prevent their use as early and sensitive indicators of fertilization-induced changes of soil quality.

The use of a long-term experiment allowed to detect modifications of slowly changing and resilient chemical parameters such as SOM content and composition in response to different fertilization regimes. As for the microbial and biochemical parameters, the most relevant and consistent changes were observed under manure, where SOM content was maximum and its relative composition was shifted toward aliphatic compounds as determined by DRIFT spectroscopy. Microbial and biochemical parameters resulted strongly correlated with SOM content and composition, indicating that microorganisms response to fertilization was mainly linked to the quantity and quality of the organic matter that have accumulated under the different treatments.

PLS regression models relating the DRIFT spectra of bulk soil with soil chemical and microbiological properties showed the potential of DRIFT spectroscopy to be used as a rapid soil testing technique for soil quality monitoring. However, the accuracy of microbial biomass content prediction based on DRIFT spectra needs further improvements.

GENERAL CONCLUSIONS

Microplate-based fluorimetric assays proved to be suitable for the simultaneous determination of multiple soil enzyme activities. Their application offer a fast and throughput tool for the study of soil functional diversity and soil quality. However, for a precise estimation of maximum potential activity assay conditions appear to require proper optimization.

The findings of this study show that in the long-term both early and slowly changing soil properties are affected by contrasting fertilization regimes and therefore represent valuable indicators of management-induced changes of soil quality status. However their combined determination offer insights into the mechanisms behind soil quality modifications that the determination of single indicators would not provide. Moreover, this study show as throughput analytical procedures as DRIFT spectroscopy and MUF fluorimetry represent promising tools for the fast monitoring of soil quality.

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